Intramuscular Lipid Content Is Increased in Obesity and Decreased by Weight Loss

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The triglyceride content of skeletal muscle samples determined by lipid extraction correlates with the severity of insulin-resistant glucose metabolism in muscle. To determine whether this reflects increased triglyceride within muscle fibers and to test the hypothesis that the lipid content in muscle fibers is increased in obesity, the present study was undertaken using quantitative histochemistry of Oil Red O staining of vastus lateralis muscle. A percutaneous muscle biopsy was performed in 9 lean subjects, 15 obese subjects without type 2 diabetes mellitus (DM), and 10 obese subjects with type 2 DM (body mass index [BMI], 23.4 ± 1.0, 33.6 ± 0.6, and 36.0 ± 1.1 kg · m^{-2} for lean, obese, and DM, respectively). Eight obese and 7 DM subjects had a weight loss and reassessment of muscle lipid content. Transverse muscle cryosections were examined by light microscopy with quantitative image analysis (grayscale images obtained by analog to digital conversion) to determine a lipid accumulation index (LAI) based on the percentage of cross-sectional fiber area occupied by lipid droplets. Muscle fiber lipid content was greater in obese individuals with DM than in lean individuals (3.62% ± 0.65% *v* 1.42% ± 0.28%, $P < .05$) but was not different in obese individuals without DM (2.53% ± 0.41%). Weight loss reduced the LAI from 3.43% ± 0.53% to 2.35% ± 0.31%. In summary, lipid accumulation within muscle fibers is significantly increased in obesity and is reduced by weight loss. This provides important information regarding the accumulation and distribution of skeletal muscle triglyceride in type 2 DM and obesity.

OBESITY causes insulin resistance,[1] a metabolic impairment that strongly implicates skeletal muscle.[2] Regional fat distribution, particularly abdominal fat, is a strong correlate of insulin resistance.[3-6] Additionally, several studies indicate that lipid content within skeletal muscle is correlated with insulin resistance.[6-9] In a recent study,[7] fat content in human skeletal muscle biopsy samples measured by lipid extraction was associated with insulin resistance independently of the effect of overall adiposity. In our laboratories, a noninvasive computed tomographic (CT) imaging method has been used to profile muscle composition,[10] demonstrating that lipid content in muscle correlates with insulin resistance independently of central and overall adiposity.[6,11] This prior investigation indicated that increased lipid content within skeletal muscle in obesity as reflected by CT is associated with decreased activity of oxidative enzymes.[11] Increased skeletal muscle lipid determined with CT can also be found in obese patients with type 2 diabetes mellitus (DM).[10] These investigations of human skeletal muscle are consistent with the findings from animal studies that fat accumulation in muscle is an important determinant of insulin resistance.[9,12,13] Weight loss can reduce or ameliorate insulin resistance associated with obesity and type 2 DM,[14-16] but the effect of weight loss on the amount of lipid stored within skeletal muscle is unknown.

An important issue that has not been fully addressed is whether these methods detect triglyceride accumulation within muscle fibers. Although muscle biopsy samples are typically trimmed of visible fat infiltration, biochemical lipid extraction does not clearly define the location of lipid inside versus outside muscle fibers. A similar restriction pertains to noninvasive imaging of muscle by CT, due to the limitations of resolution. A novel magnetic resonance spectroscopic method to assess intracellular versus extracellular fat content in muscle has been reported,[17] and although the precision of this determination has not been firmly established, the data suggest that fat accumulation within muscle is a key determinant of insulin resistance. Therefore, it is important to quantify the triglyceride within muscle fibers, and histologic methods would provide a potential method. In support of this concept, Phillips et al[8] used Oil Red O staining of neutral lipid and found that human skeletal muscle lipid content correlated with a reduced insulin-stimulated activity of glycogen synthase, an enzymatic marker of skeletal muscle insulin resistance.[18] However, the use of a subjective histological scoring system for lipid staining was a limitation in that study.[8]

The methodologic purpose of the current study was to apply the Oil Red O staining method for neutral lipid in conjunction with digital image analysis to achieve a more objective quantitation of lipid staining within skeletal muscle. This approach was used to test the hypothesis that the content of triglyceride is increased within muscle fibers in obesity and in relation to type 2 DM, and to assess whether a weight loss intervention, known to decrease adiposity, also reduces triglyceride content in muscle.

