Intramyocellular Lipid Droplet Size Rather Than Total Lipid Content is Related to Insulin Sensitivity After 8 Weeks of Overfeeding

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Objective: Intramyocellular lipid (IMCL) is inversely related to insulin sensitivity in sedentary populations, yet no prospective studies in humans have examined IMCL accumulation with overfeeding.

Methods: Twenty-nine males were overfed a high-fat diet (140% caloric intake, 44% from fat) for 8 weeks. Measures of IMCL, whole-body fat oxidation from a 24-hour metabolic chamber, muscle protein extracts, and muscle ceramide measures were obtained before and after the intervention.

Results: Eight weeks of overfeeding did not increase overall IMCL. The content of smaller lipid droplets peripherally located in the myofiber decreased, while increases in larger droplets correlated inversely with glucose disposal rate. Overfeeding resulted in inhibition of Akt activity, which correlated with the reductions in smaller, peripherally located lipid droplets and drastic increases in ceramide content. Additionally, peripherally located lipid droplets were associated with more efficient lipid oxidation. Finally, participants who maintained a greater number of smaller, peripherally located lipid droplets displayed a better resistance to weight gain with overfeeding.

Conclusions: These results show that lipid droplet size and location rather than mere IMCL content are important to understanding insulin sensitivity.

Introduction

Type 2 diabetes is a condition diagnostically characterized by hyperglycemia, with insulin resistance being an early pathological insult. Repeatedly, studies have provided evidence that higher levels of intramyocellular lipid (IMCL) in nondiabetic insulin-sensitive and insulin-resistant patients are inversely related to insulin sensitivity (1). The exact mechanisms behind this relationship are not fully understood, but much research has been directed at this phenomenon.

To further complicate this relationship, endurance-trained athletes, who are highly insulin sensitive, also possess large quantities of IMCL (2). Similarly, we have previously shown that human primary myotubes collected from physically active, insulin-sensitive donors also have a greater IMCL content compared to myotubes collected from less-insulin-sensitive, sedentary individuals of similar body weight (3). In addition, longitudinal exercise training studies, which have long been known to improve insulin sensitivity, have not
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Prior studies have utilized experimental interventions of isocaloric high-fat diet (7), intravenous lipid infusions (8), and overfeeding (9). Because the obesogenic Western diet is associated with weight gain, insulin resistance, and a higher prevalence of obesity (10), the overfeeding experimental paradigm appears to be the best way to test the mechanisms underlying the deleterious effects of Western diets (11). We therefore endeavored to overfeed 29 healthy male participants a high-fat diet (140% of nutritional calories provided compared to resting metabolic rate; 44% macronutrient content from fat) for 8 weeks and found significant weight gain and reductions in insulin sensitivity (12). Given this, we explored alterations in IMCL content from three muscles: the soleus, the tibialis anterior, and the vastus lateralis.

Methods

Participant recruitment and study design

We recruited 29 male participants (age: 26.8 ± 5.4 years; BMI: 25.5 ± 2.3 kg/m²). All participants provided informed consent, and study parameters were approved by the Institutional Review Board of Pennington Biomedical Research Center. Our study was registered on ClinicalTrials.gov (NCT01672632). Prior to the 8-week overfeeding period, participants completed a 14-day measurement of free-living energy expenditure by doubly labeled water (13) to determine baseline energy requirements. Additionally, physical activity levels were assessed using triaxial accelerometers during that time period. During the second week of doubly labeled water, participants consumed an isocaloric diet (60% carbohydrate, 25% fat, 15% protein). Participants then underwent baseline clinical procedures; the same clinical measures were also conducted after 8 weeks of overfeeding. Following baseline measures, participants consumed a diet for the next 8 weeks of 44% fat, 15% protein, and 41% carbohydrate, with the total daily intake equating to 140% of their normal caloric intake to maintain body weight. Glucose disposal rate (GDR) was determined using a euglycemic-hyperinsulinemic clamp with an insulin infusion of 50 mU/min/m² and adjusted for fat-free mass.

