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Soeters, M.R.

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2 Gender related differences in the metabolic response to fasting

Maarten R. Soeters¹, Hans P. Sauerwein¹,
Johanna E. Groener², Johannes M. Aerts²,
Mariëtte T. Ackermans³, Jan F.C. Glatz⁴,
Eric Fliers¹ and Mireille J. Serlie¹

¹Department of Endocrinology and Metabolism
²Department of Medical Biochemistry,
³Department of Clinical Chemistry, Laboratory
of Endocrinology, Academic Medical Center,
University of Amsterdam, Amsterdam, the
Netherlands, ⁴Department of Molecular
Genetics, Maastricht University, Maastricht, the
Netherlands

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Abstract

Context: Free fatty acids (FFA) may induce insulin resistance via synthesis of intramyocellular ceramide. During fasting, women have lower plasma glucose levels than men despite higher plasma FFA, suggesting protection from FFA-induced insulin resistance.

Objective: We studied whether the relative protection from FFA-induced insulin resistance during fasting in women, is associated with lower muscle ceramide concentrations compared to men.

Main Outcome Measures and Design: After a 38 h fast, measurements of glucose and lipid fluxes and muscle ceramide and fatty acid translocase/CD36 were performed before and after a hyperinsulinaemic euglycaemic clamp.

Results: Plasma glucose levels were significantly lower in women than men with a trend for a lower endogenous glucose production in women, while FFA and lipolysis were significantly higher. Insulin-mediated peripheral glucose uptake was not different between sexes. There was no gender difference in muscle ceramide in the basal state and ceramide did not correlate with peripheral glucose uptake. Muscle fatty acid translocase/CD36 was not different between sexes in the basal state and during the clamp.

Conclusion: After 38 h of fasting, plasma FFA were higher and plasma glucose was lower in women compared to men. The higher plasma FFA did not result in differences in peripheral insulin sensitivity, possibly because of similar muscle ceramide and fatty acid translocase/CD36 levels in men and women. We suggest that during fasting, women are relatively protected from FFA-induced insulin resistance by preventing myocellular accumulation of ceramide.

Introduction

The adaptive metabolic response to short-term fasting consists of integrated metabolic alterations that guarantee substrate availability for energy production and prevent hypoglycaemia. During fasting, plasma insulin levels are low and plasma concentrations of catecholamines, glucagon and growth hormone are increased, resulting in increased lipolysis and thus high plasma free fatty acid (FFA) concentrations (1-3).

It has been known for a long time that women have lower plasma glucose and higher plasma FFA concentrations than men after short-term fasting (1;4-6). However, despite extensive research on gender related distinctions in glucose and lipid metabolism, these differences in plasma glucose and FFA concentrations have not been explained in full detail so far (7-16).

In women, the combination of lower plasma glucose levels on one hand, and higher plasma FFA levels on the other hand, is intriguing, since it is generally accepted that high plasma FFA levels increase endogenous glucose production (EGP) and decrease peripheral glucose uptake (17;18). Consistently, it has been shown that women are relatively protected from FFA-induced insulin resistance (12;16).

The exact underlying mechanisms by which FFA interfere with insulin signalling have not yet been unravelled completely. One potential mechanism may involve the de novo synthesis of ceramide from palmitate, because intramyocellular ceramide was found to be increased in obese insulin-resistant patients and correlated with whole body insulin sensitivity (19;20). Moreover, in vitro studies showed that intracellular ceramide synthesis from palmitate was found to be one of the mechanisms by which palmitate interferes negatively with insulin-stimulated phosphorylation of protein kinase B (PKB) (21;22). Furthermore, metabolites from ceramide (i.e. glycosphingolipids, like glucosylceramide) might be involved in the induction of insulin resistance (23;24). Since intramyocellular ceramide concentration correlates positively with plasma FFA levels, it might be expected that the increased levels of plasma FFA in women result in higher muscle ceramide levels (20). However, this would contradict with the reported relative protection from FFA-induced insulin resistance in women.

Two mechanisms may explain this relative insensitivity to increased plasma FFA levels: firstly, lower myocellular uptake of plasma FFA and secondly, differences in muscle fatty-acid handling. Cellular uptake of plasma FFA occurs by protein-mediated transport and via flip-flop of protonated fatty acids (25-27), depending on transmembrane concentration gradients and intracellular fatty acid metabolism (25;27-29). Fatty acid

translocase (FAT)/CD36 is the main protein involved in muscle fatty acid uptake (26). Fatty-acid handling involves storage into complex lipids or oxidation of fatty acyl-CoAs. To the best of our knowledge, gender differences in muscle (glyco)sphingolipids and the fatty acid transporter CD36 content after short-term fasting in relation to glucose and lipid metabolism have not been studied before.

In this study, we measured glucose and lipid fluxes after short-term fasting in healthy lean men and women in the basal state and during a hyperinsulinaemic euglycaemic clamp (stable isotope technique). Furthermore, we assessed total muscle content of ceramide, glucosylceramide and the lipid binding protein FAT/CD36 in the basal state and during the clamp. We hypothesized that the relative protection from FFA-induced insulin resistance during fasting in women results from lower muscle ceramide or glucosylceramide levels due to lower muscle FFA uptake, subsequently resulting in higher muscle glucose uptake with lower plasma glucose concentrations.

