Effect of Weight Loss on Skeletal Muscle Bioactive Lipids in People With Obesity and Type 2 Diabetes

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Muscle *sn*-1,2-diacylglycerol (DAG) and C18:0 ceramide accumulation in sarcolemmal and mitochondrial compartments have been proposed to regulate muscle insulin sensitivity. Here, we evaluated whether weight loss-induced improvements in insulin sensitivity were associated with changes in muscle *sn*-1,2-DAG and ceramide content in people with obesity and type 2 diabetes. We measured skeletal muscle insulin sensitivity, assessed by using the hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotopically labeled glucose tracer infusion, and skeletal muscle *sn*-1,2-DAG and ceramide contents by using liquid chromatography–tandem mass spectrometry after subcellular fractionation and DAG isomer separation in 14 adults with obesity and type 2 diabetes before and after marked (18.6 ± 2.1%) weight loss. Whole-body insulin sensitivity doubled after weight loss. Sarcolemmal *sn*-1,2-DAG and C18:0 ceramide contents after weight loss were not different from values before weight loss. In contrast, mitochondrial-endoplasmic reticulum (ER) C18:0 ceramide content decreased by ∼20% after weight loss (from 2.16 ± 0.08 to 1.71 ± 0.13 nmol/g, $P < 0.005$). These results suggest a decrease in muscle mitochondrial-ER C18:0 ceramide content could contribute to the beneficial effect of weight loss on skeletal muscle insulin sensitivity.

ARTICLE HIGHLIGHTS

- Importance of changes in muscle *sn*-1,2-diacylglycerols (DAG) or C18:0 ceramide in mediating the beneficial effects of weight loss on muscle insulin sensitivity in people is not known.
- We examined whether weight loss–induced increases in muscle insulin sensitivity in people with obesity and type 2 diabetes are accompanied by changes in muscle *sn*-1,2-DAG or C18:0 ceramide.
- Weight loss improved muscle insulin sensitivity, which was accompanied by a 20% decrease in mitochondrial–endoplasmic reticulum C18:0 ceramide without a change in sarcolemmal C18:0 ceramide or *sn*-1,2-DAG.
- These results suggest that decreased muscle mitochondrial–endoplasmic reticulum C18:0 ceramide content contributes to the beneficial effect of weight loss on muscle insulin sensitivity.

Insulin resistance in skeletal muscle, defined as impaired insulin-stimulated muscle glucose uptake, is central to the pathogenesis of type 2 diabetes (T2D) (1,2). Data from studies conducted in rodent models have shown that accumulation of specific bioactive lipids, namely diacylglycerols (DAGs) and ceramides, can cause muscle insulin resistance (2). Moreover, the species and subcellular localization of these bioactive lipids are mechanistically linked with insulin resistance. The accumulation of sarcolemmal *sn*-1,2-DAG can impair insulin signaling by activating novel protein kinase C (nPKC) isoforms, which phosphorylate and inhibit insulin signaling effectors to reduce insulin receptor substrate 1–associated phosphatidylinositol-3-kinase activity (3). However, only the *sn*-1,2-DAG isomer, rather than the *sn*-1,3 or *sn*-2,3-DAG isomers, induces cellular insulin resistance through nPKC activation (4). The accumulation of sarcolemmal C18:0 ceramides can induce insulin resistance by inhibiting AKT activity, and increased mitochondrial C18:0 ceramides can induce insulin

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resistance by impairing mitochondrial respiration and increasing reactive oxygen species production (2,5–12).

Weight loss has potent beneficial effects on skeletal muscle insulin action (13). In fact, marked (>15%) weight loss causes a large increase in skeletal muscle insulin sensitivity and is associated with a high rate of diabetes remission (14,15). However, the cellular mediators responsible for enhancing skeletal muscle insulin action after weight loss are unclear. The purpose of the current study was to test the hypothesis that the improvement in skeletal muscle insulin sensitivity induced by marked weight loss in people with obesity and T2D is associated with decreases in muscle sarcolemmal *sn*-1,2-DAG, sarcolemmal C18:0 ceramide, and mitochondrial C18:0 ceramide contents, because these intramyocellular lipids impair insulin action in rodent models of obesity (3,8) and are associated with insulin resistance in people with T2D (2,6–9,16–21). Accordingly, we evaluated intramyocellular DAGs and ceramides in vastus lateralis muscle by using liquid chromatography–tandem mass spectrometry (LC-MS/MS) after subcellular fractionation and chromatographic isomer separation, and assessed skeletal muscle insulin sensitivity by using the hyperinsulinemic-euglycemic clamp procedure, in conjunction with stable isotopically labeled glucose tracer infusion, in people with obesity and T2D before and after marked (16–24%) body weight loss.

RESEARCH DESIGN AND METHODS

Participants and Study Design

A total of 14 people with obesity and T2D (6 men and 8 women, 50 ± 12 years old, 10 ± 10 years' duration of T2D) participated in this study. The data reported here are the analysis of skeletal muscle intramyocellular DAGs and ceramides present in subcellular fractions and skeletal muscle insulin sensitivity assessed before and after marked weight loss, induced by either Roux-en-Y gastric bypass surgery (n = 9) or low-calorie diet therapy (n = 5), in a subset of 22 participants that completed a study evaluating the effects of weight loss on metabolic function (14). Participants were included in the current study if they achieved >15% weight loss and had an adequate amount of skeletal muscle tissue (>100 mg) available both before and after weight loss. This weight loss target was chosen because it is associated with a high rate of remission of T2D (15). After achieving >15% weight loss, participants maintained a stable body weight (weight change ≤1% per week) for 3 weeks before postintervention metabolic studies were performed. Participants were "sedentary" at baseline (defined as <90 min of exercise/week) and were instructed to maintain their baseline level of physical activity throughout the study. All participants provided written informed consent before participating in this study, which was approved by the Washington University Institutional Review Board (St. Louis, MO) and registered in ClinicalTrials.gov (NCT02207777).

