Impact of insulin deprivation and treatment on sphingolipid distribution in different muscle subcellular compartments of streptozotocin-diabetic C57Bl/6 mice


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Meticulous glycemic control in type 1 diabetic (T1D) individuals can be achieved by administration of prandial, short-acting, and long-acting insulin to mimic β-cell secretion in response to varying nutrient levels. Metabolic control in these individuals and stringent adherence to guidelines normalize the plasma glucose and glycosylated hemoglobin levels (%Hb A1c) and allow for relatively normal life in T1D. Yet T1D individuals are at greater risk of developing cardiovascular disorders and also known to develop insulin resistance (12, 17). The molecular mechanisms responsible for insulin resistance in T1D subjects are unclear, but variable glucose concentrations (28, 70), advanced glycation end products (62, 73), or desensitization of target tissues by insulin (61) have been proposed. Subcutaneous insulin delivery using an insulin pump partially mimics β-cell insulin secretion. However, unlike in nondiabetic individuals with twofold higher hepatic insulin concentration than in the peripheral circulation, the diabetic individuals and animals receiving insulin via subcutaneous pump have similar systemic insulin levels as in liver. Although subcutaneous insulin administration suppresses hepatic gluconeogenesis, decreases protein degradation, and inhibits adipose tissue lipolysis, the lack of adequate insulin effect on triacylglycerol stores leads to the increase in free fatty acids (FFA) in the plasma of T1D individuals via the activation of hormone-sensitive lipase (32). The high FFA in plasma in turn can stimulate lipid accumulation in skeletal muscle, which is associated with insulin resistance (34, 40). Accumulation of intramyocellular ceramides (65, 66) and diacylglycerols (2, 31, 48) and their immediate precursors long-chain fatty acid-acylcoenzyme A (LCFa-CoA) (3, 21) are associated with insulin resistance (34, 40). Accumulation of long-chain ceramides (65, 66) and diacylglycerols (2, 31, 48) and their immediate precursors long-chain fatty acid-acylcoenzyme A (LCFa-CoA) (3, 21) are associated with decreased muscle glucose disposal and reduced insulin sensitivity in adults with type 2 diabetes (T2D). Because ceramide accumulation, especially palmitoyl-ceramide (C16-Cer), can induce insulin resistance in vivo (37, 71) and in vitro (53, 59), these molecules have become prime suspects in pathogenesis of muscle insulin resistance in type 2 diabetes. However, not much is known about muscle sphingolipid content and composition in T1D. Intramyocellular accumulation of ceramide was observed in the streptozotocin (STZ)-diabetic rat model (8), muscle-specific insulin receptor knockout mice, and STZ-treated mice (23). Thus, insulin deprivation or hypoinsulinemia leads to ceramide accumulation, but it has never been shown whether insulin treatment reverses the accumulation of ceramides in muscle. First, an experiment in which insulin treatment is withdrawn is important to clearly demonstrate the effect of insulin deprivation on ceramides. Long-chain hydrophobic ceramides are impermeable to the cellular membranes (4) and do not move freely between cellular compartments. This feature of long-chain ceramides underscores importance of evaluating distinct pools of ceramides found in plasma membranes, sarcoplasmic reticulum, or mitochondria, which can be regulated differently (7). Additionally, alternation in

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sphingolipid content at the level of endoplasmic reticulum (ER) can trigger ER stress response (54), whereas at the level of mitochondria it can impair mitochondrial metabolism and stimulate reactive species production (24, 76). Both processes are known to impair insulin sensitivity of target tissues. Thus it is important to evaluate sphingolipid composition of different cellular fractions.

Both plasma and intramuscular lipids with different acyl chain length and unsaturation (double bond no.) have distinctive impacts on muscle insulin sensitivity (14, 19), pancreatic insulin secretion (63, 75), and induction of metabolic disturbances. High content of long-chain saturated fatty acids compared with their unsaturated counterparts [low unsaturation index (UI)] in the Western-type diet is considered to be a major cause of both the obesity and muscle insulin resistance (13, 18). Although individual fatty acid composition of ceramides was studied in detail in animal models of T2D (50) and insulin-resistant human subjects (20), none of the studies addressed T1D models. Recent studies show that not only saturated fats (SAT) but also their monounsaturated species (MUFA) are detrimental for whole body glucose disposal (30) and can lead to insulin resistance (46) and that intramyocellular triglyceride unsaturation index and MUFA content positively correlate with estimation of insulin resistance (27). Thus it is important to evaluate not only intramuscular lipid content but also composition at the level of individual lipid species for both insulin-deprived and -treated states.

Therefore, the objective of the current investigation was to assess the impact of streptozotocin diabetes on the plasma FFA, muscle tissue LCFA-CoA and Cer, and the muscle expression of proteins implicated in lipid synthesis and insulin sensitivity in mice. To determine the degree by which insulin treatment can reverse the effects of insulin deprivation, we used long-lasting subcutaneous insulin implants to normalize diabetic phenotype in STZ T1D animals.