SUBJECTS AND METHODS

Subjects

Nine lean, 15 obese, and 10 obese subjects with type 2 DM between the ages of 25 to 45 years were recruited by public advertisement. Prior to participation, all potential research subjects underwent a medical screening evaluation, including a 2-hour 75-g oral glucose tolerance test. The nondiabetic had normal glucose tolerance. All subjects were normotensive and had fasting triglyceride and cholesterol levels less than 300 mg/dL without any antihypertensive or lipid-lowering medications. Women using oral contraceptives were excluded. DM subjects

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discontinued oral antidiabetic medications for at least 2 weeks before the study, and none previously received insulin. DM patients were excluded if they had moderate to severe complications of DM such as retinopathy or peripheral neuropathy. None of the subjects were currently engaged in exercise training, and all were weight-stable for a period of not less than 3 months. The study was approved by the University of Pittsburgh Institutional Review Board, and informed written consent was obtained from each subject.

Muscle Biopsies

Subjects were instructed not to perform physical exercise for 48 hours before the muscle biopsy procedure, to help prevent the acute effects of exercise on muscle triglyceride.[19] The subjects received a standard 10-kcal/kg meal consisting of 50% carbohydrate, 30% fat, and 20% protein on the night before the biopsy and then fasted overnight. Those participating in the weight loss program described later had a second muscle biopsy under similar conditions.

Muscle biopsies were obtained from the middle region of the vastus lateralis muscle (15 cm above the patella) and approximately 2 cm away from the fascia by the percutaneous needle-biopsy technique as described by Evans et al[20] Muscle specimens were trimmed, mounted, and frozen in isopentane cooled at −160°C by liquid nitrogen and stored at −80°C for histochemical analysis.

Histochemical Analysis

Lipid staining was performed using the Oil Red O soluble dye that stains neutral lipid (mainly triglycerides) with an orange-red tint.[21] The lipid content was determined on light-microscopic micrographs of 8-μm thick transverse cryostat sections. Initial sections from each frozen muscle block were inspected without stain to ensure that proper cross-sectional cuts were obtained, and if not, then the orientation was adjusted and this process repeated until good cross-sectional orientation was obtained. Cryosections of 8 μm were obtained at −29°C (HM505E; Micron, Walldorff, Germany), mounted on glass slides, air-dried for 15 minutes, and then immersed in a working solution of Oil Red O (Fisher Scientific, Fair Lawn, NJ) for 10 minutes. The Oil Red O solution was prepared as a stock solution (300 mg Oil Red O in 100 mL isopropanol 99%), and prior to each staining session, a working solution (12 mL Oil Red O stock + 8 mL DH_2O) was filtered (Whatman #42; Whatman, Maidstone, UK) to remove crystallized Oil Red. Slides were rinsed in DH_2O for 40 seconds (2 × 20 seconds) and then rinsed for another 10 minutes with running tap water. After air-drying, stained sections were covered with a cover slip using pure glycerol (Sigma, St Louis, MO) as an organic mounting medium, and then sealed around the edges of the cover slip using a butyl acetate–based acrylic polymer (Pavion, Nyack, NY) to prevent cover slip movement during microscopy.

An Olympus light microscope (Provis, Japan) was used to examine the stained muscle sections using a 40× oil immersion objective and bright field settings. Images were digitally captured using a CCD camera (Sony, Tokyo, Japan). Eight contiguous fields of view within the biopsy section that were free of artifact were analyzed for lipid content; quantitative image analysis was then performed on at least 80 fibers, or approximately 10 contiguous fibers per field.