Skeletal Muscle Insulin Sensitivity

Insulin sensitivity was assessed by using a 9-h, three-stage hyperinsulinemic-euglycemic pancreatic clamp procedure in conjunction with the infusion of stable isotopically labeled glucose tracer, octreotide (45 ng/kg fat-free mass/min), glucagon (1.5 ng/kg fat-free mass/min), and growth hormone (6 ng/kg fat-free mass/min), as previously described (14). Muscle insulin sensitivity was assessed as the glucose disposal rate per kilogram fat-free mass (determined by using dual-energy X-ray absorptiometry [Lunar iDXA; GE Healthcare]) divided by plasma insulin concentration during stage 3 (50 mU insulin/m^2 body surface area/min) of the clamp procedure, which provided an adequate infusion of insulin needed to assess insulin-stimulated glucose disposal.

Skeletal Muscle Lipids

Vastus lateralis muscle tissue was obtained by percutaneous biopsy during the basal period of the clamp procedure by using a Tilley Henkel forceps (Sontec Instruments, Inc., Centennial, CO). Subcellular fractions (endoplasmic reticulum [ER], lipid droplet, sarcolemma, mitochondria, and cytosol) of skeletal muscle DAGs and ceramides were processed as previously described (22). Cold-homogenized tissue lysate was subjected to differential centrifugation in sucrose-containing Tris-EDTA buffer to sequentially collect *1*) lipid droplet, *2*) plasma membrane (sarcolemma), *3*) mitochondria, *4*) ER, and *5*) cytosol fractions. Known amounts of *sn*-1,2-dinonadecanoin were added as an internal standard. Subcellular fractions were then separated on a chiral high-performance liquid chromatography column to obtain resolution of *sn*-1,2-, *sn*-1,3-, and *sn*-2,3-DAG stereoisomers. After further separation on a silica high-performance liquid chromatography column, LC-MS/MS was used to quantitate individual *sn*-1,2-DAG species in each subcellular fraction. The same five subcellular fractions were then used to quantify individual ceramide species. Ceramides were extracted in chloroform:methanol, with known amounts of N-heptadecanoyl-D-erythro-sphingosine added as an internal standard. Extracted lipids were dried under nitrogen and reconstituted in 95:4.5:0.5 hexane:methylene chloride:ethyl ether. LC-MS/MS separation of ceramide species and quantitation was performed as previously described (3). Marker protein immunoblotting of subcellular fractions was performed as described previously (23); primary antibodies are listed in Supplementary Table 1. Immunoblots revealed ER marker protein (calnexin) content in the mitochondrial fraction and mitochondrial marker protein (citrate synthase) content in the ER fraction (Supplementary Fig. 1). For this reason, the lipid contents in mitochondrial and ER fractions were summed and are presented as mitochondrial-ER lipid content.

Diabetes Medications

A composite diabetes medication score was calculated as the sum of individual diabetes medication scores determined as the daily dose relative to maximum recommended dosage, as previously described (24). Diabetes medications were adjusted every 1–2 weeks as needed after starting the study. Six of the 14 participants were taking insulin before study enrollment, and 3 were still prescribed insulin at the time of

the post–weight loss study visit. Diabetes medications were held for different durations before the baseline and post–weight loss study visit to minimize the effect of the medications on the metabolic assessments; insulin was stopped for 1 day, oral medications were stopped for 3 days, and GLP-1 receptor agonists were stopped for 2 weeks before the study visits. Ten of the 14 participants were prescribed a statin before study enrollment, and no participant started or stopped statin therapy during the study.

Sample Analyses and Calculations

Plasma glucose concentration was determined by using an automated glucose analyzer (YSI 2300 STAT plus; Yellow Springs Instruments). Plasma insulin concentration was determined by using an electrochemiluminescence assay (Elecsys 2010; Roche). Hemoglobin A_{1c} (HbA_{1c}) was measured in the Washington University Core Laboratory for Clinical Studies. Plasma glucose tracer-to-tracee ratio was determined by using gas chromatography–mass spectrometry (13), and glucose disposal rate was calculated as previously described (14). Total DAG isomer and ceramide contents were assessed as the sum of fractions.

Statistical Analysis

All data were examined for normality by using Q-Q plots before inferential analysis. Subject characteristics, insulin sensitivity, and total muscle DAG and ceramide contents within each fraction were all normally distributed and were analyzed by using two-tailed paired *t* tests. For individual lipid species, untransformed concentration data were plotted, but two-way ANOVA was used to compare individual DAG and ceramide species within each fraction after the data were $\log_2$ transformed. Šidak correction for multiple comparisons was used to identify weight loss–induced changes in individual DAGs and ceramide when a significant time × species interaction was detected. Possible interaction effects between the mode of weight loss (surgery or diet) and weight loss per se on muscle lipid content were assessed by using a two-way ANOVA. Statistical significance was set at $P < 0.05$. Sex was not considered in the statistical analyses. Statistical analyses were performed by using GraphPad Prism 10.