Subjects and methods

Subjects

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Ten male and 10 female subjects were recruited via advertisements in local magazines. Criteria for inclusion were 1) absence of a family history of diabetes; 2) age 18–35 yr; 3) Caucasian race; 4) BMI 20–25 kg/m²; 5) no excessive sport activities, i.e. < 3 times per week; and 6) no medication. Women were studied during the follicular phase of the menstrual cycle. Subjects were in self-reported good health, confirmed by medical history and physical examination. Written informed consent was obtained from all subjects after explanation of purposes, nature, and potential risks of the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

Experimental protocol

For three days before the fasting period, all volunteers consumed a weight-maintaining diet containing at least 250 g of carbohydrates per day. Then, the subjects were fasting from 2000 h two days before the start of the study until the end of the study. Volunteers were admitted to the metabolic unit of the Academic Medical Center at 0730 h. Subjects were studied in the supine position and were allowed to drink water only.

A catheter was inserted into an antecubital vein for infusion of stable isotope tracers, insulin and glucose. Another catheter was inserted retrogradely into a contralateral hand

vein and kept in a thermo-regulated (60°C) plexiglas box for sampling of arterialized venous blood. In all studies, saline was infused as NaCl 0.9% at a rate of 50 mL/h to keep the catheters patent. [6,6-²H₂]glucose and [1,1,2,3,3-²H₅]glycerol were used as tracers (>99% enriched; Cambridge Isotopes, Andover, USA).

To study total triglyceride hydrolysis, we used [1,1,2,3,3-²H₅]glycerol. This may result in underestimation of FFA release (in contrast to a fatty acid tracer). However, curves of glycerol and fatty acid tracers are very similar during fasting (2), although the latter is preferred to quantify adipose tissue lipolysis (30).

At T = 0 h (0800 h), blood samples were drawn for determination of background enrichments and a primed continuous infusion of both isotopes was started: [6,6-²H₂]glucose (prime, 8.8 μmol/kg; continuous, 0.11 μmol/kg·min) and [1,1,2,3,3-²H₅]glycerol (prime, 1.6 μmol/kg; continuous, 0.11 μmol/kg·min) and continued until the end of the study. After an equilibration period of two hours (38 h of fasting), 3 blood samples were drawn for glucose and glycerol enrichments and 1 for glucoregulatory hormones, FFA and adiponectin. Thereafter (T = 3 h), infusions of insulin (60mU/m²·min) (Actrapid 100 IU/ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, the Netherlands) and glucose 20% (to maintain a plasma glucose level of 5 mmol/L) were started. [6,6-²H₂]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to approximate the values for enrichment reached in plasma and thereby minimizing changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. Plasma glucose levels were measured every 5 minutes at the bedside. At T = 8 h, 5 blood samples were drawn at 5 minute intervals for determination of glucose and glycerol enrichments. Another blood sample was drawn for determination of glucoregulatory hormones, FFA and adiponectin.

Body composition and indirect calorimetry

Body composition was measured with bioelectrical impedance analysis (Maltron BF906, Rayleigh, UK). Oxygen consumption (VO₂) and CO₂ production (VCO₂) were measured continuously during the final 20 min of both the basal state and the hyperinsulinaemic euglycaemic clamp by indirect calorimetry using a ventilated hood system (Sensormedics model 2900; Sensormedics, Anaheim, USA).

Muscle biopsy

Muscle biopsies were performed to assess muscle content of ceramide, glucosylceramide and FAT/CD36 at the end of both the basal state and the hyperinsulinaemic euglycaemic clamp. The muscle biopsy was performed under local anaesthesia (lidocaine 20 mg/ml;

Fresenius Kabi; Den Bosch, the Netherlands) using a Pro-Mag™ I Biopsy Needle (MDTECH, Gainesville, USA). Biopsy specimens were quickly washed in a buffer (NaCl 0.9%/Hepes 28.3gr/L) to remove blood, inspected for fat or fascia content, dried on gauze swabs and subsequently stored in liquid nitrogen until analysis.

Glucose and lipid metabolism measurements

Plasma glucose concentrations were measured with the glucose oxidase method using a Beckman Glucose Analyzer 2 (Beckman, Palo Alto, USA, intra-assay variation 2-3%). Plasma FFA concentrations were determined with an enzymatic colorimetric method (NEFA-C test kit, Wako Chemicals GmbH, Neuss, Germany): intra-assay variation 1%; inter-assay variation: 4-15%; detection limit: 0.02 mmol/L. [6,6-²H₂]glucose enrichment was measured as described earlier (31). [6,6-²H₂]glucose enrichment (tracer/tracee ratio) intra-assay variation: 0.5-1%; inter-assay variation 1%; detection limit: 0.04%. [1,1,2,3,3-²H₅]glycerol enrichment was determined as described earlier (32). Intra-assay variation glycerol: 1-3%, [1,1,2,3,3-²H₅]glycerol: 4%; inter-assay variation glycerol: 2-3%; [1,1,2,3,3-²H₅]glycerol: 7%.