Images from each file were saved as grayscale images (Tagged Image File Format) and the digitized data were then analyzed with the free software NIH Image (rsb.info.nih.gov/nih-image/). Oil Red staining was quantified by establishing thresholds for the intensity of staining using the image analysis software. The full range of grayscale imaging was 0 to 255 (arbitrary units [AU]), where 255 represents white, or no intensity, and hence no staining and 0 represents black, or complete staining. Staining intensities between these two extremes represent a continuum from white to black when converted to grayscale. The field of view for each image was 750 × 550 pixels, and a scale was captured for image calibration such that each pixel represented 0.18 μm^2. To quantify Oil Red staining, pixels with intensities at least beyond the midpoint of this scale (<150 AU) were quantified. Adjusting this threshold up or down up to 30 AU had minimal impact on staining quantitation (data not shown), and thus this threshold was not varied more than this amount for any field. Once thresholds were set, quantitation then proceeded by software, and data were expressed as the number and area (square centimeters) of staining per area of the image field. The sum of stained areas for each of 8 fields was used to quantify neutral lipid content per unit area of muscle fiber. Muscle sectioning, staining, and image analysis were performed in a blinded manner with respect to group. The area occupied by lipid staining in at least 80 fibers per subject was measured with the NIH Image software. The lipid accumulation index (LAI) was calculated as LAI = total area occupied by lipid droplets of muscle fiber · 100/total cross-sectional area of muscle fiber. The LAI was calculated for each of 8 fields within the section, and a mean LAI was then calculated for each subject. A control section treated with acetone and subsequently stained showed no visible staining, and thus no background staining was observed.

Field-to-field variation in the LAI might influence the mean LAI for each individual. Each field comprised 8 to 10 fibers, and there is variation in fiber-to-fiber lipid staining (Fig 1). However, this does not appear to influence the LAI significantly. In a subset of 13 subjects (7 obese and 6 lean), the mean LAI in an analysis of 4 fields was 2.30% ± 0.55% and remained at a stable value of 2.31% ± 0.47% when 8 fields were analyzed. We conclude that the analysis of 8 fields or approximately 80 fibers provides a reliable mean value for the LAI.

Weight Loss

Eight obese subjects and 7 obese type 2 diabetics completed a 16-week medically supervised weight loss program designed to achieve at least a 10-kg weight loss. For the first 10 weeks, subjects consumed a very–low-calorie diet (800 kcal/d) consisting of liquid formula (Optifast; Novartis, Minneapolis, MN) and lean meat, fish, and fowl. During weeks 11 to 13, subjects consumed 1,200 kcal/d, with a gradual reintroduction of fruits, vegetables, and grains to the diet. During weeks 14 to 16, subjects consumed a weight-maintaining diet with 30% of calories from fat, 15% from protein, and 55% from carbohydrate. Post–weight loss assessments were performed at week 17 to avoid the confounding effects of acute fasting or caloric restriction. Whole-body fat mass and fat-free mass were assessed in lean subjects and in obese and DM subjects prior to and following weight loss with dual-energy x-ray absorptiometry (model DPX-L; Lunar, Madison, WI) using software version 1.3Z. Details of this protocol have been previously reported.[15]

Maximal Aerobic Capacity

Physical fitness was assessed before and after weight loss by measuring the maximal aerobic capacity ($\dot{V}O_2$ max) during an incremental protocol on an electronically braked cycle ergometer (Dimeq ERG 601, Delft, The Netherlands). Briefly, men and women began exercising for 2 minutes at 100 and 50 W, respectively, after which the resistance was increased 25 W every 2 minutes until volitional fatigue. Expired air was collected via a mouthpiece and 2-way breathing valve into a 5-L mixing chamber (RMC-1; Rayfield, Waitsfield, VT) containing a bidirectional turbine to measure expiratory flow. A mass spectrometer (Marquette Electronics, Milwaukee, WI) was used to analyze expired air for CO_2 and O_2 fractions for determination of oxygen consumption ($\dot{V}O_2$) every 30 seconds.