Data and Resource Availability

The data sets generated and analyzed during the current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Weight Loss, HbA_{1c}, and Insulin Sensitivity

Participants were studied before and after 18.6 ± 2.1% (range 16.0% to 24.4%) weight loss, which resulted in a marked decrease in fasting plasma glucose, fasting plasma insulin, HbA_{1c}, and use of diabetes medications, and a twofold increase in skeletal muscle insulin sensitivity (Table 1). The average duration between the start of the intervention (surgery or diet initiation) and the assessments after weight loss was 17 ± 4 weeks.

Muscle Diacylglycerol Content

The content of 11 DAG species in skeletal muscle was measured after separation by isomer (*sn*-1,2; *sn*-1,3; or *sn*-2,3) and subcellular compartment (sarcolemma, mitochondrial-ER, cytosol, lipid droplet). Weight loss did not affect total *sn*-1,2-DAG content or the content of individual *sn*-1,2-DAG species within any fraction (Fig. 1). There was no effect of the type of weight loss therapy (surgery or diet) on sarcolemmal *sn*-1,2-DAG content (two-way ANOVA, $P_{interaction} = 0.667$). Weight loss did not significantly affect skeletal muscle *sn*-1,3-DAG content (sum of species) in any compartment, with the exception of a ∼25% decrease in skeletal muscle C18:1/C18:1 *sn*-1,3-DAG content in the lipid droplet fraction ($P = 0.0002$) (Fig. 2). Total skeletal muscle *sn*-2,3-DAG content and

Table 1—Subject characteristics

	Baseline	Weight loss	*P* value
Body weight (kg)	125 ± 20	102 ± 16	<0.001
BMI (kg/m^2)	43.1 ± 5.6	35.0 ± 4.4	<0.001
Fat mass (% body weight)	47.2 ± 6.1	41.7 ± 7.0	<0.001
Fat-free mass (kg)	65.5 ± 11.7	59.4 ± 11.5	<0.001
Fasting plasma glucose (mg/dL)	117 ± 20	95 ± 14	0.001
Fasting plasma insulin (μU/mL)	25.8 ± 10.4	10.3 ± 4.6	<0.001
HbA_{1c} (%)	7.7 ± 1.9	5.9 ± 0.7	0.003
HbA_{1c} (mmol/mol)	61 ± 21	41 ± 8	0.003
Diabetes medication score	1.4 ± 1.1	0.5 ± 0.7	0.001
Glucose R_d/insulin (nmol/kg FFM/min)/(μU/mL)	259 ± 140	540 ± 175	<0.001
Clamp stage 3 plasma insulin (μU/mL)	104 ± 22	107 ± 25	0.558

FFM, fat-free mass. R_d, rate of disappearance. Data are mean ± SD. *P* values by paired two-tailed *t* test.

