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Skeletal Muscle Ceramides and Daily Fat Oxidation in Obesity and Diabetes

Nicholas T. Broskey[1], **Diana N. Obanda**[1], **Jeffrey H. Burton**[1], **William T. Cefalu**[1], and **Eric Ravussin**[1]

[1]Pennington Biomedical Research Center, Baton Rouge, LA 70808

Abstract

Background/objectives—Ectopic accumulation of lipids in skeletal muscle and the formation of deleterious lipid intermediates is thought to contribute to the development of insulin resistance and type 2 diabetes mellitus (T2DM). Similarly, impaired fat oxidation (metabolic inflexibility) are predictors of weight gain and the development of T2DM; however, no study has investigated the relation between muscle ceramide accumulation and 24-hour macronutrient oxidation. The purpose of this study was to retrospectively explore the relationships between whole body fat oxidation and skeletal muscle ceramide accumulation in obese non-diabetic individuals (ND) and in people with obesity and T2DM.

Methods—Daily substrate oxidation was measured in a respiratory chamber and skeletal muscle ceramides were measured using liquid chromatographyelectrospray ionization tandem-mass spectrometry.

Results—After adjusting for sex, age, and BMI no differences existed between the groups for fat oxidation or 24-h RQ. However, ceramides C18:1, C:20, C:22, C:24 and C:24:1 were significantly higher in people with T2DM compared to ND whereas no differences existed for C:16 and C:18. Despite low amounts of muscle ceramides, fat oxidation rates were positively associated with ceramide species concentration in ND only. Our data suggests that ceramides do not interfere with whole-body fat oxidation in ND individuals whereas a persistent lipid oversupply results in excessive ceramide muscle accumulation in people with T2DM.

Keywords

lipotoxicity; energy expenditure; type 2 diabetes

Corresponding Author: Eric Ravussin, PhD, FTOS (eric.ravussin@pbrc.edu), Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

N.T.B. researched data and wrote the manuscript. D.N.O. researched data and reviewed the manuscript. J.H.B. provided statistical support. W.T.C. researched data and reviewed/edited the manuscript. E.R. researched data and reviewed/edited the manuscript.

1. Introduction

Lipotoxicity interferes with insulin signaling and plays an important role in the development of metabolic disorders, particularly Type 2 diabetes mellitus (T2DM) [1]. Various lipid species have been measured in obese and TD2M individuals and ceramides have been proposed as one of the culprits for the induction of metabolic disorders [2]. Ceramides are bioactive precursors acting as central metabolic points in sphingolipid biosynthesis and breakdown [3] and increased muscle accumulations in obese [4] and insulin resistant [5] individuals have been hypothesized to trigger insulin resistance, even if the exact mechanisms have yet to be fully elucidated. Substrate competition between fat and glucose has long been thought to play a central role in the development of insulin resistance [6]. Dysregulation of fatty acid metabolism is a hallmark of the progression towards T2DM starting with a diminished capacity to oxidize fatty acids [7] leading with increased lipids stores and further insulin resistance [8]. Such oversupply of lipids in skeletal muscle could lead to an increased amount of ceramides and mechanistically explain the observed impaired insulin action in obese and insulin resistant subjects. We therefore conducted this cross-sectional analysis to investigate whether the variability in 24-hour substrate oxidation is associated to the variability in the concentration of ceramide species in obese individuals with or without T2DM.

2. Material and Methods

2.1 Subjects

Muscle samples primarily for the determination of the insulin signaling cascade and gene expression from three studies conducted at Pennington Biomedical Research Center from 2007–2013 were analyzed for ceramides species. Data were obtained from 106 research participants with obesity and T2DM (N=44) or obesity and non-diabetic patients (ND) (N=62) from Chromium II (NCT00398853), EAT (NCT01672632) and BARIA (NCT00936130) studies. T2DM was confirmed at screening. Only subjects on lifestyle therapy or whose fasting glucose was < 180 mg/dL were included in the T2DM group. All procedures were approved by the Pennington Biomedical Institutional Review board, and all participants provided written, informed consent.

2.2 Body Composition

Fat-free mass, fat mass and percent body fat were measured by dual-energy X-ray absorptiometry (DXA, QDR 4500A; Hologics, Bedford, MA).

2.3 Blood Analytes

Glucose was measured using a glucose oxidase electrode on a Beckman Coulter DXC600 (Brea, CA) instrument whereas insulin was assayed by enzyme immunoassay on a Siemens 2000 (Los Angeles, CA) instrument with an intra and inter-rate CV of 3.8% and 4.2%. Triglycerides were measured using an enzymatic method on a Beckman Coulter Synchron CX7 (Brea, CA).

