Sphingolipid Content of Human Adipose Tissue: Relationship to Adiponectin and Insulin Resistance

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Ceramides (Cer) are implicated in obesity-associated skeletal muscle and perhaps adipocyte insulin resistance. We examined whether the sphingolipid content of human subcutaneous adipose tissue and plasma varies by obesity and sex as well as the relationship between ceramide content and metabolic indices. Abdominal subcutaneous adipose biopsies were performed on 12 lean adults (males = 6), 12 obese adults (males = 6) for measurement of sphingolipid content and activity of the main ceramide metabolism enzymes. Blood was sampled for glucose, insulin (to calculate homeostasis model assessment-estimated insulin resistance (HOMAᵦᵢ)) adiponectin, and interleukin-6 (IL-6) concentrations. Compared to lean controls, total ceramide content (pg/adipocyte) was increased by 31% (P < 0.05) and 34% (P < 0.05) in obese females and males, respectively. In adipocytes from obese adults sphingosine, sphinganine, sphingosine-1-phosphate, C14-Cer, C16-Cer, and C24-Cer were all increased. C18:1-Cer was increased in obese males and C24:1-Cer in obese females. For women only, there was a negative correlation between C16-Cer ceramide and plasma adiponectin (r = –0.77, P = 0.003) and a positive correlation between total ceramide content and HOMAᵦᵢ (r = 0.74, P = 0.006). For men only there were significant (at least P < 0.05), positive correlations between adipocyte Cer-containing saturated fatty acid and plasma IL-6 concentration. We conclude that the sexual dimorphism in adipose tissue behavior in humans extends to adipose tissue sphingolipid content its association with adiponectin, IL-6 and insulin resistance.
inhibit GLUT4 expression is sphingomyelin hydrolysis (24). In ob/ob mice the hyperinsulinemia and elevated TNF-α associated with obesity may increase the expression of three major ceramide production enzymes: nSMase, aSMase, and SPT in adipose tissue. Despite greater mRNA of above mentioned enzymes, the ceramide and sphingomyelin content/g of adipose tissue protein in ob/ob mice is reduced compared to their lean counterparts (23). However, diet-induced obesity in mice is associated with increased adipose tissue ceramide content (27).

We found only two reports on the ceramide content of human subcutaneous adipose tissue. Ceramide concentrations were greater in adipose tissue of obese women with fatty liver than in BMI-matched obese individuals with no hepatic steatosis (22). However, another group found total adipose tissue ceramide content was less in adipose tissue from obese than lean adults despite greater mRNA levels of SMases, SPT, and C-Dases (28). The aim of the present study was to examine the effect of obesity and sex on sphingolipid content in human fat subcutaneous abdominal tissue and to understand whether relationships exist between adipokine concentrations, insulin sensitivity, and adipose tissue Cer.

**METHODS AND PROCEDURES**

Twenty-four adults participated in this study. The volunteers included six lean (BMI <25) males, six lean females (n = 6), six obese (BMI > 30) males, and six obese females. All participants were healthy, nonsmokers, and were taking no medications, including oral contraceptives. All participants gave written, informed consent before entering the study.

**Body composition**

Total body and regional fat and lean body mass were assessed using dual-energy X-ray absorptiometry (Lunar Radiation, Madison, WI) combined with a single-slice abdominal computed tomography scan at the L2-L3 interspace (29).

**Adipose tissue biopsies**

Subcutaneous abdominal adipose tissue was obtained from just lateral to the umbilicus using a needle liposuction technique with sterile conditions and local anesthesia. Samples were rinsed with saline through Nitex Nylon Fiber 250/50 and frozen at –70 °C before analysis. The volunteers were in the postabsorptive state after an overnight fast.

**Blood samples**

Arterialized venous blood was obtained using the heated hand vein technique within 30 min of the adipose tissue biopsy. We measured glucose, insulin, TNF-α, IL-6, adiponectin, free-fatty acid, triacylglycerols, total cholesterol and high-density lipoprotein-cholesterol. The plasma samples were stored at –80 °C before analysis.