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Glucoregulatory hormones and adiponectin

Insulin and cortisol were determined on an 2000 system (Diagnostic Products Corporation, Los Angeles, USA). Insulin was measured with a chemiluminescent immunometric assay, intra-assay variation: 3-6%, inter-assay variation: 4-6%, detection limit: 15pmol/L. Cortisol was measured with a chemiluminescent immunoassay, intra-assay variation: 7-8%, inter-assay variation: 7-8%, detection limit: 50nmol/L. Glucagon was determined with the Linco ¹²⁵I radioimmunoassay (St. Charles, USA), intra-assay variation: 9-10%, inter-assay variation: 5-7% and detection limit: 15ng/L. Norepinephrine and epinephrine were determined with an in-house HPLC method. Intra-assay variation norepinephrine: 2%; epinephrine 9%; inter-assay variation norepinephrine: 10%; epinephrine: 14-18%; detection limit: 0.05 nmol/L. Adiponectin was determined by a radioimmunoassay (Linco, St. Charles, USA)(Intra-assay variation: 2-7%; inter-assay variation: 16-17%; detection limit: 1 ng/mL).

Ceramide and glucosylceramide measurements

Ceramide and glucosylceramide in muscle biopsies were measured with a high performance liquid chromatography method as described (33). Muscle biopsies were weighed and homogenized in 300 µl water by sonification. 50 µl muscle homogenates

were used. All samples were run in duplicate and in every run 2 reference samples were included. CV: Inter-assay 4%, intra-assay < 14 %.

FAT/CD36 measurements

Muscle biopsies were homogenized four times 5 seconds (Ultra-Turrax®, Ika-Werke, Staufen, Germany) in 10 volumes of cold (4° C) buffer containing 250 mM sucrose, 2 mM Na-EDTA and 10 mM Tris at pH 7.4, followed by four times 5 sec ultrasonic treatment (Ultrasonic Processor, Hielsher GmbH, Teltow, Germany). Total protein was determined with the bicinchonic acid method (Pierce, Rockford IL, USA). All samples were diluted to the same protein concentration and mixed with sample buffer (4:1, vol/vol) before being subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting exactly as described before (34).

The CD36 antigen-antibody band at 88 kDa was visualized with a chemiluminescence substrate (ECL, Amersham Biosciences UK Ltd, Buckinghamshire, England) and quantified using Quantity One software (Bio-Rad, Hercules CA, USA). Rat heart and liver whole homogenates were used as positive and negative controls, respectively.

Calculations and statistics

Endogenous glucose production (EGP) and peripheral glucose uptake (rate of disappearance/Rd) were calculated using the modified forms of the Steele Equations as described previously (35;36). EGP and Rd were expressed as $\mu\text{mol}/\text{kg}\cdot\text{min}$. Glucose metabolic clearance rates (MCR) were calculated as $\text{MCR} = \text{Ra} / [\text{glucose}]$. Lipolysis (glycerol turnover) was calculated by using formulas for steady state kinetics adapted for stable isotopes (32). Lipolysis was expressed as $\mu\text{mol}/\text{kg}\cdot\text{min}$ and as $\mu\text{mol}/\text{kcal}$ as proposed by Koutsari et al (30). Resting energy expenditure (REE) and glucose and fat oxidation rates were calculated from O_2 consumption and CO_2 production as reported previously (37).

All data were analyzed with non parametric tests. Comparisons between groups (at T = 2 and 8 h) were performed using the Mann-Whitney U test. Comparisons within groups (between T = 2 and 8 h) were performed with the Wilcoxon Signed Rank test. Correlations were expressed as Spearman's rank correlation coefficient (ρ). The SPSS statistical software program version 12.0.1 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as median [minimum-maximum].

Table 1 Clinical characteristics of male and female subjects.

	men (n = 10)	women (n = 10)	p
Age(years)	21.3 [18.9 - 25.1]	22.2 [19.1 - 28.9]	0.3
Weight (kg)	74.6 [65.5 - 89.0]	63.5 [54.5 - 72.0]	0.001
Height (cm)	187 [179 - 195]	169 [160 - 177]	0.4
BMI (kg/m ²)	21.5 [19.2 - 24.7]	22.9 [18.7 - 24.2]	0.4
Lean body mass (%)	89 [77 - 91]	75 [70 - 89]	0.002
Fat mass (%)	11 [9 - 23]	25 [11 - 30]	0.002

Data are presented as median [min - max]. BMI = body mass index.

Results

Anthropometric characteristics

Men and women did not differ in age or BMI (Table 1). Weight and percentage lean body mass were higher and percentage fat mass was lower in men (Table 1).

Resting energy expenditure, glucose and lipid kinetics

Total REE was higher in men than in women, both in the basal state and during the hyperinsulinaemic clamp (Table 2a). Rates of glucose and fat oxidation did not differ between men and women in the basal state and during the clamp (Table 2a). In the female group, plasma glucose concentrations were significantly lower after 38 h of fasting compared to the male group (Table 2b). Basal EGP (and Rd) tended to be lower in women (Table 2b). Insulin-mediated peripheral glucose uptake during the clamp (Rd) did not differ significantly between men and women (Table 2b). MCR in the basal state was not different between women and men (2.0 [1.8 – 3.2] ml/kg•min vs. 2.1 [1.7 – 2.4] ml/kg•min respectively, $P = 1$). Basal plasma FFA were significantly higher in

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Table 2 REE and oxidation rates for glucose and fat.