2.4 Magnetic Resonance Spectroscopy

Intraymyocellular lipid (IMCL) and intrahepatic lipid (IHL) content were measured using ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) on a 3.0T whole body imaging and spectroscopy system (General Electric Medical Systems, Milwaukee, WI) as previously described [9, 10].

2.5 Metabolic Chamber

Twenty-four-hour energy expenditure and substrate oxidation were measured in a whole-room indirect calorimeter as previously described [11]. Energy intake was provided to match energy expenditure according to predictive equations based on fat-free mass, fat mass, age, and sex [11]. Concentrations of O_2 and CO_2 in the chamber were measured throughout the chamber stay, from which oxygen consumption, carbon dioxide production, and therefore energy expenditure and substrate oxidation were calculated every 10 s and the values were plotted at 10-min intervals. Energy expenditure was calculated from VO_2, VCO_2, and 24-hour urinary nitrogen excretion [12].

2.6 Quantification of Lipid Metabolites

Skeletal muscle tissue was collected using the technique described by Bergstrom [13], flash frozen and stored at −80 °C. Muscle tissue (~25 mg) was homogenized in deionized water and protein was determined by Bio-Rad protein assay kit (Bio-Rad laboratories, Inc. Hercules, CA). Using the Folch extraction method under acidified conditions, extraction standards (unnatural derivatives) C17:0 ceramide, dC17:1 sphingosine, and C17:0 sphingomyelin were added and each sample was subjected to a double extraction for lipids. Liquid chromatographyelectrospray ionization tandem-mass spectrometry (LC-MSMS) was used to measure intracellular levels of sphingolipids on a Waters Acquity UPLC using a Waters Aquity Xevo triple quadruple MS/MS detector with an ion source ESI operated in the positive mode. According to the retention times of standards, common product ion and ions reflecting fatty acid substituents, all target compounds and ceramides were quantified as previously described [14]. Sphingosine was quantified as a ceramide catabolism product. All values were standardized by protein level in the muscle.

2.7 Statistical analyses

All analyses were performed using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA) with a significance level set at α=0.05. Participants' characteristics were summarized as Mean ± SD for each group and group comparison was done by two-sample t-tests. All outcomes related to fat oxidation, respiratory quotient (RQ), and ceramide species content were compared between T2DM and ND groups via two-sample t-tests of least squares means from linear fixed effects models incorporating covariates for sex, age, and BMI. Additionally, within-group Spearman correlations of ceramide content with substrate oxidation and RQ were estimated and tested by one-sample t-tests.

3. Results

Subjects' characteristics are listed in Table 1. T2DM and ND groups were balanced for gender, and not significantly different in weight, BMI, and percent body fat (p=0.74, p=0.63, p=0.52, respectively). Participants with T2DM were older than ND (p<0.001). FFA and triglycerides were higher in T2DM than ND (p=0.0004 & p<.0001). As expected glucose, insulin, and subsequently HOMA-IR were higher in T2DM than ND (p<.0001, p=0.01, p=0.002, respectively). In a subset (two of the three studies that had this data) of 42 non-diabetic patients and 25 T2DM patients, although trending to be higher in T2DM than ND, similar amounts of IMCL in the soleus (p=0.34) and the tibialis (p=0.16) and IHL were present.

Group differences in ceramide species after adjusting for sex, age, and BMI are shown in Figure 1. In Figure 1 Panel A, T2DM had significantly higher amounts of species C18:1 (p=0.002), C20, C22, C24:1, C24, (all p<0.0001) than ND but not for species C16 and C18 (p=0.09 and p=0.83, respectively). In Figure 1 Panel B, total ceramide content was higher in T2DM than ND by two-fold as well as total sphingosine (p<0.0001 and p=0.003, respectively).