Ex vivo palmitate oxidation measures in skeletal muscle

Approximately 75 mg of skeletal muscle tissue was homogenized and loaded into a trapping plate apparatus to assess gas exchange for fatty acid oxidation; 0.176 μM of total palmitate (0.088 μM of [1-14C]-palmitate in 0.088 μM of nonradiolabeled palmitate) was added to the muscle homogenate. Radiolabeled palmitate was obtained from American Radiolabeled Chemicals (St. Louis, Missouri). Radiolabeled 14CO₂ and incomplete acid-soluble intermediates from palmitate oxidation were assessed using scintillation counting. Data were adjusted to total protein content obtained from muscle homogenate as determined through the bicinchoninic acid assay (Pierce BCA, Thermo Fisher Scientific, Waltham, Massachusetts).

Immunohistochemical measures in vastus lateralis

Serial transverse sections (10 μm) of mounted biopsy samples were generated using a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, Pennsylvania) at −20°C and placed on slides (Fisherfinest; Fischer Scientific, Pittsburgh, Pennsylvania). Sections were then stained in a filtered solution of Oil Red O (300 mg/mL in 36% triethyolphosphate) for 30 minutes at room temperature. Thereafter, sections were incubated with primary antibodies for antihuman myosin heavy chain (MYH7) (type I myocytes) and MYH2 (type IIa myocytes) overnight at room temperature and subsequently incubated with fluorescent (FITC) (type IIa myocytes) and rhodamine (type I myocytes) conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, California). Type IIa fibers remained unstained. Images were visualized using a Leica microscope (Leica DM4000 B; Leica Microsystems, Bannockburn, Illinois) and digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and IMCL was analyzed using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, New York). Fiber type was determined by counting the total number of fibers and assessing the percentage of those positive for MYH7 and MYH2.
Mitochondrial ROS production

Mitochondrial reactive oxygen species (ROS) production was measured from methods adapted from Seifert et al. (18). Briefly, mitochondria were extracted from 200 mg of muscle tissue using the methods of Chappell and Perry (19,20) and suspended in an incubation media containing 120 mM KCl, 5 mM KH2PO4, 3 mM HEPES, 1 mM EGTA, and 0.3% BSA (pH 7.4). Mitochondria were then infused with palmitoyl carnitine (PC) (60 μM), and ROS production was accessed fluorimetrically as a rate of H2O2 emission using p-hydroxyphenylacetate (167 μM/L) and horseradish peroxidase (9 units/mL) in mitochondria suspended in incubation media. H2O2 emission was monitored for 25 minutes at 37°C in a fluorometer at an excitation of 320 nm and emission of 400 nm.

Protein expression

Total protein for all experiments was collected using RIPA buffer (Sigma, St. Louis, Missouri) supplemented with 2% Protease Inhibitor Cocktail (Sigma, St. Louis, Missouri), 2% Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, Missouri), and 2% Phosphatase Inhibitor Cocktail 3 (Sigma, St. Louis, Missouri). Protein content was assessed from total protein extracts using Western immunoblotting with the Criterion apparatus system using 4% to 14% sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels (all from Bio-Rad, Hercules, California) and adjusted to GAPDH (Cat no. AB9484; AbCam, Cambridge, Massachusetts). Imaging of Western blots was facilitated on the Odyssey infrared imaging system (LiCor, Lincoln, Nebraska). Antibodies against total Akt (Cat no. 9272), p-S473 Akt (Cat no. 9271), total IRS1 (Cat no. 2382S), p-S1101 IRS1 (Cat no. 2385), and mTOR (Cat no. 4517) were obtained from Cell Signaling Technology (Danvers, Massachusetts). The antibodies for PLIN2 (Cat no. NB110-40877) and PLIN3 (Cat no. NB110-40764) were obtained from Novus Biologicals (Littleton, Colorado).

Lipid droplet quantification within skeletal muscle

Lipid droplet quantification was obtained using gray-scale images of Oil Red O-stained muscle sections minimally processed in Fiji (21) through background subtraction using empirically derived background images. Regions of interest representing individual muscle fibers were manually drawn, and the central and peripheral (band) regions of interest were generated using the “Enlarge” and “Make Band” tools within Fiji. Lipid droplet counts and size distribution data were determined by using the “Analyze Particles” tool with a size exclusion of 2 to 70 pixels and circularity filter setting of 0.5 to 1.00.