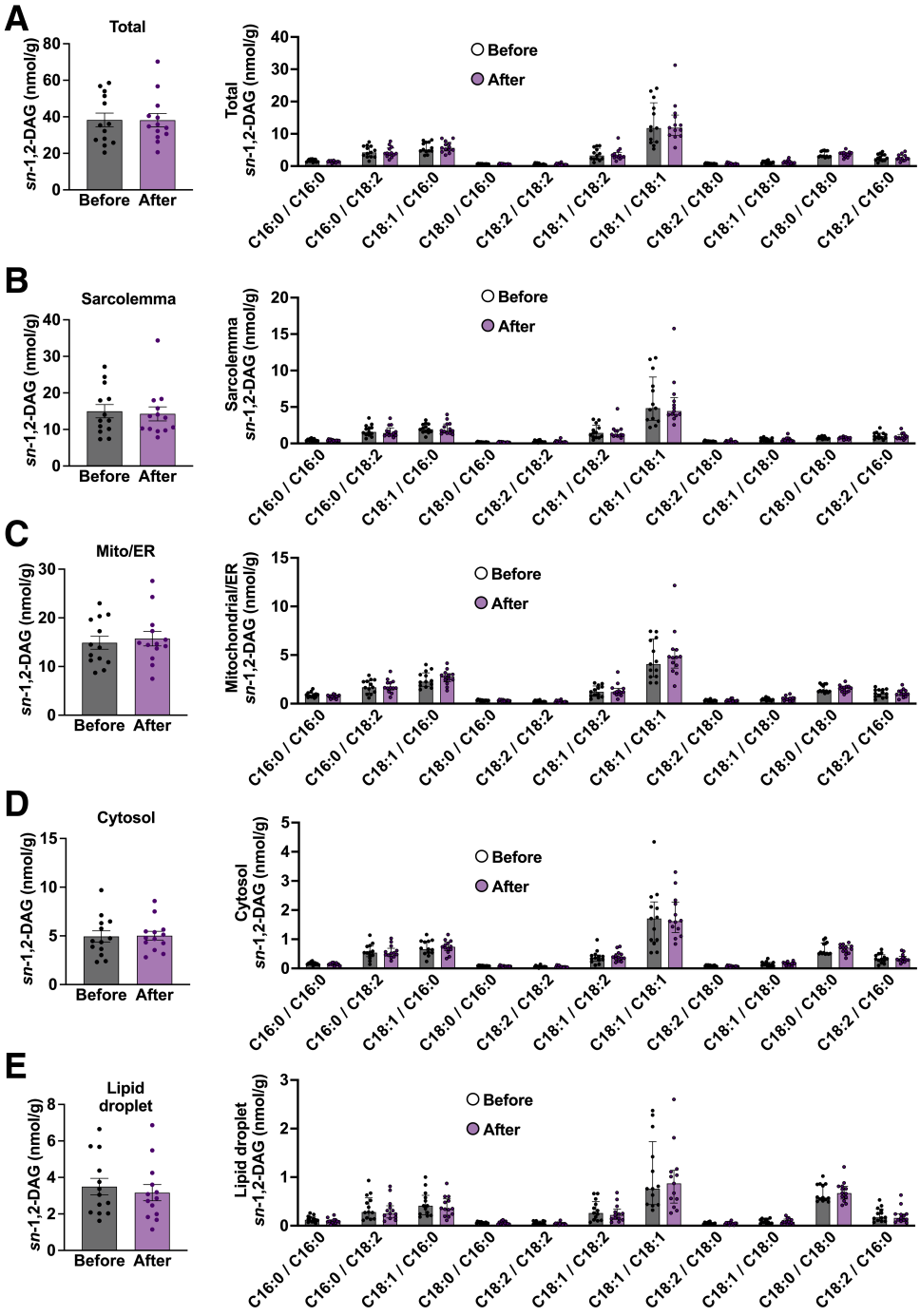

Figure 1—Skeletal muscle *sn*-1,2-DAG content before and after weight loss. Muscle *sn*-1,2-DAG content presented as sum of 11 species (left) and as individual species (right) before (gray bars) and after (purple bars) weight loss in (*A*) sum total of all subcellular fractions, (*B*) sarcolemma, (*C*) mitochondria-ER, (*D*) cytosol, and (*E*) lipid droplet fractions. In sum of species (left) plots, data are means ± SEM with individual values overlaid. For individual species (right) plots, data are median ± interquartile range with individual values overlaid. $P > 0.05$ for all comparisons. $n = 13$ (one participant was considered an outlier and excluded from the analysis because their total DAG content was >3 SD above the mean).

sn-2,3-DAG content in any compartment were not different after than before weight loss (Fig. 3).

Muscle Ceramide Content

Total muscle ceramide content (sum of six species) and total ceramide content in any subcellular compartment were not different after than before weight loss (Fig. 4). However, total C18:0, C22:0, and C24:0 ceramides decreased by ∼20%, whereas total C24:1 ceramides increased by ∼45% after weight loss (Fig. 4*A*). Several saturated and unsaturated ceramide species in different compartments changed with weight loss (Fig. 4*B*–*D*). In the sarcolemmal compartment,