	basal state		P
	men (n = 10)	women (n = 10)	
REE (kcal/day)	1995 [1840 - 2356]	1511 [884 - 1833]	0.001
Glucose oxidation (μmol/kg•min)	5.0 [0 - 18.3]	3.9 [0 - 7.8]	0.5
Glucose oxidation (μmol/kg lbm•min)	5.6 [0 - 20.6]	5.0 [0 - 10.6]	0.8
Fat oxidation (μmol/kg•min)	2.0 [0.9 - 2.8]	1.7 [1.3 - 2.1]	0.3
Fat oxidation (μmol/kg lbm•min)	2.3 [1.0 - 3.0]	2.2 [1.9 - 2.9]	0.9

Data are presented as median [minimum - maximum]. REE = resting energy expenditure, lbm = lean body mass.

women compared to men, but were equally suppressed during the hyperinsulinaemic euglycaemic clamp in both groups (Table 2b). Lipolysis expressed as $\mu\text{mol}/\text{kg}\cdot\text{min}$ was not different between men and women in the basal state and during the clamp, but when expressed in $\mu\text{mol}/\text{kcal}$ it was shown that women had significant higher lipolysis rates in the basal state, but not during the clamp (Table 2b).

Glucoregulatory hormones and adiponectin

Plasma insulin, cortisol, glucagon and norepinephrine levels were not different between sexes in the basal state and during the clamp (Table 3). Plasma epinephrine was significantly lower in females than males during the hyperinsulinaemic euglycaemic clamp (Table 3).

Adiponectin was significantly higher in females in both the basal state and during the clamp (Table 3). Adiponectin decreased significantly from baseline during the clamp, though no gender difference in relative decrease was observed (data not shown).

Ceramide and glucosylceramide measurements

Muscle ceramide concentrations in the basal state were not different between sexes (Figure 1). There was a trend to lower muscle ceramide levels in women compared to men during the hyperinsulinaemic euglycaemic clamp (Figure 1). However, the change in muscle ceramide content during the clamp from baseline did not differ between women and men (data not shown). There were no differences in muscle glucosylceramide levels between women and men (basal state: 1.9 [1.0 – 4.9] pmol/mg wet weight vs. 1.4 [1.2 – 3.0] pmol/mg wet weight respectively, $P = 0.16$ and during the clamp: 1.8 [1.3 – 6.7] pmol/mg wet weight vs. 2.2 [1.5 – 3.8] pmol/mg wet weight respectively, $P = 0.5$). In the basal state, muscle ceramide and glucosylceramide levels did not correlate with plasma FFA in women and men (ceramide (females): $\rho = 0.26$; $P = 0.47$, ceramide (males): $\rho = 0.35$; $P = 0.33$ and glucosylceramide (females): $\rho = 0.41$; $P = 0.26$, ceramide (males): $\rho =$

hyperinsulinaemic euglycaemic clamp		
men (n = 10)	women (n = 10)	P
1992 [1685 - 2177]	1467 [1150 - 1842]	0.001
20 [8.9 - 33.3]	16.1 [11.7 - 28.3]	0.4
22.2 [11.1 - 37.8]	21.7 [13.9 - 38.3]	0.9
0.7 [0 - 1.5]	0.5 [0 - 1.0]	0.4
0.8 [0 - 1.7]	0.6 [0 - 1.3]	0.7

Table 3 Glucose and lipid metabolism measurements

	basal state		P
	men (n = 10)	women (n = 10)	
Glucose (mmol/L)	4.4 [4.0-4.8]	3.9 [2.7-4.5]	0.023
EGP ($\mu\text{mol/kg}\cdot\text{min}$)	9.0 [7.3-10.3]	8.0 [7.1 - 10.1]	0.07
Rd ($\mu\text{mol/kg}\cdot\text{min}$)			
FFA (mmol/L)	0.96 [0.72-1.18]	1.26 [0.93-1.54]	0.015
Lipolysis ($\mu\text{mol/kg}\cdot\text{min}$)	4.1 [2.4-5.3]	4.1 [3.6-9.8]	0.3
Lipolysis ($\mu\text{mol/kcal}$)	194 [147-269]	259 [207-526]	0.004

Data are presented as median [minimum - maximum]. EGP = endogenous glucose production, Rd = rate of disappearance and FFA = free fatty acids. * During the clamp EGP was completely suppressed in both men and women.

Table 4 Glucoregulatory hormones and adiponectin

	basal state		P
	men (n = 10)	women (n = 10)	
Insulin (pmol/L)	18 [15-39]	19 [15-29]	0.6
Glucagon (ng/L)	75 [44-108]	66 [34-87]	0.6
Cortisol (nmol/L)	285 [207-467]	259 [177-357]	0.9
Epinephrine (nmol/L)	0.25 [0.11-0.53]	0.17 [0.08-0.30]*	0.11
Norepinephrine (nmol/L)	0.68 [0.34-1.52]	0.87 [0.54 - 4.45]*	0.17
Adiponectin ($\mu\text{g/ml}$)	7.7 [3.7-16.0]	16.0 [10.1-21.5]	0.005

Data are presented as median [minimum - maximum]. * n = 9.