Ceramide measures in skeletal muscle

Ceramide was measured using double extraction for lipids using 50 mg of skeletal muscle biopsy tissue and processed as previously shown (22). Liquid chromatography-electrospray ionization tandem mass spectrometry was used to quantify ceramides as previously described (22). According to the retention time standards of common product ions, and ions reflecting fatty acid substituents, all target ceramide species were quantified.

Limitations to measures

Because of the rigorous requirements by study participants for performing an overfeeding paradigm, as well as limitations in skeletal muscle biopsy material, it was impossible to obtain a complete data set for every data point measured in our study. Even though we recruited 29 participants, we were only able to obtain lipid droplet quantification on 25 participants. Additionally, certain procedures that required large quantities of biopsy material (ex vivo palmitate oxidation and ex vivo mitochondrial isolation for ROS generation) were only obtained on a subset of our participants.

Statistical analysis

Data were analyzed using GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, California). All data were determined to be normally distributed using the Shapiro-Wilk normality test. Data that were normally distributed were analyzed using parametric methods (Person r correlation coefficients and paired, two-way t tests), while data not normally distributed were analyzed using non-parametric methods (Spearman r correlation coefficients and Wilcoxon signed ranked paired tests). P ≤ 0.05 was considered statistically significant. All graphs are represented as mean ± SEM.

Results

Eight weeks of overfeeding altered lipid droplet size and location

Eight weeks of overfeeding in 29 males resulted in weight gain and an increase in percent fat accumulation but only a trend toward a decrease in insulin sensitivity (Table 1). Furthermore, there were no significant changes observed in the total content of IMCL in three separate muscles: soleus, tibialis anterior, or vastus lateralis (Figure 1A), nor were there differences in the fiber-type-specific content of IMCL in vastus lateralis (Figure 1B). However, we found significant reductions in the content of smaller-sized lipid droplets around the periphery of the myofiber (P = 0.005, Figure 1C) and trends toward reductions in the whole myofiber (P = 0.07, Supporting Information Figure S1A) and centrally located lipid droplets (P = 0.09, Supporting Information Figure S1B). GDR was inversely associated with the content of larger lipid droplets in the whole myofiber (r = −0.47, P = 0.02, Figure 1E) and centrally located lipid droplets (r = −0.60, P = 0.008, Supporting Information Figure S1C).

Changes in smaller lipid droplets were associated with less ROS generation from lipid oxidation and different lipid-associated proteins

Associations between lipid oxidation and lipid droplet size and localization were examined, and we found that baseline levels of smaller, peripherally located lipid droplets were associated with whole-body lipid oxidation, as indicated by the inverse trends with 24-hour RQ (r = −0.35, P = 0.09, n = 24, Figure 2A) and a significant inverse relationship with sleep RQ (r = −0.57, P = 0.004, n = 24, Figure 2B). Changes with overfeeding showed that decreases in the content of smaller lipid droplets trended toward an association with increases in whole-body lipid oxidation (r = 0.38, P = 0.07, n = 24, Figure 2C) as well as being significantly related to ex vivo lipid oxidation from the vastus lateralis muscle (r = −0.58, P = 0.04, n = 12, Figure 2D). Importantly, the production of ROS from PC using extracted mitochondria from the vastus lateralis revealed a preintervention relationship between levels of PC-induced ROS production and smaller, peripherally located lipid droplets (r = −0.72, P = 0.01, n = 11, Figure 2E). Additionally, the retention of smaller, peripherally located lipid droplets with overfeeding was
associated with less PC-induced ROS production ($r = -0.62$, $P = 0.04$, $n = 11$, Figure 2F).

Given the decline in smaller lipid droplets in our study and prior studies indicating that perilipin 2 (PLIN2) and perilipin 3 (PLIN3) were associated with larger and smaller lipid droplet sizes, respectively (23), we report a significant decrease in PLIN3 protein with overfeeding ($P = 0.04$, Figure 3A) and no change in PLIN2 protein content (data not shown). Using primary myotubes cultured from the donors from this study, we showed that the increase in PLIN2 protein content was dependent on incubation time ($200 \mu M$ palmitate, Figure 3B). In relation to this, we found a correlation between changes in PLIN2 protein content in skeletal muscle and the decrease in smaller, peripherally located lipid droplets ($r = -0.48$, $P = 0.02$, data not shown). Importantly, there was an inverse correlation between changes in PLIN2 protein content and changes in GDR ($r = -0.43$, $P = 0.03$, Figure 3C).