In ND only, there were significant positive correlations between fat oxidation and species C16 (ρ=0.54, p<.0001), C18 (ρ=0.56, p<.0001), C18:1 (ρ=0.48, p=0.0001), C:20 (ρ=0.44, p=0.001), C:22 (ρ=0.35, p=0.01), C24 (ρ =0.43, p=0.001) and total ceramides (ρ=0.48, p=0.0002), but not 24:1 (ρ=0.11, p=0.42) whereas none existed for C16 (ρ=0.11, p=0.48), C18 (ρ=0.20, p=0.21), C18:1 (ρ=0.06, p=0.71), C:20 (ρ=0.02, p=0.92), C:22 (ρ=0.01, p=0.94), C24 (ρ=0.004, p=0.98), C24:1 (ρ= −0.16, p=0.32) and total ceramides (ρ=0.002. p=0.99) in T2DM. Similarly, significant inverse relationships existed between 24-hour RQ and C16 (ρ= −0.54, p<.0001), C18 (ρ= −0.50, p<.0001), C18:1 (ρ= −0.49, p=<.0001), C:20 (ρ= −0.43, p=0.0004), C:22 (ρ= −0.30, p=0.02), C24 (ρ =−0.41, p=0.001) and total ceramides (ρ= −0.44, p=0.0003) but not C24:1 (ρ= −0.07, p=0.61) in ND, but not C16 (ρ= −0.18, p=0.23), C18 (ρ= −0.22, p=0.15), C18:1 (ρ= −0.02, p=0.91), C:20 (ρ= −0.10, p=0.50), C:22 (ρ= −0.17, p=0.26), C24 (ρ =−0.20, p=0.19), C24:1 (ρ= −0.11, p=0.49) and total ceramides (ρ= −0.19, p=0.22) in T2DM.

4. Discussion

Ceramides are thought to be involved in the development of insulin resistance and have been shown to be increased in obesity [4] and T2DM [5]. Impairments in fat oxidation are present during the development of obesity and T2DM and may contribute to lipid accumulation [7, 15]. No one has yet explored whole-body substrate oxidation in relation to muscle ceramide species accumulation. Here for the first time, we report positive associations between ceramides and whole-body oxidation in ND individuals but not in those with T2DM.

Our data supports higher amounts of total ceramides in T2DM including all species except for C16 and C18. Although we did not observe any differences in C16 and C18 in our cohort, we have previously reported higher amounts of both C16 and C18 in myotubes of T2DM donors [16]. Recently, Bergman et al [17] reported higher levels of C16 and C18 in

T2DM versus obese although these were measured in serum and not muscle. The longer chain ceramides, which were all higher in T2DM, may be the ones implicated with the development of insulin resistance and diabetes. This is supported from cross-sectional studies in humans linking longer chain ceramides as culprits for development of insulin resistance [4, 18]. Impaired skeletal muscle fat oxidation has been often described in obese and diabetic patient populations [19, 20]. In presence of established whole body insulin resistance, lipolysis remains elevated resulting in higher plasma FFA. Such excess FFA supply induces ceramide synthesis via the *de novo* pathway [21]. However, a previous study has noted a role for only palmitate (C16) to serve as a substrate for *de novo* ceramide synthesis and C16 was not different between our groups [22]. In the present study, T2DM participants had higher plasma FFA concentrations possibly leading to a higher amount of muscle ceramides. However, storage of IMCL was similar between groups with only a trend to be higher in T2DM. It is possible that in addition to the elevated FFA in T2DM, the higher levels of glucose may have be synergistically harmful, a concept known as glucolipotoxicity, which is still debated [23].

This study is not without limitations. T2DM participants were older than their ND counterparts. A strong positive relationship ($r=.76$, $p<0.0001$) existed between age and total ceramides (data not shown). For that reason, we adjusted our results for age in all statistical analyses. It has been suggested that insulin resistance is not only associated with age but importantly with obesity and physical inactivity [24]. Although no measurements of physical activity were performed, both groups were matched in terms of adiposity. Another explanation could be the transition of type 2 to type 1 fibers as one ages [25], which could explain the higher amounts of lipid in the muscle of the T2DM group, although it is also reported that type 1 fibers are lower in T2DM individuals [26]. Unfortunately, this study did not include fiber type data or direct measurements of cellular skeletal muscle fatty acid oxidation; thus, an association between whole body and muscle fat oxidation cannot be deciphered or explain the interindividual differences in fat balance.

5. Conclusion

Higher amounts of skeletal muscle ceramides, individuals with T2DM showed no associations between ceramides and whole-body 24-hour fat oxidation whereas a positive association was present in ND participants. Together, our data suggests that the elevated ceramides present in T2DM are not from hindrance of fat oxidation, at least at the whole-body level, but rather could be from a more persistent lipid oversupply. Further research is thus warranted to elucidate differences in the relationship between lipid species with whole-body and cellular fat oxidation.