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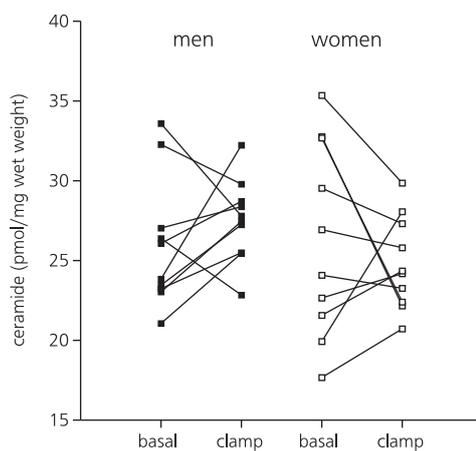


Figure 1 Muscle ceramide content in men (black boxes) and women (open boxes) in the basal state (men vs. women: P = 1.0) and during the hyperinsulinaemic euglycaemic clamp (men vs. women: P = 0.059).

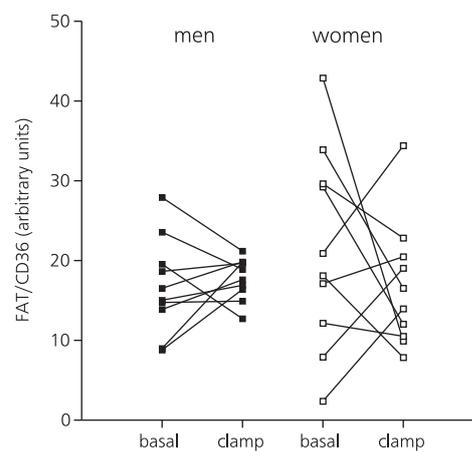


Figure 2 Protein levels of muscle FAT/CD36 content in men (black boxes) and women (open boxes) in the basal state (men vs. women: P = 0.4) and during the hyperinsulinaemic euglycaemic clamp (men vs. women: P = 0.4).

hyperinsulinaemic euglycaemic clamp		
men (n = 10)	women (n = 10)	P
4.9 [4.7-5.2]	5.1 [4.9-5.3]	0.14
_*	_*	-
46.8 [41.4-66.5]	48.5 [40.8-72.2]	0.9
<0.02	<0.02	-
0.7 [0.1-1.1]	0.9 [0.5-1.5]	0.16
39 [5-61]	50 [29-99]	0.11

hyperinsulinaemic euglycaemic clamp		
men (n = 10)	women (n = 10)	P
519 [477-624]	551 [433-644]	0.7
35 [26-86]	36 [15-51]	0.13
202 [78-311]	207 [95-278]	0.9
0.33 [0.15-0.90]	0.17 [0.05-0.26]	0.006
1.01 [0.44-3.10]	0.82 [0.56-2.12]	0.4
6.9 [3.4-15.5]	14.5 [9.3-19.7]	0.005

0.53; $P = 0.12$). No correlation was found between insulin-mediated peripheral glucose uptake and muscle ceramide or glucosylceramide levels in women and men (ceramide (females): $\rho = 0.14$; $P = 0.69$, ceramide (males): $\rho = -0.37$; $P = 0.29$ and glucosylceramide (females): $\rho = -0.05$; $P = 0.89$, glucosylceramide (males): $\rho = 0.48$; $P = 0.16$).

FAT/CD36 measurements

There were no differences in muscle FAT/CD36 content between women and men during both the basal state and the hyperinsulinaemic euglycaemic clamp (Figure 2). Also, there was no significant change in muscle FAT/CD36 content between the basal state and the end of hyperinsulinaemic euglycaemic clamp in both sexes (data not shown).

Discussion

We studied the metabolic adaptation to 38 h of fasting in healthy lean women and men to elucidate the mechanism behind the well-established finding of lower plasma glucose levels and higher plasma FFA during fasting in women.

We confirmed earlier reports on lower plasma glucose levels in women during fasting (1;4;6). The lower basal plasma glucose concentrations in women are probably attributed to the trend towards a lower EGP. Our findings are not in complete agreement with previous reports. Two studies showed equal EGP between women and men between 16 to 22 hours of fasting (11;13). In another study, the decline in EGP between 16 and 64 hours of fasting was greater in women than men but absolute EGP did not differ at both time points between sexes (4). The discrepancy with these studies in finding a trend towards a lower EGP might be explained by the fact that we studied more subjects (4;11;13). Moreover, EGP is the major determinant of basal plasma glucose concentration (38) and the MCR was not different between men and women in the basal state. This suggests that lower EGP plays a causal role in the lower plasma glucose levels in women. Notably, gender differences have been described in circulating gluconeogenic substrates during short-term fasting (1). The difference in EGP between men and women may be caused by differences in plasma concentrations of ovarian steroids since it was shown that in female ovariectomized mice, gluconeogenesis was higher compared to intact control mice (39). To our knowledge, there have been no other reports on gender differences in EGP after 38 h of fasting.

A remarkable finding in our study was the absence of a difference in peripheral insulin sensitivity, despite significantly higher plasma FFA in females after fasting for 38 h. So far, there have been no reports on gender differences regarding insulin sensitivity after short-term fasting. However, equal or higher insulin sensitivity in women after an overnight fast has been found (10;15;16;40).