Smaller lipid droplet depletion was associated with reduced Akt phosphorylation and increased ceramides

While seeking to understand the mechanisms responsible for the interaction between glucose disposal and lipid droplet redistribution, we found a significant reduction in the phosphorylation of protein kinase B (PKB/Akt) at S473 ($P = 0.05$, Figure 4A) after overfeeding, which was associated with the depletion of smaller, peripherally located lipid droplets ($r = 0.47$, $P = 0.04$, data not shown). There were no differences in the phosphorylation of insulin receptor substrate 1 (IRS1) at S1101 (data not shown). Given that a high ceramide content has been shown to reduce Akt phosphorylation independent of altering other aspects of the insulin signaling cascade (24), we measured the muscle content of ceramides and found it to be increased with overfeeding ($P < 0.001$, Figure 4B) regardless of the subspecies (Figure 4C). Ceramide content was inversely related to S473-Akt phosphorylation ($r = -0.47$, $P = 0.04$, Figure 4D). In addition, mammalian target of rapamycin (mTOR) protein content increased in skeletal muscle with overfeeding ($P = 0.05$, Figure 4E).

Retention of smaller lipid droplets was associated with higher physical activity and resistance to weight gain induced with overfeeding

Prospectively, we found that those with higher baseline physical activity levels ($r = 0.64$, $P = 0.002$), higher metabolic equivalents ($r = 0.47$, $P = 0.03$), and higher total daily energy expenditure

## Table 1: Study characteristics of the 25 male participants who had lipid droplet analysis performed at baseline and after 8 weeks of overfeeding

| Characteristic              | Baseline, mean ± SD | Post-overfeeding, mean ± SD |
|----------------------------|---------------------|-----------------------------|
| **Anthropometric characteristics** |                     |                             |
| Weight (kg)                | 82.8 ± 9.2          | 89.7 ± 9.4<sup>a</sup>     |
| % Fat                      | 19.7 ± 4.9          | 22.5 ± 5.2<sup>a</sup>     |
| FM (kg)                    | 16.4 ± 4.8          | 20.4 ± 5.6<sup>a</sup>     |
| FFM (kg)                   | 66.0 ± 7.4          | 69.5 ± 7.4<sup>a</sup>     |
| SAT (kg)                   | 4.1 ± 1.4           | 5.3 ± 1.7<sup>a</sup>      |
| VAT (kg)                   | 0.59 ± 0.50         | 0.95 ± 0.59<sup>a</sup>    |
| **Metabolic characteristics** |                     |                             |
| 24-hour RQ                 | 0.90 ± 0.02         | 0.88 ± 0.03<sup>a</sup>    |
| GDR (mg/min/EMBS)          | 10.92 ± 2.17        | 10.48 ± 2.39<sup>a</sup>   |
| EGP (mg/min/kg)            | 0.06 ± 0.16         | 0.28 ± 0.30<sup>a</sup>    |
| EGP % suppression          | 96 ± 10             | 82 ± 20<sup>a</sup>        |
| **Serum measures**         |                     |                             |
| Glucose (mg/dL)            | 90.4 ± 5.2          | 91.5 ± 7.2                 |
| Insulin (µU/mL)            | 6.6 ± 4.1           | 7.1 ± 5.2                  |
| FFA (nmol/L)               | 0.27 ± 0.09         | 0.30 ± 0.10                |
| Triglycerides (mg/dL)      | 87.3 ± 61.5         | 100.0 ± 73.0<sup>a</sup>   |
| Total cholesterol (mg/dL)  | 176.6 ± 20.9        | 196.5 ± 30.0<sup>a</sup>   |
| HDL-C (mg/dL)              | 56.1 ± 13.0         | 56.9 ± 11.1                |
| LDL-C (mg/dL)              | 103.1 ± 20.0        | 119.6 ± 26.4<sup>a</sup>   |
| Cholesterol/HDL            | 3.34 ± 1.00         | 3.58 ± 0.93<sup>a</sup>    |
| HDL/LDL                    | 0.57 ± 0.20         | 0.50 ± 0.16<sup>a</sup>    |

<sup>a</sup>P < 0.05.