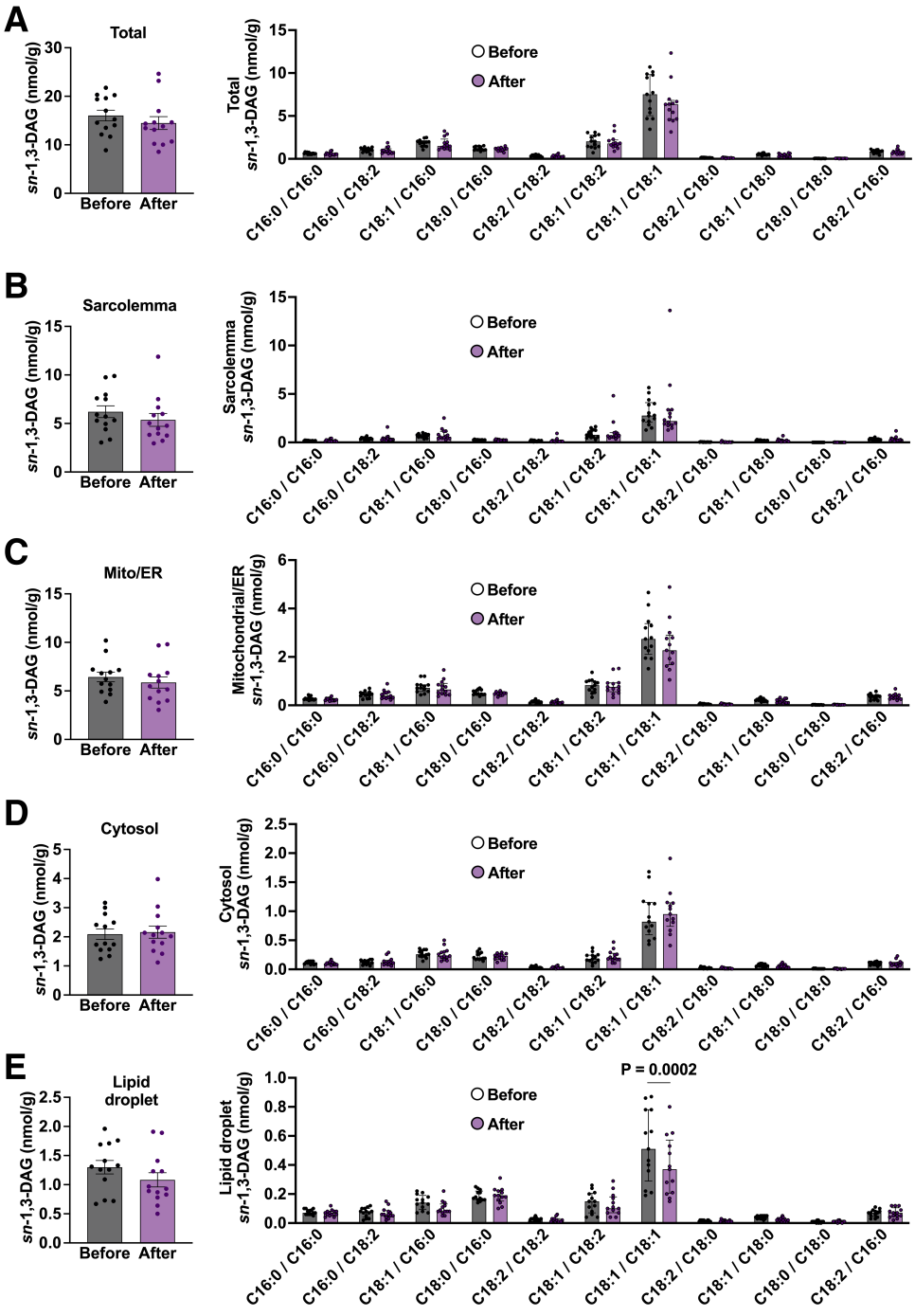

Figure 2—Skeletal muscle *sn*-1,3-DAG content before and after weight loss. Muscle *sn*-1,3-DAG content presented as sum of 11 species (left) and as individual species (right) before (gray bars) and after (purple bars) weight loss in (*A*) sum total of all subcellular fractions, (*B*) sarcolemma, (*C*) mitochondria-ER, (*D*) cytosol, and (*E*) lipid droplet fractions. In sum of species (left) plots, data are means ± SEM with individual values overlaid. For individual species (right) plots, data are median ± interquartile range with individual values overlaid. $P > 0.05$ for all comparisons except lipid droplet *sn*-1,3 C18:1/C18:1 DAG. $n = 13$ (one participant was considered an outlier and excluded from the analysis because their total DAG content was >3 SD above the mean).

C24:1 ceramide content increased by ∼50% after weight loss ($P < 0.0001$) (Fig. 4*B*), whereas there were no weight loss–induced changes in any of the saturated ceramides. In the mitochondrial-ER compartment, C18:0, C22:0, and C24:0 ceramides decreased by ∼20% (all $P < 0.005$), and mitochondrial-ER C24:1 ceramide content increased by ∼40% after weight loss ($P < 0.0001$) (Fig. 4*C*). Cytosolic C24:1 ceramide content increased by ∼50% ($P = 0.015$); however, total cytosolic ceramide content was much lower than the values observed in other fractions (Fig. 4*D*). The content of all ceramide species located in lipid droplets did not change with weight loss (Fig. 4*E*). There was no effect of the type