The finding of higher plasma FFA probably results from a higher lipolytic flux. Lipolysis per kg body weight did not differ between sexes. However, if expressed as flux per REE, women displayed a higher rate of lipolysis (30;41). The most appropriate way to express lipolysis is still under discussion (30). The FFA flux can be seen as a function of adipose tissue mass, but also as a function of fatty acid-consuming tissue requirements. Koutsari et al. argue that expressing lipolysis per REE is a better alternative, since resting energy requirements are a major factor determining the rate of FFA release in resting humans (30).

Despite higher plasma FFA levels in the basal state, women were equally insulin-sensitive compared to men, suggesting a relative protection from FFA-induced insulin resistance. This finding confirms other reports. Perseghin et al. found no differences in insulin sensitivity between sexes, despite higher levels of plasma as well as intramyocellular triglycerides in women after an overnight fast (16). Frias et al. demonstrated that women were less sensitive to FFA-induced insulin resistance during a lipid infusion (12). The higher plasma FFA levels during short-term fasting in the women we studied, may result from a decreased FFA-uptake in skeletal muscle, caused by differences in skeletal muscle fatty acid transporter proteins. The uptake of FFA is thought to depend on, besides transmembrane concentrations, intracellular fatty acid metabolism, i.e. storage or oxidation (28;29). We did not find differences in fat oxidation (whole body or per kilogram lean body mass), which makes increased fatty acid oxidation in skeletal muscle in our female subjects less likely.

To detect potential differences in FFA-uptake and fatty acid handling we assessed muscle concentrations of ceramide and glucosylceramide. Ceramide and glucosylceramide levels in skeletal muscle did not differ between females and males in the basal state, suggesting that these are not causally involved in the finding of lower fasting plasma glucose in women. During the clamp, muscle ceramide levels (but not glucosylceramide) tended to be lower in women compared to men, despite similar Rd. Accordingly; we found no correlations between muscle ceramide or glucosylceramide and FFA or Rd.

There were no differences between women and men in total muscle FAT/CD36 content in the basal state and during the clamp, which is remarkable since it has been described earlier that women have higher levels of this lipid-binding protein after an overnight fast (8) which is in concordance with the findings that the intramyocellular lipid content during the post absorptive period in women is increased compared to men (16). Our data suggest that the initial differences in intramyocellular lipids(16) and fatty acid transporters after an overnight fast (8) are abolished after 38 h of fasting.

Intracellular stored FAT/CD36 is translocated to the plasma membrane, thereby promoting sarcolemmal long chain fatty acid uptake and this effect is stimulated by insulin (26). Whether a difference in FAT/CD36 activity (translocation, upregulation) during fasting exists between males and females is not known, but our data imply such differences exist. Unfortunately we did not measure the intracellular localization or activity of the FAT/CD36 protein, which could have clarified higher plasma FFA levels despite similar total levels of this transporter in skeletal muscle. (25-27;29).

Since we did find higher lipolysis rates in women without an increase in whole body fat oxidation, the key question remains how the increased plasma FFA in women are

disposed. Uptake of plasma FFA predominantly occurs in liver, adipocytes and muscle. Shadid et al showed that FFA recycling occurs in the post-absorptive state and that women display greater efficiency in direct FFA uptake in subcutaneous tissue (femoral area) (42). The exact role of the adipocyte in FFA uptake under fasting conditions is currently unknown. The liver may be another site of increased non-oxidative FFA disposal. Although livers of females do not contain more fat than male livers (43), it is known that female livers contain more FAT/CD36 and have a greater capacity for FFA uptake and synthesis of ketone bodies and very low-density lipoproteins-triglycerides (1;14;44). Despite our data on muscle ceramide and FAT/CD36, we cannot completely rule out increased FFA disposal in muscle of our female subjects because of earlier mentioned arguments on possible differences in FAT/CD36 location and activity.

It is unlikely that the difference in plasma epinephrine levels during the clamp can explain our findings, since the concentration threshold for an effect of epinephrine on glycaemia has been reported to be between 0.55 and 1.0 nmol/L (45-47).

Finally, women had higher plasma adiponectin levels than men, which may be another mechanism by which women are relatively protected from FFA-induced insulin resistance. It has been shown *in vitro* that adiponectin can stimulate muscle fatty acid oxidation via stimulation of AMP-kinase, which hypothetically may result in lower intramyocellular lipid-content with beneficial effects on peripheral insulin sensitivity (48). However, whole body fat oxidation did not differ between sexes. This challenges the hypothesis of an important role for adiponectin as protecting factor.

In conclusion, women display lower plasma glucose concentrations and higher plasma FFA concentrations after 38h of fasting. The former is at least in part explained by lower endogenous glucose production and the latter by higher rates of lipolysis. Moreover, women are relatively protected from FFA-induced insulin resistance. This protection does seem to result neither from differences in fat oxidation rates, muscle concentrations of ceramide and glucosylceramide, nor differences in total amount of FAT/CD36 in skeletal muscle. However, a difference in activity of this fatty acid binding protein or intracellular localization between fasting men and women may result in lower plasma FFA uptake in women, thereby increasing plasma FFA and decreasing deleterious effects of long chain fatty acids on insulin signaling.