<sup>b</sup>P < 0.10.

FM, fat mass; FFM, fat-free mass; SAT, abdominal subcutaneous adipose tissue; VAT, visceral adipose tissue; RQ, respiratory quotient; GDR, glucose disposal rate (from a 50 mIU/min/m² insulin infusion); EGP, endogenous glucose production ($n = 19$); EMBS, estimated mean body size (FFM + 17.7); FFA, free fatty acids; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
were relatively protected against a decrease in smaller, peripherally located lipid droplets in response to 8 weeks of overfeeding. Importantly, participants with higher levels of smaller, peripheral lipid droplets were also more resistant to weight gain during overfeeding ($r = -0.44$, $P = 0.03$, Figure 5).

**Discussion**

Our study highlights for the first time that prospective examination of IMCL content, on average, does not change with 8 weeks of overfeeding. The participants of this study gained 7.6 kg on average over the 8-week period, and our original hypothesis was that overfeeding would result in increased IMCL. Surprisingly, there were no differences in IMCL measured in the soleus, tibialis anterior, and vastus lateralis muscles (Figure 1A), regardless of fiber type examined in the vastus lateralis (Figure 1B). These results led us to investigate how alterations in lipid organization within muscle might influence insulin sensitivity.

IMCL is stored in the form of lipid droplets, thus implicating lipid droplet size and subcellular localization to be important in understanding skeletal muscle insulin resistance. Prior reports have shown reductions in lipid droplet size with weight loss associated with increased insulin sensitivity (25). Subcellular localization of lipid

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**(Figure 1)** Overfeeding-associated increases in the content of large myofiber lipid droplets are related to reductions in insulin sensitivity. (A) Total intramyocellular lipid (IMCL) content in the soleus and tibialis anterior via magnetic proton resonance spectroscopy and (B) in the vastus lateralis using Oil Red O staining in frozen tissue from biopsy did not increase with overfeeding ($n = 29$ soleus and vastus lateralis, $n = 28$ tibialis anterior). (C) Lipid droplet size from the vastus lateralis was determined as centrally vs. peripherally located droplets, and the content of small lipid droplets located peripherally decreased ($P = 0.005$, $n = 25$). (D) Representative image of histological cross sections of the myofibers stained with Oil Red O from the same participant before and after overfeeding. Borders indicate the demarcation between central and peripheral location and the outer border of the myofiber. Arrows indicate the large lipid droplets that accumulated with overfeeding in this participant. (E) Inverse relationship between the percent change in larger lipid droplets throughout the entire fiber and the percent change in glucose disposal rate with overfeeding ($r = -0.47$, $P = 0.02$, $n = 23$). Graphs represents mean ± SEM. *$P < 0.05$. 

$(r = 0.50, P = 0.01)$
droplets has been shown to be different based on physical activity with highly active individuals having more lipid droplets around the periphery of the myofiber (26). Furthermore, stimulated contraction of muscle shows a preference for depleting lipid droplets around the outer bands of the myofiber with increased lipid oxidation (27). These insights led us to hypothesize that overfeeding would result in a reduction in the content of smaller, peripherally located lipid droplets, and that these reductions would be associated with worsening in insulin sensitivity. Indeed, there were significant reductions in the content of smaller-sized lipid droplets around the periphery of the myofiber (Figure 1B), as well as trends toward reductions of both centrally located lipid droplets (Supporting Information Figure S1A) and globally throughout the whole myofiber (Supporting Information Figure S1B). Our present study highlights a relationship between the change in glucose infusion rates with the change in the content of larger lipid droplets in the whole myofiber (Figure 1E) and centrally located lipid droplets (Supporting Information Figure S1C).

