Assessment of Skeletal Muscle Triglyceride Content by ^{1}H Nuclear Magnetic Resonance Spectroscopy in Lean and Obese Adolescents

Relationships to Insulin Sensitivity, Total Body Fat, and Central Adiposity

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The metabolism and composition of skeletal muscle tissue is of special interest because it is a primary site of insulin action and plays a key role in the pathogenesis of insulin resistance. Intramyocellular (IMCL) triglyceride stores are an accessible form of energy that may decrease skeletal muscle glucose utilization, thereby contributing to impaired glucose metabolism. Because of the invasive nature of muscle biopsies, there is limited, if any, information about intramuscular lipid stores in children. The development of ^{1}H nuclear magnetic resonance (NMR) spectroscopy provides a unique noninvasive alternative method that differentiates intracellular fat from intercellular fat in muscle tissue. The present study was performed to determine whether IMCL and extramyocellular (EMCL) lipid contents are increased early in the development of juvenile obesity and to explore the relationships between IMCL and EMCL to in vivo insulin sensitivity, independently of total body fat and central adiposity in obese and nonobese adolescents. Eight nonobese (BMI 21 kg/m^2, age 11–16 years) and 14 obese (BMI 35 ± 1.5 kg/m^2, age 11–15 years) adolescents underwent *1*) ^{1}H-NMR spectroscopy to noninvasively quantify IMCL and EMCL triglyceride content of the soleus muscle, *2*) a 2-h euglycemic-hyperinsulinemic clamp (40 mU · m^{-2} · min^{-1}) to assess insulin sensitivity, *3*) a dual-energy X-ray absorptiometry scan to measure total percent body fat, and *4*) magnetic resonance imaging to measure abdominal fat distribution. Both the IMCL and EMCL content of the soleus muscle were significantly greater in the obese adolescents than in the lean control subjects. A strong inverse correlation was found between IMCL and insulin sensitivity, which persisted and became even stronger after controlling for percent total body fat and abdominal subcutaneous fat mass (partial correlation $r = -0.73$, $P < 0.01$) but not when adjusting for visceral fat ($r = -0.54$, $P < 0.08$). In obese adolescents, increase in total body fat and central adiposity were accompanied by higher IMCL and EMCL lipid stores. The striking relationships between both IMCL and EMCL with insulin sensitivity in childhood suggest that these findings are not a consequence of aging but occur early in the natural course of obesity. *Diabetes* 51:1022–1027, 2002

Juvenile obesity is the most prevalent nutritional disorder, affecting one in five children in the U.S. (1). It is associated with hyperinsulinemia/insulin resistance, hyperlipidemia, elevated blood pressure (2–4), and an alarming increase in the frequency of type 2 diabetes (5,6). As in adults, insulin resistance may explain the increased incidence of type 2 diabetes in obese adolescents as well as the constellation of other metabolic problems these youngsters face. Indeed, previous studies from our group suggested that increased visceral fat, hyperinsulinemia, and insulin resistance are closely linked abnormalities that are expressed early in the natural history of juvenile obesity (3). Whereas many adult studies have focused on the metabolic impact of intra-abdominal fat versus subcutaneous fat, the deposition of fat within muscle cells may be another important aspect of body composition that is altered in obesity and associated with insulin resistance (7–9). In humans, the deposition of triglycerides as energy stores and the generation of fatty acids (FAs) via lipolysis during increased energy demands have been thought to occur only in adipose tissue (10). More recently, however, FAs derived from skeletal muscle lipolysis have been recognized as an important energy source (11,12), and abnormalities in insulin signaling may arise as a result of an overaccumulation of various lipids in skeletal muscle cells (13,14). These lipid stores have been found to be a potent marker of insulin resistance in obese (7,8) and nonobese adults (15) as well as in nondiabetic offspring of type 2 diabetic subjects (16,17). Muscle fat deposition has been measured either by muscle biopsies

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DEXA, dual-energy X-ray absorptiometry; EMCL, extramyocellular; FA, fatty acid; IMCL, intramyocellular; IRS, insulin receptor substrate; NMR, nuclear magnetic resonance.

TABLE 1
Clinical and biochemical characteristics of study groups

	Nonobese adolescents	Obese adolescents
n (M/F)	5/3	11/3
Age (years)	14 ± 0.6 (12–16)	13 ± 0.6 (10–16)
Ethnicity (white/African-American)	6/2	9/5
Height (cm)	167 ± 3 (159–178)	162 ± 2 (150–168)
Weight (kg)	60 ± 4 (45–76)	93 ± 4† (66–113)
BMI (kg/m^2)	21 ± 0.8 (18–25)	35 ± 1.5† (25–44)
% Fat mass	18 ± 1.6 (12–24)	41 ± 1.6† (28–52)
Fat-free mass (kg)	45 ± 3 (34–37)	50 ± 2 (38–68)
Fasting glucose (mmol/l)	4.8 ± 0.11 (4–5)	5.04 ± 0.22 (4.2–5.5)
Fasting insulin (pmol/l)	66 ± 9 (30–108)	180 ± 12† (66–480)
Insulin-to-glucose ratio	0.11 ± 0.01 (0.06–0.14)	0.32 ± 0.04† (0.11–0.98)
Fasting C-peptide (pmol/ml)	0.40 ± 0.10 (0.20–0.70)	0.72 ± 0.09* (0.50–1.50)
Fasting triglyceride (mmol/l)	8 ± 2 (4–19)	18 ± 4 (4–60)
Fasting FAs (μmol/l)	415 ± 55 (256–640)	517 ± 50 (258–823)

Data are means ± SE (range) unless otherwise indicated. *$P < 0.05$; †$P < 0.001$.

or estimated by computed axial tomography (CAT scan). The invasive nature of muscle biopsies and safety issues (ionizing radiation) have limited the determination of the lipid stores, particularly in children. Moreover, these techniques lack the accuracy and sensitivity to distinguish between intramyocellular (IMCL) and extramyocellular (EMCL) lipid content (18,19). These problems have been overcome by the recent use of ^{1}H nuclear magnetic resonance (NMR) spectroscopy, which provides a unique noninvasive method that differentiates intracellular fat from intercellular fat in muscle tissue. We used this technique for the first time in a pediatric group to determine whether *1*) IMCL lipid content and EMCL content are increased in obese adolescents and are related to in vivo insulin sensitivity and *2*) these associations are independent of other factors known to alter insulin sensitivity, such as total body fat and central adiposity.