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Reference List

1. Haymond MW, Karl IE, Clarke WL, Pagliara AS, Santiago JV. Differences in circulating gluconeogenic substrates during short-term fasting in men, women, and children. *Metabolism* 1982; 31(1):33-42.
2. Klein S, Sakurai Y, Romijn JA, Carroll RM. Progressive alterations in lipid and glucose metabolism during short-term fasting in young adult men. *Am J Physiol Endocrinol Metab* 1993; 265(5):E801-E806.
3. Norrelund H, Nielsen S, Christiansen JS, Jorgensen JO, Moller N. Modulation of basal glucose metabolism and insulin sensitivity by growth hormone and free fatty acids during short-term fasting. *Eur J Endocrinol* 2004; 150(6):779-787.
4. Clore JN, Glickman PS, Helm ST, Nestler JE, Blackard WG. Accelerated decline in hepatic glucose production during fasting in normal women compared with men. *Metabolism* 1989; 38(11):1103-1107.
5. Hojlund K, Wildner-Christensen M, Eshoj O, Skjaerbaek C, Holst JJ, Koldkjaer O, Moller Jensen D, Beck-Nielsen H. Reference intervals for glucose, {beta}-cell polypeptides, and counterregulatory factors during prolonged fasting. *Am J Physiol Endocrinol Metab* 2001; 280(1):E50-E58.
6. Merimee TJ, Fineberg SE. Homeostasis during fasting. II. Hormone substrate differences between men and women. *J Clin Endocrinol Metab* 1973; 37(5):698-702.
7. Binnert C, Koistinen HA, Martin G, Andreelli F, Ebeling P, Koivisto VA, Laville M, Auwerx J, Vidal H. Fatty acid transport protein-1 mRNA expression in skeletal muscle and in adipose tissue in humans. *Am J Physiol Endocrinol Metab* 2000; 279(5):E1072-E1079.
8. Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, Nielsen JN. Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. *J Appl Physiol* 2004; 97(4):1209-1218.
9. Blaak E. Gender differences in fat metabolism. *Curr Opin Clin Nutr Metab Care* 2001; 4(6):499-502.
10. Borissova AM, Tankova T, Kirilov G, Koev D. Gender-dependent effect of ageing on peripheral insulin action. *Int J Clin Pract* 2005; 59(4):422-426.
11. Corssmit EP, Stouthard JM, Romijn JA, Endert E, Sauerwein HP. Sex differences in the adaptation of glucose metabolism to short-term fasting: effects of oral contraceptives. *Metabolism* 1994; 43(12):1503-1508.
12. Frias JP, Macaraeg GB, Ofrecio J, Yu JG, Olefsky JM, Kruszynska YT. Decreased susceptibility to fatty acid-induced peripheral tissue insulin resistance in women. *Diabetes* 2001; 50(6):1344-1350.
13. Mittendorfer B, Horowitz JF, Klein S. Gender differences in lipid and glucose kinetics during short-term fasting. *Am J Physiol Endocrinol Metab* 2001; 281(6):E1333-E1339.
14. Mittendorfer B. Insulin resistance: sex matters. *Curr Opin Clin Nutr Metab Care* 2005; 8(4):367-372.
15. Paula FJ, Pimenta WP, Saad MJ, Paccola GM, Piccinato CE, Foss MC. Sex-related differences in peripheral glucose metabolism in normal subjects. *Diabetes Metab* 1990; 16(3):234-239.
16. Perseghin G, Scifo P, Pagliato E, Battezzati A, Benedini S, Soldini L, Testolin G, Del Maschio A, Luzi L. Gender factors affect fatty acids-induced insulin resistance in nonobese humans: effects of oral steroidal contraception. *J Clin Endocrinol Metab* 2001; 86(7):3188-3196.
17. Boden G. Interaction between free fatty acids and glucose metabolism. *Curr Opin Clin Nutr Metab Care* 2002; 5(5):545-549.