To understand the mechanisms responsible for the interaction between glucose infusion rates and lipid droplet redistribution, we first examined the canonical insulin signaling pathway, which showed a significant reduction in the phosphorylation of Akt-S473 (Figure 2A) but not phosphorylation of IRS1-S1101. Given this observation, we aimed specifically to understand why Akt might be altered without changes in IRS1, focusing on ceramide targets that are known to disrupt insulin signaling at the Akt level independent of altering other aspects of the signaling cascade (24). Additionally, studies in humans have shown that increased ceramide content in muscle is associated with insulin resistance (28). Our study did show that ceramide content significantly increased in muscle following overfeeding (Figure 2B–2C); however, it should also be noted that though the average levels of ceramides increased significantly, insulin sensitivity only trended toward a decrease (Table 1). Ceramide content, though, was inversely related to S473-Akt phosphorylation (Figure 2D). Given recent investigations implicating mTOR signaling in ceramide-induced inhibition of Akt

Figure 2 Alterations in lipid droplet size are related to changes in lipid oxidation. (A) Baseline content of small, peripherally located lipid droplets was more associated with whole-body fat oxidation, as evident by inverse relationships with 24-hour respiratory quotient (RQ, \( r = -0.36, P = 0.09, n = 24 \)) and (B) sleep RQ (\( r = -0.57, P = 0.004, n = 24 \)). (C) Changes in smaller lipid droplet content tended to correlate positively with changes in 24-hour RQ (\( r = 0.38, P = 0.07, n = 23 \)) and (D) correlated inversely with ex vivo total palmitate oxidation (\( r = -0.58, P = 0.04, n = 12 \)). (E) Smaller, peripherally located lipid droplets at baseline were inversely associated with palmitate-produced mitochondrial reactive oxygen species (ROS) production (\( r = -0.72, P = 0.01, n = 11 \)). (F) Changes in the content of smaller, peripherally located lipid droplets inversely correlated with changes in palmitate stimulated ROS production ex vivo from isolated mitochondria (\( r = -0.65, P = 0.03, n = 11 \)).

Figure 3 Alterations in lipid droplet size are related to changes in perilipin 2 (PLIN2) and perilipin 3 (PLIN3) protein expression with overfeeding. (A) PLIN3 protein content decreased with overfeeding (\( P = 0.04, n = 27 \)). (B) Baseline human primary myotubes treated with 200 \( \mu \text{M} \) of palmitate showed that PLIN2 is expressed later over a time course of 2 hours. (C) Changes in PLIN2 protein content correlated inversely with glucose disposal rate (GDR; \( r = -0.43, P = 0.03, n = 25 \)). Graphs represent mean ± SEM. *\( P < 0.05 \).
activity (29), we investigated levels of mTOR and found a significant increase in mTOR protein content (Figure 2E) after overfeeding. Based on these results, we postulate that increases in intramyocellular ceramide content were responsible for the inhibition of insulin signaling. In addition, because smaller, peripherally located lipid droplets are preferentially oxidized (27), we speculate that the depletion of these lipid droplets would be linked to either reductions in lipid oxidation, inefficiencies in lipid oxidation, or both, which in turn would contribute to the accumulation in ceramide content.

Isoenergetic high-fat consumption has been shown to increase lipid oxidation (30), and thus we examined any associations between lipid oxidation and lipid droplet size or localization. Baseline levels of smaller, peripherally located lipid droplets were associated with whole-body lipid oxidation, as indicated by the inverse correlations with sleep RQ. However, we found that decreases in the content of smaller lipid droplets after overfeeding were associated with increased whole-body lipid oxidation as well as ex vivo lipid oxidation in vastus lateralis muscle. In connection to this, an increase in lipid oxidation after the high-fat diet is often incomplete, resulting in the production of oxidative lipotoxic species (31,32), and smaller lipid droplets are more associated with less lipotoxic fat oxidation. To explore this further, we measured the production of ROS from PC using extracted mitochondria from the vastus lateralis and found that preintervention levels of PC-induced ROS production were inversely associated with smaller, peripherally located lipid droplets.
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induced alterations in skeletal muscle lipid droplet morphology. Peripherally located lipid droplets are resistant to weight gain with overfeeding. Gained less weight with overfeeding, indicating that participants who had higher preintervention levels of smaller, peripherally located lipid droplets were being depleted, perhaps lipid droplets that remained were being packaged with PLIN2. Though we did not see a change in PLIN2 protein content with overfeeding, we did see an inverse correlation between a decrease in smaller, peripherally located lipid droplets and changes in PLIN2 protein content.

Additionally, the retention of smaller, peripherally located lipid droplets with overfeeding was associated with less PC-induced ROS production, suggesting that smaller, peripherally located lipid droplets produce more efficient, less lipotoxic oxidation with overfeeding.