**Adipose tissue and plasma sphingolipids**

The content of sphingolipids was measured using a UPLC/MS/MS approach. Briefly, the adipose tissue samples (40mg) were homogenized in a solution composed of 0.25 mol/l sucrose, 25 mmol/l KCl, 50 mmol/l Tris, and 0.5 mmol/l EDTA, pH 7.4. Immediately afterwards 10 µl of the internal standard solution (17C-sphingosine and 17C-SIP, and 17C16-Cer Avanti Polar Lipids) as well as 1.5 ml of an extraction mixture (isopropanol:water:ethyl acetate, 30:10:60; vol:vol:vol) were added to each homogenate. The mixture was vortexed, sonicated, and then centrifuged for 10 min at 4,000 rpm (Sorvall Legend RT). The supernatant was transferred to new tube and pellet was re-extracted. After centrifugation, supernatants were combined and evaporated under nitrogen. The dried sample was reconstituted in 100 µl of LC Solvent A (2 mmol/l ammonium formate, 0.15% formic acid in methanol) for UPLC/MS/MS analysis. Plasma sphingolipids were measured using a similar approach requiring 100 µl of plasma.

**Enzyme activity assays**

The activities of nSMase and aSMase were determined according to Liu and Hannun (30) using [N-methyl-1-14C]-sphingomyelin (Perkin-Elmer Life Sciences, Waltham, MA) as the radiolabeled substrate. The product of reaction—14C-choline phosphate—was extracted with CHCl3/methanol (2:1, vol/vol), transferred to scintillation vials and counted using a Packard TRI-CARB 1900 TR scintillation counter. The activity of neutral (nCDase), and acid (aCDase) ceramidase was measured by the method of Nikolova-Karakashian and Merrill (31) using radiolabeled [N-palmitoyl-1-14C]—sphingosine (Moravek Biochemicals, Brea, CA) as a substrate. Unreacted Cer and liberated 14C-palmitate were separated with basic Dole solution (isopropanol/heptane/1 mol/l NaOH, 40:10:1, vol/vol/vol). Radioactivity of the 14C-palmitate was measured by scintillation counting. SPT activity was measured as described by Merrill (32) using [1H]-L-serine (Moravek Biochemicals) as the labeled substrate. Briefly, microsomal fraction was obtained by ultracentrifugation at 150,000g for 40 min. Microsomes were incubated for 10 min at 37 °C in the reaction buffer (100 mmol/l HEPES (pH 8.3), 5 mmol/l DTT (dithiothreitol), 2.5 mmol/l EDTA (pH 7.0), 50 mmol/l pyridoxal phosphate, 200 mmol/l palmitoyl-CoA, and 2 mmol/l L-serine, 44,000 dpm/nmol). The labeled lipid product 3-ketosphinganine was extracted with CHCl3/methanol (1:2, vol/vol), and the radioactivity was measured by scintillation counting.

**Adipocyte size**

Adipocyte size (µg lipid/cell) was assessed using the approach of Di Girolamo et al. (33) which involves collagenase digestion of the adipose tissue sample, separation of adipocytes by centrifugation, methylene blue staining to identify nuclei, and measurement of cell diameter (33).

**Adipose tissue total cell number**

The total number of cells per gram of tissue was assessed using the DNA content of tissue, assuming that 1 ng of DNA corresponds to 150 cells. Frozen, powdered fat tissue was homogenized in 800 µl ice-cold radioimmunoprecipitation assay buffer composed of 25 mmol/l Tris–HCl, pH 7.6, 150 mmol/l NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS. The homogenate was centrifuged for 5 min at 10,000 rpm/min and lipid layer was removed by aspiration. DNA concentration was measured in homogenate using Quant-it Pico-Green (Invitrogen, Carlsbad, CA) according manufacturer’s instructions. DNA concentrations were calculated using a standard curve of fluorescence from known concentration of the bacteriophage lambda DNA.