Statistical Analysis

Data are presented as the mean ± SE unless otherwise indicated. One-way and 2-way ANOVA was used to compare muscle lipid data from lean and obese individuals, and to determine an interaction with

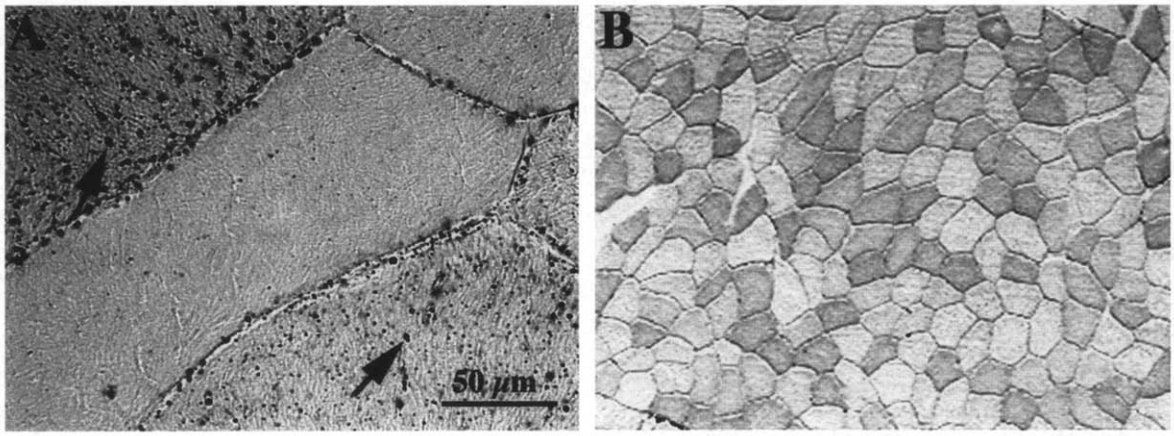

Fig 1. Oil Red O staining of neutral lipid within skeletal muscle at (A) 60× and (B) 10× magnification. 40× images were converted to grayscale for quantification of lipid staining. The arrows are directed at lipid droplets viewed as distinct spots of stain of approximately 1 μm in diameter. Heterogeneity in lipid content with respect to muscle fiber type is shown in A and B.

type 2 DM. Paired *t* tests were used to compare the effects of weight loss on skeletal muscle lipid content. All statistical analyses were performed using JMP for Macintosh (SAS Institute, Cary, NC).

RESULTS

Clinical Characteristics

The lean group consisted of 3 women and 6 men. The group of 25 obese subjects included 15 subjects without DM, consisting of 5 women and 10 men, and 10 subjects with type 2 DM, consisting of 5 women and 5 men. Subject characteristics are presented in Table 1. According to the study design, obese subjects had a higher body mass index (BMI) and a greater percentage of body fat than the lean subjects. There were no differences in aerobic capacity ($\dot{V}O_2max$) among these sedentary groups. The obese DM group was slightly older than the lean subjects ($P < .05$), but age was not different in obese subjects without DM.

Muscle Lipid Content in Lean, Obese, and Obese Type 2 DM

Oil Red staining of vastus lateralis muscle fibers, performed on cross-sections of percutaneous biopsy samples, ranged from 0.6 to 1.3 μm in diameter (Fig 1). The quantitative data on Oil Red O staining of muscle are shown in Table 2. There were significant group differences ($P < .05$) for the number of lipid-stained spots within muscle fibers, for the total area of lipid staining, and for the LAI, which is the percent of fiber area occupied by lipid staining. In pairwise post hoc comparisons, the differences between muscle from lean and type 2 DM subjects were significant, while lean compared with obese nondiabetic subjects did not quite achieve a statistical difference ($P = .07$). In addition, the LAI was positively associated with the BMI ($r = .42$, $P < .01$) but was not related to age ($r = .30$, $P = .13$) or $\dot{V}O_2max$ ($r = .29$, $P = .2$). There was no significant difference in quantitative Oil Red O staining between muscle from obese and type 2 DM subjects ($P = .2$).