MATERIALS AND METHODS

Animal model. Experiments were conducted using 13-wk-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). Mice were housed individually with free access to water and chow (TD.10112; Harlan Laboratories, Indianapolis, IN), with a 12:12-h light-dark cycle and temperature and humidity control. Mice were acclimated for 1 wk prior to the beginning of the experiment. The protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee. Following a 6-h fast, mice were given intraperitoneal injections of STZ (125 mg/kg; in sodium acetate buffer, pH = 4.5) (67). Injections were repeated on the following day. Control animals received intraperitoneal injection of vehicle. Only mice that displayed blood glucose ≥300 mg/dl and an increase in blood ketones (both values by Precision Xtra glucometer; Abbott Laboratories, Abbott Park, IL), hyperphagia, and polyuria and were positive for urine glucose presence via dipstick (Uristix, Bayer, Pittsburgh, PA) on day 7 after the first STZ dose were included in the experiment. Animals that were positive for STZ diabetes received LinBit subcutaneous insulin implant (LinShin Canada, Toronto, ON, Canada) (79) under pentobarbital sodium anesthesia (Nebutal, 40 mg/kg of body wt) according to the manufacturer’s protocol. Each animal received two subcutaneous implants (total dose: 0.2 U/24 h for ≥30 days, 10 U/kg for 20-g mice). Insulin treatment was continued for 3 wk. Control animals (C; n = 13) received blank implants. Diabetic control was confirmed by biweekly measurements of blood and urinary glucose. In some cases, when urine glucose was present and blood glucose was ≥288 mg/dl, the animal received a third implant. The insulin treatment was continued until initially lower plasma glucose content in diabetic animals reached control values. Three weeks following implantation, diabetic mice were divided randomly into diabetic-treated (D + I; n = 13) and diabetic-deprived (D – I; n = 13) groups. Insulin implants were removed from the D – I group under pentobarbital anesthesia, which led to the return of the diabetic phenotype within 24 h. Animals from the D + I group continued on insulin treatment (Fig. 1). At the age of 18 wk, animals from all groups were analyzed for body composition by an Echo-MRI Body Composition Analyzer (EchoMRI, Houston, TX) and euthanized by decapitation 5 wk after the initial STZ or
vehicle dose. Figure 1 depicts the timeline of the experiment and blood glucose profiles for each experimental group. Additional animals were used for estimation of skeletal muscle insulin sensitivity by acute insulin stimulation. The mice were divided into the C (n = 6), D − 1 (n = 7), and D + 1 (n = 7) groups and followed appropriate experimental treatment, except for acute insulin stimulation 10 min prior to euthanization by pentobarbital overdose.

Isolation of muscle sarcoplasmic fraction and purification of mitochondria. Mitochondria were isolated from quadriceps muscle by differential centrifugation, as described previously (38). Briefly, quadriceps muscle samples were homogenized on ice using a motor-driven Potter-Elvejhem tissue grinder. After initial centrifugation, the supernatant containing the mitochondrial and sarcoplasmic fraction was transferred to a chilled microcentrifuge tube and centrifuged at 10,000 g for 2 min to pellet mitochondria. The supernatant containing sarcoplasmic fraction was frozen for further analysis. Mitochondrial pellet was washed twice by resuspending/centrifugation and finally suspended in a mitochondrial storage buffer. The levels of both the LCFA-CoA and sphingolipids in homogenates and various muscle fractions were normalized to total protein content, as measured by 660 nm Protein Assay (Thermo Scientific; Pierce Protein Biology Products, Rockford, IL).

Measurement of plasma FFA and tissue LCFA-CoA content by LC/MS/MS. Plasma free fatty acid concentrations were measured by liquid chromatography/mass spectrometry (LC/MS), as described previously (51). Briefly, 50 μl of plasma was spiked with heptadecanoyl-CoA internal standard (ISTD) and analyzed with Applied Biosystems (Foster City, CA) API5000 mass spectrometer coupled with a Cohesive (Franklin, MA) TX2 liquid chromatography system. Concentration of individual FFA was measured against a six-point standard curve prepared for each analyte. Both the ISTD and individual FFA standard curves were prepared in 2% fatty acid-free human albumin solution. All analytes were monitored as their [M − H]– ions.

LCFA-CoA esters were estimated using the LC-MS/MS method (9). After extraction in the presence of internal standard (20 ng of heptadecanoyl-CoA), samples were analyzed by UHPLC-ESI-MS/MS operating in multiple reaction monitoring mode [Waters Acquity UHPLC, C8 UPLC BEH column 2.1 × 150 mm, 1.7 μm (Waters, Milford, MA) and TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA)]. All standard curves were prepared using chemicals from Avanti Polar Lipids.