In order to further understand connections between lipid droplet size and lipid oxidation, we examined the alterations in key lipid droplet-associated proteins within the skeletal muscle. Lipid droplet-associated proteins, particularly the perilipin family (23), have been shown to coat lipid droplets based on their size (33) and have also been shown to play a role in lipid oxidation. Recently, we showed that PLIN3 was positively associated with both whole-body in vivo and skeletal muscle ex vivo lipid oxidation (34). Given that our study shows a decline in smaller lipid droplets after overfeeding and prior studies have shown an association between PLIN3 and smaller lipid droplets (23), a significant decrease in PLIN3 protein content with overfeeding (Figure 3A) is in line with present literature. Additionally, the expression of PLIN2 is associated with larger lipid droplets (23). Though prior studies have shown PLIN2 to become expressed with increasing duration of lipid incubation using mice and mouse cell lines (35), we show for the first time using primary myotubes cultured from the donors from this study that the increase in PLIN2 protein is dependent on incubation time (200 μM palmitate, Figure 3B). Based on these data, we speculate that as smaller lipid droplets were being depleted, perhaps lipid droplets that remained were being packaged with PLIN2. Though we did not see a change in PLIN2 protein content with overfeeding, we did see an inverse correlation between changes in PLIN2 protein content and changes in GDR (Figure 3C), suggesting that larger, PLIN2-coated lipid droplets may be associated with incomplete lipid oxidation, thus resulting in the accumulation of ceramides and ROS production and the disruption of insulin signaling.

Finally, given the connections between lipid droplet size and lipid oxidation as well as the increase in lipid oxidation response with physical fitness (36), we aimed to determine whether physical fitness is related to lipid droplet size. Individuals with higher preintervention physical activity level, metabolic equivalents, and total daily energy expenditure were protected against a decrease in the number of smaller, peripherally located lipid droplets with 8 weeks of overfeeding. Furthermore, a higher content of smaller, peripheral lipid droplets prior to overfeeding was negatively associated with an overfeeding-induced weight gain (Figure 5).

One of the limitations of this study, given the translational nature of this investigation, is that our data rest on correlational analyses between lipid droplet size and location within the myofiber and various components of human metabolism. However, this limitation should not by itself make the data that we have presented any less valuable, given the fact that all our data are based exclusively in human tissue collected from a clinical investigation. In addition, our study was only performed in healthy men and thus not completely translatable to the population as a whole. We originally aimed to recruit an equal proportion of men and women to our study, but we were not able to recruit and retain enough female participants to this high-fat overfeeding study, and thus we restricted our focus to only male participants. Finally, this study was conducted in healthy individuals without insulin resistance or type 2 diabetes, which could limit our understanding of the progressive pathophysiology relating to individuals who already have type 2 diabetes and still maintain unhealthy eating practices.

Overall, our investigation in lean men indicates that the size and location of lipid droplets, rather than the total IMCL content, are determining factors for the magnitude of the increase in insulin resistance and the resistance to weight gain with obesogenic overfeeding. We show that smaller, peripherally located lipid droplets are reflective of better physical fitness and efficient lipid oxidation, which mirrors prior investigations of myofiber lipid droplet location in trained athletes (26). Given the athlete’s paradox, whereby highly

Figure 5 Preintervention levels of smaller, peripherally located lipid droplets in muscle are associated with less weight gain during overfeeding. (A) Participants who had higher preintervention levels of smaller, peripherally located lipid droplets gained less weight with overfeeding, indicating that participants who have smaller, peripherally located lipid droplets are resistant to weight gain with overfeeding (r = −0.44, P = 0.03, n = 25). (B) Schematic diagram examining the effects of diet-induced alterations in skeletal muscle lipid droplet morphology.
trained athletes possess large quantities of IMCL and yet possess high insulin sensitivity (2). IMCL alone is not the only culprit behind skeletal muscle insulin resistance. Our data suggest that packaging of lipid into smaller, peripherally located lipid droplets may hold more benefit for improving insulin sensitivity in individuals with type 2 diabetes (Figure 5B). Future investigations should be aimed at remodeling lipid droplet size and location to treat insulin resistance, as well as other factors that regulate lipid droplet size and packaging in relation to lipid oxidation in skeletal muscle.

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