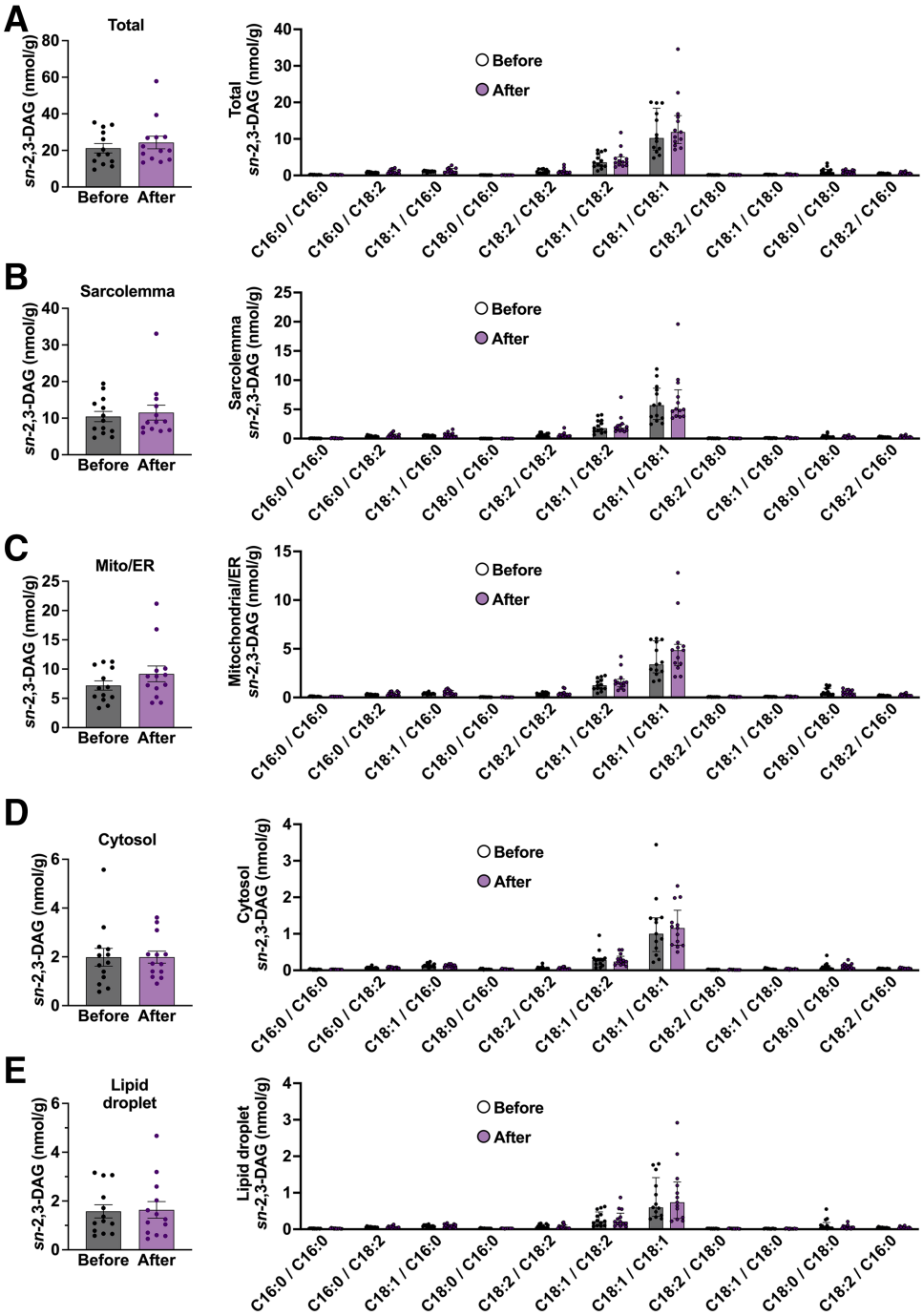

Figure 3—Skeletal muscle *sn*-2,3-DAG content before and after weight loss. Muscle *sn*-2,3-DAG content presented as sum of 11 species (left) and as individual species (right) before (gray bars) and after (purple bars) weight loss in (*A*) sum total of all subcellular fractions, (*B*) sarcolemma, (*C*) mitochondria-ER, (*D*) cytosolic, and (*E*) lipid droplet fractions. In sum of species (left) plots, data are means ± SEM with individual values overlaid. For individual species (right) plots, data are median ± interquartile range with individual values overlaid. $P > 0.05$ for all comparisons. $n = 13$ (one participant was considered an outlier and excluded from the analysis because their total DAG content was >3 SD above the mean).

of weight loss therapy (surgery or diet) on sarcolemmal (two-way ANOVA, $P_{interaction} = 0.182$) or mitochondrial-ER (two-way ANOVA, $P_{interaction} = 0.714$) C18:0 ceramide contents. There was no significant association between the percent change in mitochondrial-ER C18:0 ceramide content and the percent change in skeletal muscle insulin sensitivity ($r = 0.16$, $P = 0.611$).

DISCUSSION

Weight loss increases skeletal muscle insulin sensitivity in people with obesity and T2D (14). In the current study, we evaluated whether changes in skeletal muscle DAGs and ceramides are potentially involved in the beneficial effect of marked (16–25%) weight loss on skeletal muscle insulin sensitivity in people with obesity and T2D. We