Measurement of sphingolipid content by LC-MS/MS. Quantity of sphingoid backbones [i.e., sphingosine (SpH) and sphinganine (dHSp)], together with individual molecular species of ceramide (Cer), was measured with the use of LC-MS/MS (10). Briefly, pure homogenates and sarcoplasmic and mitochondrial fraction aliquots were spiked with internal standards for sphingoid backbones and ceramides [dihydro 17C-sphingosine (d17:1-Sph) and margaric ceramide (C17:1-Cer)]. After extraction, samples were analyzed by UHPLC-ESI-MS/MS (Waters Acquity UHPLC, C8 UPLC BEH column 2.1 × 150 mm, 1.7 μm, Waters, and TSQ Quantum Ultra triple-quad mass spectrometer) working in MRM mode. All standard curves were prepared using standards obtained from Avanti Polar Lipids. All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Immunoblotting. Frozen muscle tissue was pulverized in liquid nitrogen and sonicated on ice (Model 100 Sonic Dismembrator; Fisher Scientific) in RIPA buffer (Sigma-Aldrich) containing 5 mM TCEP (Thermo Fisher Scientific, Waltham, MA) and protease and phosphatase inhibitors cocktail tablets and PhosphoSTOP phosphatase inhibitor cocktail tablets; Roche Applied Science, Indianapolis, IN). After incubation at 4°C for 30 min, samples were centrifuged at 15000g to remove cell debris. Protein content in the resulting supernatant was measured using Pierce 660nm Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Samples were prepared for SDS-PAGE in NuPAGE LDS Sample Buffer with 5 mM TCEP at a final protein concentration of 3 μg/μl and denatured by heating at 70°C for 10 min. A total of 45 μg of protein (15 μl total) was added to each well of precast gels (4–12% NuPAGE Novex Bis-Tris Midi Gels; Invitrogen, Carlsbad, CA). Proteins were separated by electrophoresis and blotted to nitrocellulose membranes. Membranes were blocked with 5% fat-free milk before incubating overnight with primary rabbit anti-mouse antibodies for proteins of intracellular fatty acid uptake (CD36, ab64014; Abcam, Cambridge, MA), acyl-CoA synthesis (FATP1/ACSVL5, M-100; Santa Cruz Biotechnology, Dallas, TX), sphingolipid de novo synthesis [serine palmitoyltransferase (SPT) 10005260; Cayman Chemical, Ann Arbor, MI], ceramide synthesis (CerS1-NBP1–59733 and CerS5-NBP1–76964; Novus Biologicals, Littleton, CO), insulin sensitivity [Akt (Pan)] no. 2920, phospho-Akt (Ser473) no. 4060, GSK-3α/β no. 5676, phospho-GSK-3β (Ser9) no. 5558 (Cell Signaling Technology, Danvers, MA), and housekeeping protein (Vinculin, AB6039; Merck, Darmstadt, Germany). Proteins were detected using infrared fluorescent detection (Li-Cor Odyssey, Lincoln, NE) using appropriate anti-mouse and anti-rabbit secondary antibodies. Proteins were normalized to muscle vinculin expression (a focal adhesion plaque protein), which compared with GAPDH did not display variability between experimental groups (data not shown), and expressed as fold change over control group values.

Acute insulin stimulation. For acute insulin stimulation, the mice were fasted for 6 h and given human recombinant insulin (1 U/kg) by intraperitoneal injection, as described by Zong et al. (80). Ten minutes later the animals were euthanized by pentobarbital sodium overdose, and tissue samples were collected and subjected to immunoblotting and immunofluorescence analysis.

Gene transcript expression. Muscle tissue RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines. Taqman Reverse transcription kit (Life Technologies, Carlsbad, CA) was used to prepare cDNA according to manufacturer’s instructions. Real-time PCR was performed on the Viaa7 Real Time PCR system (Life Technologies) using the Taqman Gene Expression Assays for glucose transporter type 4 (GLUT4; Mm01245502_m1), glycerone synthase 1 (GYS1; Mm01962575_s1), and insulin sensitivity (Akt) no. 5558 (Cell Signaling Technology, Danvers, MA), and housekeeping protein (Vinculin, AB6039; Merck, Darmstadt, Germany). Proteins were detected using infrared fluorescent detection (Li-Cor Odyssey, Lincoln, NE) using appropriate anti-mouse and anti-rabbit secondary antibodies. Proteins were normalized to muscle vinculin expression (a focal adhesion plaque protein), which compared with GAPDH did not display variability between experimental groups (data not shown), and expressed as fold change over control group values.

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Principal component analysis. We employed multivariate modeling in form of principal component analysis (PCA) to estimate interdependence between variables and groups. Analysis was performed using Statistica 10.0 software package, using noniterative partial least squares algorithm. Noniterative partial least squares algorithm maximum number of iterations and convergence criterion were set at 50 and 0.0001, respectively. The number of principal components was determined using the Krzansowski cross-validation method. Results were presented as both the scores biplot to visualize relationships between individual animals and loadings plot to visualize relationships between variables. To prevent an artificial increase in the PCA model strength, we excluded a majority of closely interdependent variables. Analysis was performed using Statistica 10.0 software package, using noniterative partial least squares algorithm. Noniterative partial least squares algorithm maximum number of iterations and convergence criterion were set at 50 and 0.0001, respectively. The number of principal components was determined using the Krzansowski cross-validation method. Results were presented as both the scores biplot to visualize relationships between individual animals and loadings plot to visualize relationships between variables. To prevent an artificial increase in the PCA model strength, we excluded a majority of closely interdependent variables.

Statistical analysis. Statistical significance between groups was estimated using ANOVA with the Tukey honestly significant difference post hoc test. We used Pearson’s approach to establish relationships between selected variables chosen on the basis of PCA to estimate statistical relationship. Significance level was set to P < 0.05.

RESULTS

Anthropometric parameters. When compared with control animals, insulin-deprived diabetic mice displayed significantly lower total body weight, fat and lean tissue mass, and total body water (P < 0.001 in all cases; Table 1). The drop in total body water as seen in D − 1 group and increase in bladder water volume had no impact on body hydration ratio, which was not different from the C group. When values were ex-
pressed in percentage of total body mass, only the decrease in fat content and increase in bladder water volume were significantly different between the D − I and C groups. Insulin deprivation significantly elevated plasma glucose, ketones, and %Hb A_{1c} values compared with the C group (P < 0.001 in all cases). Insulin treatment normalized all body composition and blood parameters in D + I animals except total body mass and Hb A_{1c} value.