Effects of Weight Loss on Muscle Lipid Content

Eight obese subjects lost a mean of 15.0 ± 1.5 kg body weight during the 12-week weight loss program, whereas 7 obese DM subjects lost a mean of 14.2 ± 1.9 kg while participating in an identical program. Expectedly, the proportion of total body fat decreased from 38.1% ± 1.3% to 31.5% ± 1.5% in the obese group and from 40.8% ± 2.4% to 33.5% ± 2.2% in the obese DM group. $\dot{V}O_2max$ did not change following weight loss in either obese subjects (2.31 ± 0.9 *v* 2.22 ± 0.9 $L \cdot min^{-1}$) or obese DM subjects (1.96 ± 0.25 *v* 2.00 ± 0.22 $L \cdot min^{-1}$).

The weight loss intervention reduced the number of Oil Red O staining spots, the total Oil Red O staining area, and the fat content relative to fiber area (LAI, $P < .01$; Table 3). The effect of weight loss was similar in obese and type 2 DM subjects, although the weight loss reduction in muscle lipid did not quite reach significance in the obese subjects ($P = .07$; Fig 2). Weight loss reduced the lipid content within muscle fibers by a mean value of 31% (23% and 41% for obese and DM, respectively).

Table 1. Descriptive Characteristics of the Lean, Obese, and DM Subjects

Characteristic	Lean (n = 9)	Obese (n = 15)	DM (n = 10)
Age (yr)	30 ± 2.0	36 ± 1.3	45 ± 2.2*
Body mass (kg)	68 ± 5	105 ± 4*	103 ± 6*
BMI (kg/m^2)	23.4 ± 1.1	33.6 ± 0.8	36.0 ± 0.9
Body fat (%)	14.9 ± 1.8	35.3 ± 1.9*	40.2 ± 2.2*
$\dot{V}o_2$ max ($L \cdot min^{-1}$)	1.99 ± 0.16	2.39 ± 0.10	1.96 ± 0.25

NOTE. Values are the mean ± SE.
*$P < .05$ *v* lean.

Table 2. Parameters of Fat Staining for the Lean, Obese, and DM Groups

Parameter	Lean (n = 9)	Obese (n = 15)	DM (n = 10)
No. of spots	1,278 ± 205	1,985 ± 216*	2,445 ± 309*
Total fat (μm^2)	964 ± 182	1,641 ± 248*	2,431 ± 438*
Mean area of spots (μm^2)	0.74 ± 0.04	0.78 ± 0.05	0.93 ± 0.07
LAI (%)	1.42 ± 0.28	2.53 ± 0.41	3.62 ± 0.65*

NOTE. Values are the mean ± SD.
*$P < .05$ *v* lean.