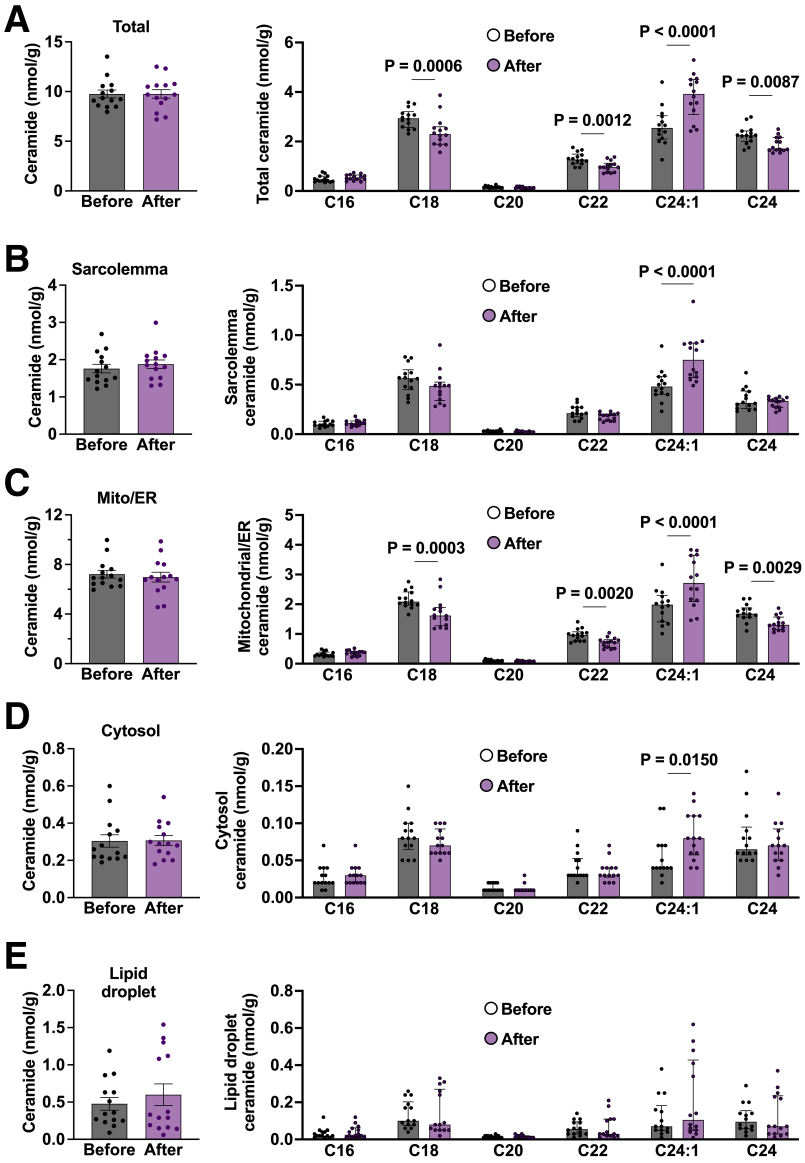

Figure 4—Skeletal muscle ceramide content before and after weight loss. Muscle ceramide content presented as sum of six species (left) and as individual species (right) before (gray bars) and after (purple bars) weight loss in (*A*) sum total of all subcellular fractions, (*B*) sarcolemma, (*C*) mitochondria-ER, (*D*) cytosol, and (*E*) lipid droplet fractions. In sum of species (left) plots, data are means ± SEM with individual values overlaid. For individual species (right) plots, data are median ± interquartile range with individual values overlaid. $n = 14$.

hypothesized that sarcolemmal *sn*-1,2-DAG, sarcolemmal C18:0 ceramide, and mitochondrial C18:0 ceramide contents would decrease after weight loss, because these lipids have been implicated as mediators of muscle insulin resistance (2,3,6–9,11,12,16,17). We found that marked weight loss, which caused a doubling in skeletal muscle insulin sensitivity, was associated with a 20% decrease in mitochondrial-ER C18:0 ceramide content, without a change in sarcolemmal C18:0 ceramide or *sn*-1,2-DAG contents. In addition, mitochondrial-ER C22:0 and C24:0 ceramides decreased by ∼20% and sarcolemmal, mitochondrial-ER and cytosolic C24:1 ceramides increased by ∼40–50% after weight loss. Lipid droplet C18:1/C18:1 *sn*-1,3-DAG decreased by ∼25%, but weight loss did not alter *sn*-1,2-DAG or *sn*-2,3-DAG contents in any subcellular compartment. These results demonstrate that a decrease in mitochondrial-ER saturated ceramides, including C18:0, are associated with weight loss–induced improvements in skeletal muscle insulin sensitivity, but suggest changes in sarcolemmal *sn*-1,2-DAGs and ceramides are not responsible for the doubling of skeletal muscle insulin sensitivity induced by marked weight loss (decrease in mean BMI from 43.1 kg/m^2 to 35.0 kg/m^2) in people with obesity and T2D.

Skeletal muscle DAGs cause muscle insulin resistance in rodents and are associated with insulin resistance in people (7,16,18,25). Three DAG isomers (*sn*-1,2-DAG, *sn*-1,3-DAG, and *sn*-2,3-DAG) are present in cells, but only the *sn*-1,2-DAG isomers are capable of nPKC isoforms, such as PKCθ and PKCε (4). Activated nPKC isoforms translocate to the sarcolemma,

where they phosphorylate and inhibit insulin signaling effectors, thereby decreasing insulin receptor substrate 1–associated phosphatidylinositol-3-kinase activity and downstream insulin signaling (2,3,26). Skeletal muscle membrane-associated DAGs and nPKC activation are greater in people with T2D than in people who are normal weight (7,18,25). In addition, muscle insulin resistance induced by an acute lipid infusion in healthy lean adults is associated with an increase in membrane-associated DAG content and nPKC activation (18,19). Weight loss in people with obesity is associated with decreased muscle content of long-chain acyl-CoAs, which are DAG precursors (27). However, the effect of weight loss on skeletal muscle DAG content in people with obesity is unclear because of studies that showed an increase in several membrane-associated DAG species (28,29) and either no change (30,31) or a decrease (32) in total muscle DAG content after weight loss. A study that evaluated the effect of weight loss induced by bariatric surgery on muscle insulin sensitivity and muscle bioactive lipids found a threefold increase in membrane-associated C18:1/C18:1 DAG content at 12 and 24 weeks after surgery in conjunction with an increase in whole-body insulin sensitivity (28). Membrane-associated C18:1/C18:1 DAG content returned to presurgical levels at 52 weeks. Our data extend these previous observations by studying people with obesity and T2D before and after a marked increase in weight loss–induced muscle insulin sensitivity and evaluating multiple subcellular compartments, isomers, and species of DAGs to enhance our ability to detect whether total or individual DAG content changed after weight loss. The only DAG pool that decreased after weight loss was lipid droplet C18:1/C18:1 *sn*-1,3-DAG, which suggests adipose triglyceride lipase (ATGL) activity decreased after weight loss because ATGL acts at the lipid droplet to hydrolyze triacylglycerols and generate the *sn*-1,3-DAG isomer (33). These results are consistent with previous studies that found muscle ATGL activity is increased in people with obesity (25) and muscle ATGL protein content is negatively associated with insulin sensitivity (34).