**Impact of STZ diabetes on plasma fatty acid composition.** Compared with control, total content of plasma FFA doubled under the insulin deprivation state (P < 0.01; Fig. 2A). The most prominent increase was noted for all 18-carbon chain FFAs (P < 0.01 in each case) and was responsible for ∼80% of the total FFA concentration change. Percentage composition of plasma FFAs was also affected, with all non-18-carbon FFA displaying significant decreases in their percentage yield (P < 0.01 in each case), with linoleate (C18:2) and linolenate (C18:3) showing significant increase (P < 0.001 in both cases; Table 2). Those changes increased FFA UI by 15% (P < 0.001). Insulin treatment completely reversed the changes in plasma FFA concentration introduced by insulin deprivation (Fig. 2A). Despite insulin treatment, the percentage yield of C14, C16, and C16:1 FFA in D + I group was still significantly lower (P < 0.01), whereas the percentage yield of C18:2 FFA was significantly higher (P < 0.001). FFA UI was still significantly higher in D + I group compared with control (by 7%, P < 0.05; Table 2).

**Insulin deprivation increases the content and UI of LCFa-CoAs in skeletal muscle.** Insulin deprivation more than doubled the total content of LCFa-CoA esters in mouse quadriceps compared with control animals (P < 0.001; Fig. 2B, black bars). Differences were most prominent for C16:1-CoA, C18:1-CoA, C18-CoA, and C18:2-CoA esters (P < 0.001 in all cases). Percentage of monounsaturated palmitoleoyl and oleoyl ester was significantly higher (by ∼10%, P < 0.001 in both cases), whereas percentage of saturated palmitoyl and arachidonoyl esters was significantly lower (by 27% P < 0.001 in both cases; Table 2), compared with the control values. Overall LCFa-CoA UI increased by 4% (P < 0.001; Table 2). Insulin treatment normalized the content of almost all LCFa-CoAs.
Table 2. Impact of insulin deprivation and treatment on composition of plasma FFA, skeletal LCFa-CoA and Cer molecular species in homogenate, and sarcoplasmic and mitochondrial fraction

<table>
<thead>
<tr>
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<th>Plasma FFA (%)</th>
<th>Homogenate Cer (%)</th>
<th>Mitochondrial Cer (%)</th>
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<tr>
<td></td>
<td>LCFa-CoA (%)</td>
<td>Cer (%)</td>
<td>Cer (%)</td>
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<tr>
<td></td>
<td>D</td>
<td>C</td>
<td>D+I</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.00 ± 0.18</td>
<td>0.77 ± 0.03</td>
<td>0.70 ± 0.08</td>
</tr>
<tr>
<td>C14</td>
<td>0.82 ± 0.08</td>
<td>0.67 ± 0.02a</td>
<td>0.64 ± 0.02a</td>
</tr>
<tr>
<td>C16</td>
<td>0.63 ± 0.05</td>
<td>0.57 ± 0.01b</td>
<td>0.53 ± 0.01b</td>
</tr>
<tr>
<td>C18</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
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<tr>
<td></td>
<td>0.76 ± 0.07</td>
<td>0.74 ± 0.01</td>
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Effect of diabetes on mitochondrial sphingolipids. The modest changes in the content of mitochondrial ceramide species were not significantly different between experimental groups, nor was the ceramide percentage composition, UI, or dhSph content. The only exception was the elevation in the percentage yield of palmitoyl-ceramide in both the D − I and D + I groups (by ∼65%, P < 0.05 in both cases; Table 2) and significant decrease in the total content of mitochondrial ceramide in the D + I group compared with control (by 24%, P < 0.01; Fig. 3E). Sph was the sole sphingolipid affected by insulin deprivation in mitochondrial fraction, with the level nearly 50% higher in the mitochondria of the D − I group compared with the C group values (P < 0.05; Fig. 3F). Insulin treatment decreased the level of mitochondrial Sph to a level that was not significantly different from either the C or diabetic-deprived groups.

STZ diabetes increases expression of proteins implicated in de novo ceramide synthesis in mouse skeletal muscle. Consistent with the overall increase in the content of both LCFa-CoAs and ceramides, the protein expression of both the multifunctional fatty acid transporter/acyl-CoA synthase protein (FATP1/ACSVL5) and major enzymes of sphingolipid synthesis was elevated in the muscle of diabetic animals (Fig. 4). Although the protein content of CD36 fatty acid transporter was not affected by insulin deprivation in our study, the FATP1/ACSVL5 level increased significantly (by 39%, P < 0.05) compared with control (Fig. 4A). The expression of SPT increased by 34% and was at the edge of significance (P = 0.08; n = 8) compared with the control group (Fig. 4B). Insulin deprivation almost doubled the protein content of the CerS1 and CerS5 ceramide synthases compared with the C group value (P < 0.01 in both cases; Fig. 4B).

Insulin treatment decreased the expression of SPT proteins in skeletal muscle significantly compared with insulin deprivation and control animals (Fig. 4A and B). Under insulin treatment, the protein content of the CerS1 ceramide synthase decreased compared with the D − I group (by 20%, P < 0.05) yet was still significantly higher than C group values (P < 0.01 vs. C). A similar trend was noted for CerS5, although the changes were at the edge of significance (Fig. 4B).