18. Boden G, Chen X, Ruiz J, White JV, Rossetti L. Mechanisms of fatty acid-induced inhibition of glucose uptake. *J Clin Invest* 1994; 93(6):2438-2446.
19. Straczkowski M, Kowalska I, Nikolajuk A, Dzienis-Straczkowska S, Kinalska I, Baranowski M, Zendzian-Piotrowska M, Brzezinska Z, Gorski J. Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle. *Diabetes* 2004; 53(5):1215-1221.
20. Adams JM 2nd, Pratipanawat T, Berria R, Wang E, DeFronzo RA, Sullards MC, Mandarinog LJ. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes* 2004; 53(1):25-31.
21. Powell DJ, Turban S, Gray A, Hajdich E, Hundal HS. Intracellular ceramide synthesis and protein kinase C ζ activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochem J* 2004; 382(Pt 2):619-629.
22. Chavez JA, Knotts TA, Wang LP, Li G, Dobrowsky RT, Florant GL, Summers SA. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *J Biol Chem* 2003; 278(12):10297-10303.
23. Tagami S, Inokuchi Ji J, Kabayama K, Yoshimura H, Kitamura F, Uemura S, Ogawa C, Ishii A, Saito M, Ohtsuka Y, Sakaue S, Igarashi Y. Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J Biol Chem* 2002; 277(5):3085-3092.
24. Aerts JM, Ottenhoff R, Powlson AS, Grefhorst A, van Eijk M, Dubbelhuis PF, Aten J, Kuipers F, Serlie MJ, Wennekes T, Sethi JK, O'rahilly S, Overkleeft HS. Pharmacological inhibition of glucosylceramide synthase enhances insulin sensitivity. *Diabetes* 2007;db06-1619.
25. Hamilton JA. Fast flip-flop of cholesterol and fatty acids in membranes: implications for membrane transport proteins. *Curr Opin Lipidol* 2003; 14(3):263-271.
26. Koonen DPY, Glatz JFC, Bonen A, Luiken JJFP. Long-chain fatty acid uptake and FAT/CD36 translocation in heart and skeletal muscle. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2005; 1736(3):163-180.
27. Schaffer JE. Fatty acid transport: the roads taken. *Am J Physiol Endocrinol Metab* 2002; 282(2):E239-E246.
28. Mashek DG, Coleman RA. Cellular fatty acid uptake: the contribution of metabolism. *Curr Opin Lipidol* 2006; 17(3):274-278.
29. Stremmel W, Pohl L, Ring A, Herrmann T. A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. *Lipids* 2001; 36(9):981-989.
30. Koutsari C, Jensen MD. Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. *J Lipid Res* 2006; 47(8):1643-1650.
31. Ackermans MT, Pereira Arias AM, Bisschop PH, Endert E, Sauerwein HP, Romijn JA. The quantification of gluconeogenesis in healthy men by (2)H₂O and [2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H₂O are higher than those measured with [2-(13)C]glycerol. *J Clin Endocrinol Metab* 2001; 86(5):2220-2226.
32. Ackermans MT, Ruiter AF, Endert E. Determination of glycerol concentrations and glycerol isotopic enrichments in human plasma by gas chromatography/mass spectrometry. *Anal Biochem* 1998; 258(1):80-86.
33. Groener JEM, Poorthuis BJHM, Kuiper S, Helmond MTJ, Hollak CEM, Aerts JMFG. HPLC for Simultaneous Quantification of Total Ceramide, Glucosylceramide, and Ceramide Trihexoside Concentrations in Plasma. *Clin Chem* 2007;([Epub ahead of print]):PMID: 17332150 [PubMed - as supplied by publisher].
34. Bonen A, Luiken JJFP, Arumugam Y, Glatz JFC, Tandon NN. Acute Regulation of Fatty Acid Uptake Involves the Cellular Redistribution of Fatty Acid Translocase. *J Biol Chem* 2000; 275(19):14501-14508.
35. Finegood DT, Bergman RN, Vranic M. Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps. Comparison of unlabeled and labeled exogenous glucose infusates. *Diabetes* 1987; 36(8):914-924.
36. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 1959; 82:420-30.:420-430.



37. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; 55(2):628-634.
38. Fery F. Role of hepatic glucose production and glucose uptake in the pathogenesis of fasting hyperglycemia in type 2 diabetes: normalization of glucose kinetics by short-term fasting. *J Clin Endocrinol Metab* 1994; 78(3):536-542.
39. Ahmed-Sorour H, Bailey CJ. Role of ovarian hormones in the long-term control of glucose homeostasis, glycogen formation and gluconeogenesis. *Ann Nutr Metab* 1981; 25(4):208-212.
40. Yki-Jarvinen H. Sex and insulin sensitivity. *Metabolism* 1984; 33(11):1011-1015.
41. Nielsen S, Guo Z, Albu JB, Klein S, O'Brien PC, Jensen MD. Energy expenditure, sex, and endogenous fuel availability in humans. *J Clin Invest* 2003; 111(7):981-988.
42. Shadid S, Koutsari C, Jensen MD. Direct Free Fatty Acid Uptake Into Human Adipocytes In Vivo: Relation to Body Fat Distribution. *Diabetes* 2007; 56(5):1369-1375.
43. Westerbacka J, Corner A, Tiikkainen M, Tamminen M, Vehkavaara S, Hakkinen AM, Fredriksson J, Yki-Jarvinen H. Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: implications for sex differences in markers of cardiovascular risk. *Diabetologia* 2004; 47(8):1360-1369.
44. Magkos F, Patterson BW, Mohammed BS, Klein S, Mittendorfer B. Women Produce Fewer but Triglyceride-Richer Very Low-Density Lipoproteins than Men. *J Clin Endocrinol Metab* 2007; 92(4):1311-1318.
45. Chu CA, Sindelar DK, Neal DW, Cherrington AD. Direct effects of catecholamines on hepatic glucose production in conscious dog are due to glycogenolysis. *Am J Physiol* 1996; 271(1 Pt 1):E127-E137.
46. Clutter WE, Bier DM, Shah SD, Cryer PE. Epinephrine plasma metabolic clearance rates and physiologic thresholds for metabolic and hemodynamic actions in man. *J Clin Invest* 1980; 66(1):94-101.
47. Cryer PE. Adrenaline: a physiological metabolic regulatory hormone in humans? *Int J Obes Relat Metab Disord* 1993; 17 Suppl 3:S43-6; discussion S68.:S43-S46.
48. Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH, Kim JB. Adiponectin Increases Fatty Acid Oxidation in Skeletal Muscle Cells by Sequential Activation of AMP-Activated Protein Kinase, p38 Mitogen-Activated Protein Kinase, and Peroxisome Proliferator-Activated Receptor { alpha }. *Diabetes* 2006; 55(9):2562-2570.