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List of Abbreviations

FFA	Free fatty acids
IHL	Intrahepatic lipids
IMCL	Intramyocellular lipids
RQ	Respiratory Quotient
T2DM	Type 2 Diabetes Mellitus

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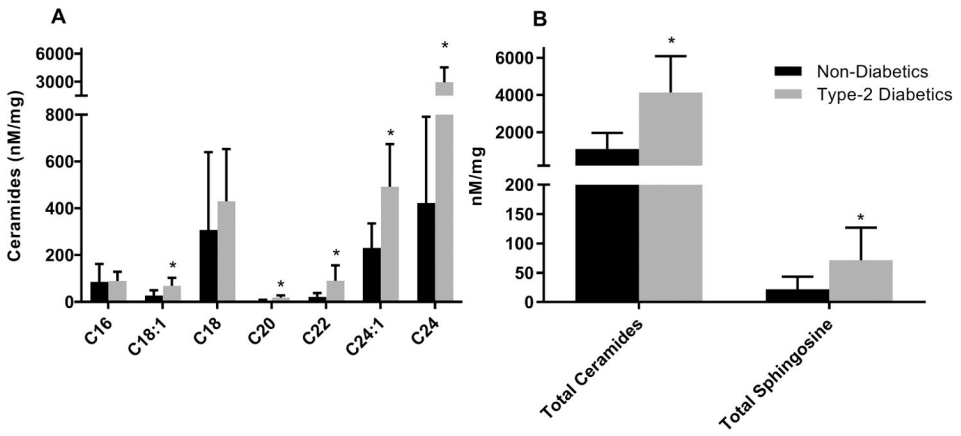

Figure 1. Comparison of ceramides between non-diabetic (black) and type 2 diabetic patients (gray) (mean ± SD)

Panel A: Comparison of ceramide species between diabetics and non-diabetics. **Panel B:** Comparison of total ceramides and total sphingosine between diabetics and non-diabetics. All values were normalized for protein content. Statistical significance (*= $p<0.05$) was assessed after adjustment for sex, age, and BMI. FFA = free-fatty acid, IMCL = intramyocellular lipids.

Table 1

Subjects' characteristics, respiratory chamber, and magnetic resonance imaging data.

	Non-Diabetics Patients	Type 2 Diabetics Patients
Gender (Male/Female)	31/31	23/21
Race (%White/Black/Other)	47/50/3	84/16/0
Age (years)	32.7 ± 10.80	57.7 ± 7.9 *
Weight (kg)	97.8 ± 31.8	95.9 ± 23.4
Height (cm)	171.4 ± 9.9	170.2 ± 10.0
BMI (kg/m^2)	33.6 ± 11.8	32.7 ± 7.2
Fat mass (kg)	31.6 ± 22.7	33.0 ± 14.7
Fat-free mass (kg)	59.3 ± 11.0	61.8 ± 11.8
Percent body fat (%)	32.2 ± 15.6	34.0 ± 9.2
Free fatty acids (mmol/L)	0.421 ± 0.241	0.578 ± 0.142 *
Triglycerides (mg/dL)	93.0 ± 51.4	179.0 ± 123.0 *
Total Cholesterol (mg/dL)	184.0 ± 29.4	170.2 ± 10.0
Glucose (mg/dL)	94.0 ± 8.3	116.6 ± 22.2 *
Insulin (mU/L)	11.8 ± 10.7	19.4 ± 18.3 *
HOMA-IR	2.8 ± 2.6	5.9 ± 6.4 *
24 hour RQ	0.889 ± 0.045	0.865 ± 0.032
Sleep RQ	0.874 ± 0.055	0.850 ± 0.038
Fat oxidation (g/24hr/kgFFM)	0.95 ± 0.87	1.29 ± 0.48
Carbohydrate oxidation (g/24hr/kgFFM)	5.15 ± 1.51	4.41 ± 1.11
Soleus IMCL (% of oil signal)	0.46 ± 0.25	0.71 ± 0.60
Anterior Tibialis IMCL (% of oil signal)	0.38 ± 0.20	0.79 ± 1.01
Intrahepatic lipids (% of oil signal)	0.02 ± 0.03	0.09 ± 0.12

Absolute unadjusted values are shown in the table. For statistical analyses, all estimates were adjusted for sex, age, and BMI and presented as mean ± SD. G=grams, hr=hour. kgFFM= kilograms of fat-free mass. IMCL = intramyocellular lipids. Data presented as mean ± standard deviation.

* $p<0.05$