STZ diabetes decreases expression of genes implicated in skeletal muscle insulin sensitivity. Insulin deprivation decreased gene expression of muscle GLUT4 and GYS1 significantly compared with control (by ∼60%, P < 0.01 in both cases; Fig. 4C). A similar decrease was observed for IRS-1 gene expression, yet a 30% decrease did not gain significance. Insulin treatment had no effect on the gene expression of IRS-1 or GYS1 compared with both control and insulin-deprived
animals, whereas GLUT4 mRNA level increased only moderately (Fig. 4C).

Insulin signaling in STZ-diabetic animals is inhibited in both insulin-deprived and insulin-treated states. To evaluate how the alternations at the muscular lipid level influence skeletal muscle insulin sensitivity, we performed acute insulin stimulation in an additional group of animals. Both diabetic-deprived and diabetic-treated animals displayed decreased phosphorylation of protein kinase B (p-Akt/Akt, by ~40%, \( P < 0.05 \); Fig. 4D) and glycogen synthase kinase-3\( \beta \) (p-GSK-3\( \beta \)/GSK-3\( \beta \), by ~20%, \( P < 0.05 \)). This indicates lower insulin sensitivity or insulin resistance in both experimental groups.

PCA and correlation analysis reveal close relationship between sarcoplasmic ceramide levels, expression of ceramide synthesis enzymes, and diabetic phenotype parameters. Three major principal components identified were responsible for a total of 59.3% of cumulative variance within the data set (pc1 = 39.1%, pc2 = 10.4%, pc3 = 9.67%; cumulative R\(^2\)X = 0.593,

Fig. 3. Insulin deprivation increases the content of ceramide in mouse skeletal muscle homogenates and sarcoplasmic fraction but not in mitochondria. Shown is the impact of insulin deprivation (black bars) and insulin treatment (gray bars) on the content of individual ceramide (Cer) molecular species (left) and sphingoid bases (right) in mouse quadriceps muscle homogenates (A and B), sarcoplasmic fraction (C and D), and mitochondrial fraction (E and F). Values were normalized to protein content in appropriate fraction and represent the mean ng/mg of protein content ± SE; \( n = 13 \)/group; \(^a^P < 0.05 \) vs. C group; \(^b^P < 0.05 \) vs. D + I group.
Q2 = 0.319). Score biplot shows wide separation between D−I and D + I animals (Fig. 5). Diabetic-treated animals clustered near control ones, yet they did not create a single cluster, suggesting that despite similar behavior of variables in the D + I and C groups, insulin treatment did not fully rescue the phenotype. Overlay of variable vectors shows that factors that mostly differentiate the D−I group from D + I and C groups (along the t1 axis; Fig. 5) were diabetes-related variables such as %Hb A1c, plasma FFA, body fat content, and muscular lipid-related variables such as the content of LCFA-CoA and sarcoplasmic ceramide (both total and C18-FA-containing species), the content of lipid synthesis enzymes (SPT, FATP1/ACSVL5, CerS), and mRNA expression of proteins implicated in muscle insulin resistance. Mitochondrial sphingolipids (total and individual molecular species) and CD36 protein expression had the least impact on between-group differentiation but were the major factors describing within-group differentiation (along the t2 axis; Fig. 5).

Loadings scatter plot revealed clustering of D−I group with features characteristic for diabetic phenotype (plasma glucose, %Hb A1c) together with sarcoplasmic ceramide and homogenate LCFA-CoA content (both the total and 18-carbon molecular species content). Those variables were the members of feature cluster 1 (Fig. 6), which also included other molecu-
ular species of sarcoplasmic Cer and LCFa-CoA and the proteins involved in their synthesis. Other notable clusters were plasma ketones and the FFA cluster (cluster no. 2; Fig. 6), which contained total content and a majority of molecular species of FFA together with 18:1-Cer in sarcoplasmic fraction. All the above-mentioned features were in opposition to each other and opposite to both the D\textsuperscript{I}/H11001 and C group representations (along the first-principle component axis). Both the C and D\textsuperscript{I}/H11001 groups were the members of features cluster no. 3 (Fig. 6\textbf{A}), which included variable characteristics of nondiabetic phenotypes such as high mRNA expression of genes implicated in muscle insulin sensitivity (IRS-1, GLUT4, GYS1), major body composition parameters (fat, lean tissue, and total body weight) and %SAT in plasma FFA and tissue LCFa-CoA. This suggests negative correlation of the above-mentioned variables with diabetic phenotype and positive correlation with control or diabetic-treated groups. Except for the plasma FFA UI, this parameter in LCFa-CoA and Cer in all fractions did not cluster with features of the diabetic phenotype. The same was observed for mitochondrial sphingolipid parameters and CD36 protein content.

Results of PCA suggest a close relationship between the plasma content of FFA, muscular content of LCFa-CoA, and ceramides in both the total homogenate and sarcoplasmic fractions. We used Pearson approach to further confirm interdependence of those variables. LCFa-CoA in homogenates showed a strong, positive correlation with total ceramide content in both the homogenates (r = 0.780, P < 0.001; Fig. 7\textbf{B}) and sarcoplasmic fraction (r = 0.726, P < 0.001; Fig. 7\textbf{C}) and weaker yet still significant positive correlation with total plasma FFA (r = 0.560, P < 0.05; Fig. 7\textbf{A}). At the level of proteins, FATP1/ACSVL5 and SPT protein content were highly correlated with each other (r = 0.867, P < 0.001) and with both total and 18-carbon molecular species of plasma FFA (mean r = 0.7, both the FATP1/ACSVL5 and SPT, P < 0.001 in both cases). SPT, CerS1, and CerS5 protein expression displayed significant, positive correlation with sarcoplasmic ceramide content (mean r = 0.6, P < 0.01).