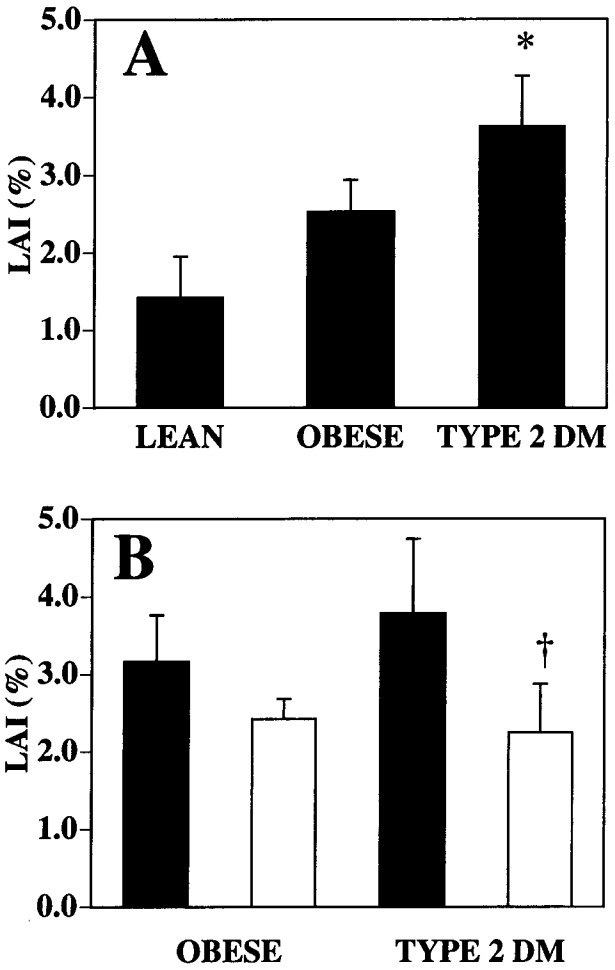

Fig 2. (A) Skeletal muscle lipid content represented by the mean LAI in lean, obese, and obese type 2 DM subjects. Values are the mean ± SE. *$P < .05$ *v* lean. Difference in LAI between obese and lean, $P = .07$. (B) Muscle lipid content (LAI) in obese subjects with weight loss (WL). (■) Pre-WL values, (□) post-WL values. †$P < .05$ *v* pre-WL. Change in the mean LAI in obese group, $P = .07$.

Reproducibility of Lipid Staining Quantification

To address the reproducibility of the LAI, we examined a test-retest paradigm of lipid staining and quantitation. The results are presented in Table 4. Lipid staining was performed on separate sections of the same biopsy specimen from 5 subjects on two separate occasions by separate microscopists. The variation in the LAI for the mean of two measures averaged 0.48% for the 5 subjects. Thus, the interassay, interobserver variance is substantially less than group differences in the LAI.

Table 3. Parameters of Fat Staining in 15 Obese and DM Subjects Following Weight Loss

Parameter	Pre-WL	Post-WL
No. of spots	2,321 ± 232	1,908 ± 169*
Total fat (μm^2)	2,175 ± 333	1,552 ± 199*
LAI (%)	3.43 ± 0.53	2.35 ± 0.31*

NOTE. Values are the mean ± SD.
Abbreviation: WL, weight loss.
*$P < .01$ *v* pre-WL.

Table 4. Results of a Simple Replication Reliability Study in Which the Oil Red O Staining Procedure Was Performed and the LAI Was Measured in Five Subjects by Separate Microscopists

Subject No.	Measure 1	Measure 2	Mean ± SE
1	0.80	0.70	0.75 ± 0.05
2	5.23	7.58	6.40 ± 1.17
3	1.03	1.32	1.17 ± 0.14
4	5.31	6.27	5.79 ± 0.48
5	1.43	1.34	1.38 ± 0.04
Mean	2.76	3.44	3.10 ± 0.34

DISCUSSION

Recent studies have demonstrated that accumulation of lipid within human skeletal muscle is associated with obesity-related insulin resistance[6-8] and type 2 DM.[10] The current study was undertaken to address the extent of lipid accumulation within muscle fibers. Oil Red O staining of lipid within vastus lateralis skeletal muscle fibers was measured in lean and obese nondiabetic subjects and obese individuals with type 2 DM. The results indicate that there is increased triglyceride within muscle fibers in obesity associated with type 2 DM. Another important and novel observation of the current study is that following weight loss, there was a reduction in muscle lipid content. These findings affirm that the increase in muscle lipid content associated with obesity occurs within muscle fibers and this lipid pool is reduced with weight loss.

Applications of the Oil Red O staining method to identify neutral lipids within tissue have included examination of muscle lipid content in humans.[22-24] However, these early investigations of lipid content in skeletal muscle have largely been qualitative in nature, describing lipid deposition in various myopathic diseases[23,24] and with respect to muscle fiber type.[22,25] Odusote et al[25] found that type 1 and type 2A muscle fibers contained the greatest amount of neutral lipid and type 2B fibers the least, and type 1 fibers varied considerably in lipid content. Moreover, Askanas and Engel[22] were able to identify subtypes of type 1 muscle fibers in humans according to lipid content. Although lipid content was not quantified with respect to fiber type in the current investigation, Fig 1 illustrates that, in accordance with these studies,[22,25] there is heterogeneity in skeletal muscle lipid content among muscle fibers. Other studies have subjectively examined Oil Red O staining in obese[26] and insulin-resistant[9] rats. In accordance with our results in humans, Campion et al[26] found greater skeletal muscle lipid content using Oil Red O staining in obese compared with lean Zucker rats. Moreover, the current observations in human muscle are consistent with other animal models of increased muscle lipid in obesity and insulin resistance.[9,13] The findings from the current study indicate that the objective quantitation of histologic staining of lipid content in skeletal muscle can be a useful method for the investigation of lipid metabolism in skeletal muscle and body composition changes attendant to obesity.