**Number of adipocyte and nonadipocyte cells**

The number of adipocytes was calculated by dividing the fat content per gram adipose tissue by the mean adipocyte lipid content (µg/cell). The number of nonadipocytes was calculated by subtracting the number of adipocytes from the total cell number.

**Plasma metabolites**

Plasma glucose concentrations were measured using a glucose analyzer (Beckman Instruments, Fullerton, CA). Insulin concentrations were measured using a chemiluminescence method (Access Immuneassay, Beckman, Chaska, MN). Total cholesterol, high-density lipoprotein-cholesterol, and triglyceride concentrations were measured with enzymatic colorimetric methods (Roche Diagnostics, Indianapolis, IN). Plasma adiponectin concentrations were measured using the human adiponectin double antibody radioimmunooassay kit (Linco Research, St Louis, MO). Plasma IL-6 and TNF-α concentrations were measured using a quantitative two-site enzyme immunoassays from R&D Systems (Minneapolis, MN).
Statistical analysis
The statistics were performed with PASW Statistics 17.0 software (SPSS, Chicago, IL). All data are presented as means ± SD. Data were analyzed by one-way analysis of variance, followed by Tukey–Kramer post hoc test or Games–Howell post hoc test (in the case of uneven variances). P values <0.05 were taken to indicate statistical significance.

RESULTS

Subject characteristics
The characteristics of the participants are provided in Table 1. As expected, percent body fat, regional fat masses, and fat cell size were greater in obese female and male groups compared to the corresponding lean groups. We also saw the well-known sex differences in body composition with respect to regional fat masses. In addition, abdominal adipocytes were significantly larger in lean women than lean men. Plasma insulin concentrations were significantly greater in obese than lean females and males. There were no sex differences in fasting plasma insulin concentrations and no statistically significant differences in plasma glucose or total plasma cholesterol concentrations between any of the groups. High-density lipoprotein-cholesterol and adiponectin concentration were reduced whereas plasma triglyceride and IL-6 concentrations were significantly greater in the obese compared to lean groups. Adiponectin was significantly less and plasma IL-6 was significantly greater in men than women (lean or obese). There were no significant differences in plasma TNF-α concentration among groups. Lean men and women were more insulin sensitive than the corresponding obese groups as assessed by HOMA.

Adipose sphingolipids
Adipocyte sphinganine, sphingosine and sphingosine-1-phosphate (S1P) content was greater (P < 0.001) in obese than lean volunteers (Table 2). The C14-Cer and C16-Cer content was significantly greater in obese than lean males and females counterparts. The adipocyte C18:1-Cer content was greater in obese than lean males (P < 0.01), but it was lower in obese females than obese males (P < 0.001). The C18-Cer content of adipocytes did not differ significantly between obese and lean groups, however, it was significantly lower in obese females than obese males. Adipocyte C20-Cer content was not significantly different between any of the groups, whereas C24:1-Cer was greater (P < 0.01) in obese than lean females. C24:1-Cer was greater (P < 0.05) in lean females than lean males and greater (P < 0.001) in obese females than obese males. The content of C24:1-Cer increased in obese males (P < 0.05) and females (P < 0.01) compared to lean men and women. As expected from the analysis of individual Cer, the total ceramide content was greater in obese females (P < 0.05) and males (P < 0.05) than their lean counterparts.

Plasma sphingolipids
Plasma sphingosine-1-phosphate content was greater in obese males and females (both P < 0.01) compared to their lean counterparts.