**Fig. 5.** Scores scatter plot for 1st and 2nd components of principal component analysis: •, individual D = I animals; gray circles, individual D = I animals; ○, individual C animals. Ovals represent clustering of animals from respective experimental groups. Arrows indicate directions of major variables that are responsible for between- and within-group differences. S-Cer, total sarcoplasmic ceramide; M-Cer, total mitochondrial ceramide; M-dhSPH, mitochondrial sphinganine.

**Fig. 6.** Loadings scatterplot for first 2 components show clustering of the diabetic phenotype, muscle lipid-related variables, and representation of a diabetic group (D = I). A: scatterplot of all variables. The variables grouped in 3 major clusters (encircled) are described in RESULTS. B: for better visibility, the variables from clusters 1 and 2 are displayed. PC1, principal component 1 axis; PC2, principal component 2 axis.
Because daily plasma glucose values depend on food intake, we used %Hb A1c level in establishing a relationship between glycemic control and lipid parameters. %Hb A1c displayed the strongest positive correlation with total muscle content of LCFa-CoA, total muscle ceramide, and sarcoplasmic ceramide, with Pearson’s correlation $r = 0.908$, $r = 0.732$, and $r = 0.716$ respectively ($P < 0.001$ in all cases; Fig. 7, D, E, and F, respectively). Notably, all of the 18-carbon molecular species of FFA, LCFa-CoA, and ceramides had the strongest correlation with %Hb A1c values, ranging from $r = 0.890$ for C18:1 LCFa-Coa to $r = 0.690$ for sarcoplasmic C18:0-Cer (mean: $r = 0.736$, $P < 0.001$ in all cases), which was not pronounced for palmitate-containing lipids (mean $r = 0.589$, $P < 0.05$ for palmitoyl-CoA and sarcoplasmic palmitoyl-ceramide only). Although plasma FFAs were only weakly, positively correlated with %Hb A1c values ($r = 0.593$, $P < 0.05$), both total and
percentage body fat had strong, negative correlations ($r = -0.773$ and $r = -0.754$, respectively, $P < 0.001$ in both cases), and ketones had a strong positive correlation ($r = 0.812$, $P < 0.001$) with glycylated hemoglobin level. It has to be noted that although the above-mentioned variables displayed high reciprocal correlation, the effect was visible only when all three groups were introduced into correlation analysis but were absent when only the values from single group were taken into consideration. Lipids containing stearate or oleate moieties displayed strong reciprocal interdependence between different extra- and intracellular pools and across measured compounds ranging from $r = 0.826$ for plasma stearate and sarcoplasmic stearoyl-ceramide to $r = 0.524$ for plasma oleate and tissue oleoyl-CoA (mean: $r = 0.782$ for stearate-containing lipids and $r = 0.694$ for oleate-containing lipids). This was not observed for lipids with palmitate moiety.

**DISCUSSION**

The major findings of the current study are that 1) insulin deprivation elevates plasma FFA, which correlates with the increase in the muscle LCFA-CoA and ceramide content in both the total homogenates and sarcoplasmic fraction; 2) the changes were accompanied by an increase in the protein expression of lipid-synthesizing enzymes and a decrease in the expression of genes implicated in muscle insulin sensitivity; 3) lipid species based on stearic and oleic fatty acids display the greatest degree of change both in content and in percentage; 4) insulin treatment normalizes both plasma FFA and sphingolipid alterations in muscle but has lesser influence on muscular LCFA-CoA content and composition, protein expression of CerS, gene expression of GYS1, and Akt-dependent insulin signaling; 5) insulin deprivation and treatment has substantial impact on sphingolipid content and composition in sarcoplasmic fraction, but less so in the mitochondrial fraction; and 6) principal component and correlation analysis reveals a close relationship between features of diabetic phenotype such as %Hb A1c and both the muscle LCFA-CoA and ceramide content in the sarcoplasmic fraction but not in the mitochondrial one. Altogether, these results offer a mechanistic link between sphingolipids, LCFA-CoAs, and the diabetic phenotype, which is a potential explanation for the predisposition to insulin resistance in type 1 diabetes.

The substantial decline in fat mass observed in the current study during insulin deprivation could be explained by both the lack of insulin action on adipose tissue hormone-sensitive lipase and fatty acid esterification (32). The insulin-deprived animals displayed specific increases in all 18-carbon plasma FFAs, which can be explained by dietary fat composition. Oleate and linoleate fatty acids account for 80% molar content of soybean oil used in TD.10112 chow, with palmitate, stearate, and linoleate accounting for the remaining 20%. It has been reported that increased intracellular drive of FFAs during the insulin-deprived state is connected not only with passive, diffusion-based flip-flop mechanism but also with increased expression and membrane translocation of fatty acid transporters CD36 and FABPpm, as seen in T1D individuals (25, 44). Although in the current study we did not observe alternations in CD36 expression, the protein content of acyl-CoA synthase FATP1/ACSVL5 was significantly elevated during insulin deprivation. Intracellular FFAs are transformed to acyl-CoA esters by acyl-CoA synthetase activity, and our results demonstrate a close relationship between plasma FFAs and muscular LCFA-CoA level, a feature most prominent for their 18-carbon species. An increase in the content of LCFA-CoA was observed previously in rat heart from STZ T1D animals (47), in skeletal muscle of insulin-resistant diabetic Zucker rats (5), and in soleus muscle preincubated with oleate and linoleate (69).