Although sarcolemmal C18:0 ceramides in skeletal muscle are associated with muscle insulin resistance in both rodents and people (6–8,17), weight loss did not change sarcolemmal C18:0 ceramides in our participants. However, weight loss caused a 20% decrease in skeletal muscle mitochondrial-ER C18:0 ceramide content. This decline in mitochondrial-ER C18:0 ceramide is potentially physiologically important; we recently found that mitochondrial C18:0 ceramide content was 50% lower in people with "metabolically healthy obesity" than in people with "metabolically unhealthy obesity" and marked skeletal muscle insulin resistance (35). Our study cannot determine whether the decrease in mitochondrial-ER C18:0 ceramide content contributed to the weight loss–induced improvement in insulin sensitivity, was caused by the improvement in insulin sensitivity, or is simply an associated finding. Impaired mitochondrial function is strongly associated with insulin resistance in people (36–39), and expression of genes involved in mitochondrial structure and function is negatively correlated with mitochondrial ceramide content (9,35). Therefore, it is possible that the decrease in mitochondrial-ER C18:0 ceramide could have contributed to an increase in skeletal muscle insulin sensitivity by improving mitochondrial function, because mitochondrial C18:0 ceramide inhibits ADP-stimulated mitochondrial respiration and increases oxidative stress by depleting coenzyme Q (7,9). Moreover, blocking de novo ceramide synthesis via adenoviral *SPTLC1* silencing increases maximal mitochondrial respiration in primary human myoblasts from aged donors and increases muscle mitochondrial respiration in aged mice (40). Beyond effects on mitochondrial respiration and oxidative stress, the molecular mechanisms that could link mitochondrial ceramide accumulation to impaired insulin-stimulated muscle glucose transport remain elusive (9,41).

Weight loss also caused a marked increase in C24:1 ceramide content in multiple muscle compartments, including sarcolemmal, mitochondrial-ER, and cytosolic pools. The physiological importance of this observation is unclear because of conflicting results from previous studies that found total skeletal muscle C24:1 ceramide content increased after ∼8% diet-induced weight loss (32) but decreased after ∼20% bariatric surgery–induced weight loss (30). Unlike C18:0 ceramide, C24:1 ceramide does not impair mitochondrial ADP-stimulated respiration (7,42). The six ER-resident ceramide synthases (CerS1–CerS6) have different acyl chain substrate preferences, with C24:1 generation carried out by CerS2 and C18:0 synthesized by CerS1 (42–44). The reciprocal changes in C18:0 and C24:1 ceramide observed in the mitochondrial-ER fractions after weight loss suggest an increase in the ratio of CerS2 activity to CerS1 activity.

Our study was designed to maximize our ability to determine whether alterations in skeletal muscle DAGs or ceramides might be involved in mediating the beneficial effects of weight loss on muscle insulin sensitivity. Accordingly, we evaluated DAG isomers and ceramides, located in specific subcellular compartments, before and after marked weight loss and a twofold increase in muscle insulin sensitivity in people with obesity and T2D. However, our study also has some important limitations. Adequate muscle tissue for lipid analysis was only available from a subset (14 of 22) of participants who completed a previous weight loss study, limiting the statistical power to detect small weight loss–induced changes in muscle bioactive lipid content. In addition, we were unable to assess the key metabolic pathways that are regulated by muscle DAGs and ceramides (e.g., nPKC activation, insulin signaling, and mitochondrial respiration), because of the amount of muscle tissue needed to reliably conduct those analyses. We were also unable to achieve robust separation of mitochondrial and ER compartments in these samples, which is consistent with other reports (7) and likely caused by the presence of mitochondrial-associated membranes linking these organelles (45). Moreover, although the sarcolemmal

fraction was the only fraction containing the sarcolemmal marker (Na-K ATPase), we detected other subcellular marker proteins in the sarcolemmal fraction. We therefore cannot exclude the possibility that differences in sarcolemmal lipids were obscured by the presence of nonsarcolemmal compartments in that fraction. Although participants were instructed to maintain their usual sedentary physical activity, it is possible that some participants began to exercise after initiating the intervention, which could have independent effects on muscle lipids and insulin sensitivity (30). A control group of weight-stable participants were not studied, so we cannot exclude the possibility that day-to-day variability in the outcome measures or other behavioral changes associated with study participation affected the results. Finally, although body weight decreased and muscle insulin sensitivity markedly improved, the participants still had obesity, and mean HbA_{1c} was above the normal range, demonstrating metabolic function was not completely normalized.

In conclusion, marked weight loss and an increase in muscle insulin sensitivity in people with obesity and T2D were associated with a decrease in skeletal muscle mitochondrial-ER C18:0 ceramide content and increases in skeletal muscle sarcolemmal, mitochondrial-ER, and cytosolic C24:1 ceramides. However, changes in sarcolemmal *sn*-1,2-DAG and sarcolemmal C18:0 ceramide, or other putative lipid mediators of insulin resistance, were not detected, suggesting these lipids are not involved in mediating the beneficial effects of weight loss on muscle insulin sensitivity. These findings suggest muscle mitochondrial-ER C18:0 ceramides could be involved in regulating insulin action and support the need for further mechanistic investigations.

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Duality of Interest. S.K. serves on scientific advisory boards for Merck, Abbvie, Alnylam, and Boehringer Ingelheim. M.Y. is a current employee of Eli Lilly Japan. G.I.Sh. serves on the scientific advisory boards for OrsoBio, Novo Nordisk, AstraZeneca, Equator Therapeutics, iMBP, Forrest Research Institutes, Levels, Kriya, Arrowhead Pharmaceuticals, and Esperion. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. M.C.P. analyzed data and wrote the paper. M.Y. performed clinical studies. G.I.Sm. performed clinical studies, analyzed data, and edited the paper. R.C.G., M.K., and G.I.Sh. performed and supervised subcellular fractionation procedures and lipid measurements. D.S. analyzed data. S.K. designed the study and edited the paper. All authors approved the final version of the manuscript. S.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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