Table 1. Anthropometric and biochemical parameters in the studied groups

|                      | Lean females | Lean males | Obese females | Obese males |
|----------------------|--------------|------------|---------------|-------------|
| Number               | 6            | 6          | 6             | 6           |
| Age                  | 37 ± 11      | 31 ± 9     | 41 ± 4        | 37 ± 7      |
| Body mass (kg)       | 66.4 ± 8.5   | 77.7 ± 12.7| 89.5 ± 12.2   | 111.2 ± 14.2|
| BMI (kg/m²)          | 23 ± 1.7     | 24 ± 1.7   | 33 ± 3.7d     | 34 ± 2.4d   |
| Body fat%            | 32 ± 5       | 19 ± 6***  | 45 ± 6d       | 33 ± 55a    |
| UBSQ fat (kg)        | 11.3 ± 2.2   | 7.2 ± 3.3* | 19.4 ± 3.8d   | 19.8 ± 3.6d |
| Visc fat (kg)        | 1.1 ± 1.0    | 1.7 ± 0.4**| 4.3 ± 2.8d    | 5.6 ± 2.0a  |
| FFM (kg)             | 44.5 ± 6.6   | 62.5 ± 7.8*| 48.3 ± 8.8    | 72.8 ± 9.2* |
| FCS (µg/cell)        | 0.44 ± 0.23  | 0.35 ± 0.16*| 0.62 ± 0.26c  | 0.69 ± 0.18d|
| Insulin (µU/ml)      | 3.6 ± 1.8    | 3.6 ± 1.6  | 9.7 ± 3.5c    | 10.5 ± 7.1c |
| Glucose (mg/dl)      | 89 ± 5       | 87 ± 6     | 93 ± 7        | 100 ± 9     |
| Cholesterol (mg/dl)  | 164 ± 15     | 163 ± 21   | 175 ± 42      | 172 ± 41    |
| HDL-cholesterol (mg/dl) | 70 ± 13   | 54 ± 6*    | 56 ± 18a      | 43 ± 15c**  |
| TG (mg/dl)           | 67 ± 12      | 59 ± 6     | 133 ± 70i     | 139 ± 42i   |
| FFA (µmol/l)         | 414 ± 90     | 389 ± 151  | 495 ± 137a    | 429 ± 134   |
| TNF-α (pg/ml)        | 1.06 ± 0.50  | 0.82 ± 0.3 | 0.74 ± 0.08   | 0.85 ± 0.15 |
| ADIP (ng/ml)         | 10,443 ± 5,078 | 8,319 ± 3,628** | 6,684 ± 2,736c | 3,297 ± 1,509i*** |
| IL-6 (pg/ml)         | 1.17 ± 0.17  | 1.92 ± 1.25*** | 1.62 ± 0.28i  | 2.2 ± 1.56*** |
| HOMAIRα              | 1.26 ± 0.72  | 0.81 ± 0.40 | 3.48 ± 2.32   | 2.96 ± 1.68  |

Values are expressed in pg per adipocyte (mean ± SD).
ADIP, adiponectin; FCS, fat cell size; FFA, free-fatty acid; FFM, fat free mass; HOMAIR, homeostasis model assessment-estimated insulin resistance; IL-6, interleukin-6; TG, plasma triglyceride concentrations; TNF-α, tumor necrosis factor-α; UBSQ, upper body subcutaneous fat mass.
*P < 0.05; **P < 0.02; ***P < 0.01; ****P < 0.001 vs. the same sex lean group; "P < 0.05; ""P < 0.01; """"P < 0.001 vs. lean or obese group but opposite sex.
counterparts (Table 3). Total plasma ceramide content was greater in both obese males and females (all \( P < 0.001 \)) compared to the lean groups. Specifically, the concentrations of C14-Cer (\( P < 0.001 \)), C16-Cer, C18-Cer, C18:1-Cer (all \( P < 0.01 \)), and C24:1-Cer (\( P < 0.001 \)) in plasma were significantly greater in obese men and women (all \( P < 0.05 \)) than in their lean counterparts.

Ceramide metabolism enzyme activity
SPT activity (males \( P < 0.05 \), females \( P < 0.02 \)) and nSMase activity (both sexes \( P < 0.05 \)) were significantly greater in both obese groups as compared to the same sex lean group (Table 4). There were no significant differences in aSMase activity between lean and obese groups. The activity of nCDase increased in the obese females (\( P < 0.05 \)) compared to lean females. There were no significant differences in aCDase activity between lean and obese groups.