Accumulation of muscular LCFA-CoA can contribute to muscle insulin resistance, as shown by Ellis et al. (21), where high-fat diet-induced insulin resistance was accompanied by the increase in activity of acyl-CoA synthase and elevation in rat gastrocnemius muscle LCFA-CoA. Similar to the current study, the most affected molecular species of acyl-CoAs were 18-carbon unsaturated CoAs (e.g., 18:1-CoA and 18:2-CoA). Acyl-CoAs diminish the effect of insulin on muscle glucose uptake by inhibiting hexokinase activity (68) and by activation of various PKC isoenzymes, which interferes with insulin signaling (57). Activation of PKCε and PKCθ concomitant with the elevation of muscular LCFA-CoAs was observed by Laybutt et al. (39) in skeletal muscle of rats made insulin resistant by chronic glucose infusion or high-fat diet (58).

Other groups reported that 4 h of lipid infusion in rats leads to accumulation of 18:2-CoA, PKCθ membrane translocation, inhibition of insulin-signaling cascade at IRS-1 level, and a decrease in muscle glucose uptake (42, 77), whereas PKCθ knockout mice are resistant to fat-induced insulin resistance (35). In vitro studies confirmed that both the palmitoyl-CoA and oleoyl-CoA can directly activate various PKC isoforms with similar potency as classic PKC activators, Ca2+, diacylglycerol, and phosphatidylcholine (74). The current study demonstrated for the first time, to the best of our knowledge, that the insulin deprivation-related increase in plasma FFA is accompanied by LCFA-CoA accumulation in skeletal muscle, which is most visible for oleoyl-CoA. Interestingly, the current study has also shown that insulin deprivation-related alternations in muscular LCFA-CoA content and composition are not completely reversed by insulin treatment, which suggests that a similar mechanism could potentially occur in long-term insulin-treated T1D subjects and can contribute to muscle insulin resistance.

Ceramides are also recognized as potent inducers of muscle insulin resistance. Ceramides are released as products of complex sphingolipid hydrolysis or synthetized from sphingoid base (Sph or dhSph) and LCFA-CoA at the level of sarcoplasmic reticulum (45). The rise in circulating FFA and elevated muscle ceramide levels was observed previously in soleus and red gastrocnemius skeletal muscle of streptozotocin-diabetic rats (8, 16) and in muscle-specific insulin receptor knockout mice and streptozotocin-treated mice (23). De novo sphingolipid synthesis depends on the supply of LCFA-CoA for both the sphingosine backbone formation by SPT and subsequent sphenosine acylation to yield ceramide. The latter is catalyzed by various isoforms of ceramide synthase CerS. CerS1 and CerS5 account for ~50 and 25% of total ceramide synthase activity, respectively, in skeletal muscle and in vitro synthesizes ceramides with C14 to C20 acyl chain length (49). CerS1 and -5 display the highest substrate specificity toward Sph, C18-CoAs, and C16-CoAs. Upregulation of both CerS isoforms by insulin deprivation resulted in the acute increase in C16, C18, and C18:1 ceramides in mouse quadriceps, which was most prominent in the muscle sarcoplasmic fraction, the place of
ceramide synthesis. The strong, positive interdependence between muscle SPT, the content of ceramide, and insulin resistance was reported previously by Holland et al. (29) in various models of insulin resistance in mice. The current study shows for the first time that muscular ceramide content is highly correlated with the plasma FFA, muscular LCFa-CoAs, and muscle SPT and CerS protein expression in streptozotocin-diabetic mice when all three experimental groups are included in correlation analysis. Ceramide accumulation in muscle can be reversed by insulin treatment. This relationship is more prominent for sarcoplasmic than total muscle ceramide and is not observed in mitochondria. Mitochondria contain several sphingolipid-metabolizing enzymes and are not directly connected with sarcoplasmic sphingolipid pool by Golgi membrane vesicular transport (7, 26). The current data suggest distinct regulation of mitochondrial sphingolipid metabolism in muscle from that of sarcoplasm, whereas sphingolipid content and composition followed the alternations in LCFa-CoA level. Alteration of lipid synthesis is one of the events observed in ER stress response (22). Lipid-induced ER stress is known to be involved in both liver (43) and muscle (56, 78) insulin resistance. Ceramide accumulation with triggering of ER stress markers and inhibition of insulin signaling was observed in liver tissue biopsies from alcohol-related liver disease (ALD) patients (43) and in a rat model of ALD (54). Significant accumulation of sarcoplasmic ceramide observed in our study can promote muscle insulin resistance through ER stress response, yet elucidation of this mechanism requires further investigation.