Despite the broad use and usefulness of the Oil Red O method to stain neutral lipid within skeletal muscle, there has

been a more limited application of objective methods for the quantitation of skeletal muscle staining of lipid content. Odusote et al[25] were perhaps the first to quantify lipid staining in skeletal muscle using electronic image analysis, observing 2- to 2.5-fold greater muscle lipid accumulation within the muscle of the diaphragm compared with the biceps or soleus of guinea pigs. Indeed, the range for the amount of lipid staining in their study[25] is similar to the 1% to 5% range of lipid staining found in the current study. In a recent study, Phillips et al[8] found that lipid staining in vastus lateralis muscle was not increased in obesity but was associated with an enzyme marker of insulin resistance. These results are at variance with those in the current study, as we observed an increased lipid content within muscle in obese subjects and a positive association between the BMI and muscle lipid content. It is likely that a wider range of obesity in our subjects contributed to the significant association between obesity and muscle lipid content. Moreover, since a semiquantitative score was used in that study, it is difficult to directly compare those results with our study. Our results further indicate that neither age nor physical fitness were associated with muscle lipid content in our cohort. Similarly, differences in lipid contained within muscle fibers between lean and obese DM groups were not influenced by age. Perhaps the quantification of lipid staining within muscle fibers in older individuals or across a broader range of physical fitness levels would yield important information regarding the influence of age or patterns of physical activity on muscle lipid content.

There has been a renewed interest in ascertaining muscle lipid content in relation to insulin resistance and obesity. A variety of techniques have been used, including biochemical lipid extraction[7,8] and noninvasive imaging.[6,27] However, if triglyceride is extracted from biopsy samples of skeletal muscle, it is less than certain that the results accurately reflect lipid contained within muscle fibers. Pan et al[7] found that muscle triglyceride content in percutaneous biopsy samples of vastus lateralis muscle correlated with insulin resistance. However, a limitation of this method is a relatively high between-sample variability,[28] and it is not possible, using a biochemical extraction from muscle homogenate, to completely discern intramyocellular versus extramyocellular lipid. The within-sample variability of lipid staining within muscle fibers has been demonstrated to be low in the current study, although future studies should examine the between-biopsy variability in lipid staining. A single magnetic resonance spectroscopic study published in abstract form has found that intramyocellular lipid is increased in insulin-resistant humans,[17] but this potentially useful method needs to be confirmed.

Another important finding in the current investigation is that weight loss reduced the lipid content within muscle fibers. Weight loss is known to improve insulin sensitivity in both obese[14,15] and type 2 DM[16] subjects. However, little is known regarding the effects of weight loss on skeletal muscle lipid content. In the current study, following weight loss in obese subjects and in those with type 2 DM, muscle was reexamined for potential changes in lipid content. Following a large weight loss (approximately 14 kg) in which there were no concomitant changes in physical fitness, muscle lipid content decreased by 32%. It is interesting to note, as previously reported for a large cohort in which these subjects are included,[15] that the percent loss of systemic fat mass was 29%. Thus, muscle lipid content can be significantly altered by a diet-induced weight loss, and the quantitation of Oil Red O staining of lipid within muscle is useful for assessing the magnitude of change during a clinical intervention. Future studies should address whether these changes in muscle lipid content with weight loss mediate known concomitant changes in insulin action, or whether exercise training independently of weight loss alters lipid within muscle fibers.

In summary, these results show that obese individuals possess greater lipid content within skeletal muscle than lean individuals. We also find that a weight loss intervention substantially reduces muscle lipid content, with the magnitude of reduction being similar to the loss of systemic fat mass. These findings indicate the value of image analysis methods to quantify Oil Red O lipid staining of human muscle and to study the effects of obesity and weight loss interventions. Potential further applications of this method to determine specific localities of lipid within muscle, eg, proximity to the sarcolemma, and differences across fiber types and in relation to oxidative enzyme capacities may provide important information regarding how muscle lipid accumulation impairs metabolic function.

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