In general, the activities of adipose tissue SPT, nSMase, nCDase, and aCDase were correlated (all \( r > 0.6 \), \( P < 0.005 \)). We found a strong positive correlation between total ceramide content and both SPT (\( r = 0.72 \), \( P < 0.0002 \)) and nSMase (\( r = 0.59 \), \( P < 0.004 \)) activities. Surprisingly, similar positive relationships were observed between total adipose tissue ceramide and nCDase (\( r = 0.72 \), \( P < 0.0002 \)) and aCDase (\( r = 0.56 \), \( P < 0.006 \)) activities. When tested separately by sex, the correlations between total ceramide content and enzyme activities were stronger in females than males.

Associations between sphingolipids and obesity markers
We found a negative correlation (\( r = -0.73 \), \( P = 0.007 \)) between total adipocyte ceramide and plasma adiponectin concentration in females (Figure 1a). The strongest correlation was noticed with C16-Cer (\( r = -0.77 \), \( P = 0.004 \)). There was also a negative correlation between adipocyte total ceramide content and fasting plasma insulin concentrations (\( r = 0.85 \), \( P = 0.0005 \)) as well as ceramide and homeostasis model assessment-estimated insulin resistance (HOMA\(_{IR}\)) (\( r = 0.74 \), \( P = 0.006 \)) in females (Figure 1b). No such associations were detected adipocyte ceramide content in men. There were positive correlations between Cer containing saturated fatty acid (C16:0-Cer, C18:0-Cer and C20:0-Cer) and plasma IL-6 concentration (\( r = 0.57 \), \( P = 0.05 \); \( r = 0.65 \), \( P = 0.02 \) and \( r = 0.62 \), \( P = 0.03 \) respectively) in males, but not females.

There was a positive correlation between plasma total ceramide and IL-6 concentrations (\( r = 0.68 \), \( P = 0.02 \)) and between total ceramide content in plasma and insulin concentration (\( r = 0.65 \), \( P = 0.03 \)) in females. For men, there was a significant correlation between plasma total ceramide and TNFα concentrations (\( r = 0.59 \), \( P = 0.05 \)) and total plasma ceramide concentration and HOMA\(_{IR}\) (\( r = 0.61 \), \( P = 0.04 \)).

**DISCUSSION**
Our goal was to understand whether adipocyte ceramide content is altered in human obesity and, if so, whether there was any association between adipocyte Cer and adipokines or insulin resistance. We collected abdominal subcutaneous
adipose tissue from 24 lean and obese adults for measurement of fat cell size and number, total cell number, as well as ceramide content. Ceramide content was significantly greater in obese than lean men and women. In women, adipocyte ceramide content correlated with hyperinsulinemia and adiponectin, whereas in men it correlated with IL-6. It is possible that accumulation of Cer in adipocytes in obese humans contributes to adipocyte dysfunction, perhaps in a sex-specific manner. An association between adiponectin and ceramide metabolism has been reported (34,35), but the directionality is not entirely clear. For example, progestin and adiponectin receptors are responsible for production of sphingoid bases through the activation of ceramidase in yeast (34) and transfection with cDNAs encoding adiponectin receptors into HEK-293-T enhaced ceramidase activity. Moreover, adiponectin reduces ceramide and glucosylceramide content in the liver of mice and a negative correlation between plasma and cardiomyocyte ceramide concentrations and adiponectin concentration has been reported (35). In these experiments, we found a negative relationship between adipocyte ceramide content and plasma adiponectin concentrations in woman. The apparent co-regulation of enzymes involved in both ceramide synthesis and degradation makes it difficult to speculate on a causal relationship between adiponectin production and action as regards ceramide metabolism in human adipocytes.