Another intriguing observation is the finding that mutual interdependence of plasma FFA, muscular LCFa-CoA, and sarcoplasmic ceramides was most prominent for their 18-carbon molecular species, rather than for 16-carbon ones, as shown by correlation analysis. Until now, most studies have focused on palmitate and its involvement in intramuscular ceramide accumulation and induction of insulin resistance (1, 19, 53, 64), the rationale being that it can form both the sphingoid backbone (dhSph, through condensation of palmitoyl-CoA and l-serine by sarcoplasmic SPT) and palmitoyl-ceramide (through the activity of sarcoplasmic ceramide synthase), thus increasing ceramide content by two parallel mechanisms. Palmitate, but not oleate, was shown to increase ceramide accumulation in muscle myotubes (11), decrease phosphorylation of Akt (55), and induce muscle insulin resistance (52), the latter even improving palmitate-induced insulin resistance (6, 14, 55). On the contrary, a study by Thompson et al. (69) reported that oleate and linoleate were more potent inhibitors of insulin-stimulated glucose uptake, glucose phosphorylation, and glycogen synthesis in isolated soleus muscle than palmitate. Oleate and linoleate were also reported by Schmitz-Peiffer et al. (59) to inhibit both basal and insulin-stimulated glycogen synthesis, glucose uptake, and glucose phosphorylation. In the saturated form, 18-carbon stearate was shown to be as potent as palmitate in stimulation of ceramide synthesis. (11). In the current study, plasma stearate and oleate had greater degrees of correlation than palmitate, with the respective molecular species of LCFa-CoA and ceramide in both the total homogenates and sarcoplasmic fraction. Our results suggest that 18-carbon saturated and unsaturated fatty acids are more important in T1D-induced LCFa-CoA and muscle sphingolipid accumulation and display stronger correlation with diabetic phenotype descriptors such as %Hb A1c. This relationship is further reinforced by the results of PCA analysis, where lipid species containing stearic, oleic, or linoleic fatty acid moiety were among the top 10 variables that were most influential on PCA analysis outcome (data not shown).

We have reported in the current study the mRNA expression of proteins that are involved in insulin signaling. Because of the continuous release of insulin in implanted animals and long insulin washout time after implant removal (due to minute debris of implant material still present under the skin), the employment of the hyperinsulinemic euglycemic clamp was not feasible in our experimental model. A decrease in the gene and/or protein expression of IRS-1 with concomitant insulin resistance was observed in muscle biopsies of insulin-resistant Pima Indians (36), muscle of T2D subjects (33), and muscle biopsies from women with gestational diabetes (15). Similar concordance of mRNA gene expression of GLUT4 in the skeletal muscle of rats (41) and humans (15, 33) and for GYS mRNA expression in the muscle of T2D subjects (72) and insulin resistance has been reported. The decline observed in GLUT4 and GYS1 gene expression in the diabetic animals during insulin deprivation (D − I) indicates decreased muscle insulin sensitivity, which was confirmed by the status of Akt-related signaling under acute insulin stimulation. Insulin treatment of the diabetic mice (D + I) failed to normalize these declines in signaling protein mRNA expression or improve Akt signaling, which supports the finding that these animals remained insulin resistant despite peripheral insulin administration. The current study supports the notion that insulin resistance may contribute to the complication of type 1 diabetes even after replacing insulin.

The measure of glycosylated hemoglobin level (%Hb Ahc) is regarded as a gold standard to assess glycemic control in type 1 and type 2 diabetes (60). An increase in %Hb Ahc correlates with the prevalence of diabetic complications. Therefore, long-term normalization of %Hb Ahc level is a key goal in T1D treatment. Our data show that %Hb Ahc value is tightly correlated with muscle content of both the LCFa-CoA and ceramides in both total homogenates and sarcoplasmic fraction. Both the PCA results and correlation analysis also confirm the importance of stearate- and oleate-containing lipids, as those molecular species displayed the greatest correlation with the %Hb Ahc value and were major sphingolipids clustered with features of the diabetic phenotype. Finally, we were able to demonstrate that most of the lipid alternations introduced by insulin deprivation could be reversed by long-term insulin treatment. Animals from the D + I group did not differ from controls in total and percent fat content, plasma FFA, total homogenate, or sarcoplasmic ceramide levels. These data suggest insulin inhibition of hormone-sensitive lipase and stimulation of fat tissue triglyceride synthesis channeled an excess of FFA toward storage and prevented intramyocellular sphingolipid accumulation. Of interest is that the muscle content of LCFa-CoA, although suppressed by insulin treatment, was still significantly higher in the D + I group. The insulin had no influence on percentage distribution or unsaturation index of LCFa-CoAs and only partially affected unsaturation of plasma FFA. These results suggest that many of the factors not corrected by insulin treatment may play important roles in developing insulin resistance in T1D individuals even when tight glycemic control is maintained. However, it is important
to recognize that STZ-diabetic mice are not true T1D individuals and that the pathophysiology of the two are different, although both result in insulin deficiency.

In summary, we demonstrate that insulin deprivation significantly affects both the content and percentage composition of skeletal muscle ceramide through an increase in the protein expression of enzymes involved in sphingolipid synthesis and is connected with reduced gene expression of proteins involved in muscle insulin sensitivity and Akt-related insulin signaling. PCA and correlation analysis revealed that alterations were connected with plasma FFA and muscular LCFa-CoA increase and were most visible for 18-carbon containing sphingolipids in sarcoplasmatic fraction of the muscle. Although insulin treatment normalized many of the observed derangements in sphingolipids in diabetic mice, there were persistent changes in LCFa-CoA levels, ceramide synthesis enzymes, and insulin signaling proteins following insulin treatment that provide a potential explanation for the insulin resistance that manifests even after exogenous insulin treatment in type 1 diabetic individuals.

REFERENCES


MUSCLE SPHINGOLIPID DISTRIBUTION IN DIABETES

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