There are few reports on the sphingolipid content of human adipose tissue (22). It was reported that ceramide (C22:0-Cer and C24:1-Cer) concentration/g of subcutaneous adipose tissue in women with fatty liver was greater than in BMI-matched women with no liver steatosis (22). Another study indicated that obesity with and without diabetes is associated with decreased adipose tissue ceramide content (28). Moreover, a positive correlation between ceramide content and plasma adiponectin concentrations and negative correlation between total ceramide content and HOMA IR has been demonstrated (28). In ob/ob mice total adipose tissue ceramide content was reduced compared to the lean animals (23). However, the means of data expression may well explain the discrepancy between our findings and those mentioned above. Previous investigators express adipose tissue sphingolipid content per mg of adipose tissue protein, whereas we expressed it per adipocyte. It may be that, because the protein content of adipose tissue is an imperfect reflection of the number of adipocytes, previous investigators underestimated the ceramide burden of adipocytes. Our findings are consistent with those of Wu et al. (36), who reported total adipocyte ceramide (expressed pmol/µg DNA) was increased in old, insulin resistant mice compared to young insulin sensitive animals. We suggest that expressing ceramide content and enzyme activity per adipocyte may be the most appropriate, because numbers and size of adipocytes differs in obese and lean subjects. Because obese adults typically have fewer but larger adipocytes per mg tissue (or per mg of tissue protein), the ceramide content from these lesser number of cells would be diluted even though the content per cell was greater.
The greater ceramide content in adipocytes from obese volunteers that we observed is likely the result of increased HOMAIR, homeostasis model assessment-estimated insulin resistance. Total ceramide content in white adipose tissue (lower panel) in females.

In both obese men and women, the Cer species that were most increased were those containing saturated fatty acids: C14-Cer, C16-Cer and C24-Cer. Of interest, there were a number of subspecies of Cer that displayed sex differences as regards the difference between obese and lean participants. It is possible that the greater C18:1-Cer concentrations offset potential downregulation of adiponectin by other species (obese men) or greater C24:1-Cer is uniquely disadvantageous (obese women). We could find no data regarding specificity of the various species of Cer with regards to regulation of adipocyte metabolism or the association between adipocyte Cer and adiponectin. However, the relationship between adipocyte Cer and IL-6 in men is consistent with the observation that 3T3-L1 adipocytes treated with Cer show significant induction of IL-6 expression (23). Our observations raise the possibility that the secretion of adipokines is regulated differently in men and women, and that these processes may be related to adipose tissue saturated fatty acid ceramide content.

Greater total ceramide content in plasma and adipose tissue of obese mice has been reported (27), although the predominant species that were increased were the long chain Cer. Similarly, we noticed the greatest elevation in C14-Cer, C16-Cer and C24-Cer in adipocytes and C14-Cer, C16-Cer, C18:1-Cer, C18-Cer, and C24:1-Cer in plasma.

Obesity is associated with a state of chronic low-level inflammation (37,38), which likely contributes to ceramide accumulation. Our findings that plasma Cer are elevated in obese adults (Table 3) is consistent with previous studies (23,39). There is some evidence for causal, not mere correlational links, between Cer and obesity-related disorders. The administration of myriocin (an inhibitor of de novo ceramide biosynthesis) decreased plasma Cer, body fat mass and improved metabolic and inflammatory parameters in obese mice (40).

In conclusion, this is the first report on ceramide metabolism in human subcutaneous adipose tissue of lean and obese males and females. We found elevated adipocyte content of particular ceramide species, sphinganine, sphingosine, and sphingosine-1-phosphate. In females, but not males, there was a strong negative correlation between total adipocyte ceramide content and plasma adiponectin concentrations and a positive correlation between total adipocyte ceramide content and HOMA_m. Taken together elevated adipose tissue sphingolipid content is associated with hypo-adiponectinemia and insulin resistance in women. However, it remains to be shown whether the increased ceramide content in adipocyte from obese adults are responsible for adipose tissue dysfunction. The ability to specifically modulate ceramide content, and especially those of various subspecies, would allow investigators to address this question.
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DISCLOSURE
The authors declared no conflict of interest. See the online ICMJE Conflict of Interest Forms for this article.

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