RESEARCH DESIGN AND METHODS

Subjects. We studied 8 nonobese and 14 obese adolescents. Their clinical and laboratory characteristics are shown in Table 1. All obese adolescents had a BMI >95th percentile specific for sex and age (20), whereas lean adolescents had a BMI between the 50th and 75th percentile (20). Total body fat mass measured by dual-energy X-ray absorptiometry (DEXA) was more than twofold greater in the obese adolescents. Pubertal stage of development was similar in both groups and was assessed by physical examination according to the criteria of the Tanner stage for breast development in girls and genital development in boys. In both groups, the Tanner stage ranged from III to IV. All subjects were in good health and taking no medications. There was no decrease in body weight in any subject before the study. Basal fasting insulin and triglycerides, but not FA levels, were greater in obese adolescents than in lean adolescents. All obese subjects were recruited from the Yale Pediatric Obesity Clinic. The nonobese adolescents were recruited from either the Yale Pediatric Diabetes Clinic or the Obesity Clinic; therefore, they were siblings of obese or diabetic patients or had a parent with obesity or diabetes. The nature and purpose of the study were carefully explained to both parents and to every adolescent before written consent from parents and voluntary assent from the child were obtained. The study protocols were approved by the Human Investigation Committee of the Yale University School of Medicine.

In vivo ^{1}H-NMR spectroscopy. Localized ^{1}H-NMR spectra of the soleus muscle were acquired on a 2.1 T/a.m. Biospec spectrometer (Bruker Instruments, Billerica, MA) by using a coil assembly consisting of two circular hydrogen-1 coil loops (13.0 cm diameter each) arranged spatially to generate a quadrature field (21). During the measurements, the subjects remained supine, with the gastrocnemius-soleus complex of the right leg positioned within the homogeneous volume of the magnet. Scout images were acquired to position the volume of interest. Volumes of interest (typically 15 × 15 × 25 mm^3) were centered within the soleus muscle and placed to avoid vascular structures and gross adipose tissue deposits. Localized shimming in the soleus muscle was performed using FASTERMAP (21) with typical line widths of ~10 Hz being obtained. Localized proton spectra were collected using a point resolved spectroscopy sequence with the following parameters: repetition time = 3 s, echo time = 21.1 ms, 8,192 data points over 5,000 Hz spectral width, 128 scans. Signals in the time domain were multiplied by a Gaussian function before Fourier transformation and manual phase correction (the correction factor used is 0.61). ^{1}H resonances were assigned to water and methyl-methylene of triglycerides from their chemical shift and were line fitted using the Mac-Nuts-PPC software package (Acorn NMR, Freemont, CA). IMCL and EMCL lipid content were calculated from the peak areas of IMCL CH_2 (methylene) at 1.3 ppm and EMCL CH_2 at 1.5 ppm, respectively, with respect to the water peak area and were corrected for T_1 (longitudinal relaxation) and T_2 (transverse relaxation) relaxation effects (Table 2). These relaxation times are close to those reported by Szczepaniak et al. (22). IMCL and EMCL content were then expressed as a percentage of water content.

Euglycemic-hyperinsulinemic clamp. In the morning at 0800, after an overnight fast of 10–12 h, total body insulin sensitivity was measured by the euglycemic-hyperinsulinemic clamp, during which insulin was administered as a prime continuous infusion of 40 mU · m^{-2} · min^{-1} for 120 min, as previously described (23). Two intravenous catheters were inserted before the clamp studies: one in an antecubital vein for administration of test substances and the other in a vein of the hand or distal forearm of the contralateral arm for blood sampling. The hand chosen for blood sampling was placed in a heated box (~65°C) to facilitate blood sampling and to arterialize blood. Videotaped movies entertained the children and kept them relaxed before and during the infusion studies. Clamp studies were performed in all nonobese adolescents and six obese adolescents.

Anthropometric measurements, assessment of fat distribution and total body composition. Weight (to the nearest 0.1 kg) and height (to the nearest 0.5 cm) were measured while the subjects were fasting and wearing only their undergarments. Magnetic resonance imaging was used to assess directly intra-abdominal fat deposition, as previously described (3). This procedure was obtained in all nonobese control subjects and 11 obese subjects because three adolescents did not give their assent for this part of the study because of claustrophobia. Total body composition (fat mass and fat-free mass) was measured by DEXA using a Hologic scanner (Hologic, Boston, MA). DEXA scans were performed and analyzed using pediatric software (Version 1.Se).

Analytical procedures and calculations. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Plasma insulin was determined by a double-antibody radioimmunoassay (Linco RIA Laboratories, St. Charles, MO). Fasting plasma triglyc-

TABLE 2
T_1 and T_2 relaxation times in soleus muscle

	H_2O skeletal muscle (ms)	IMCL (CH_2) (ms)	EMCL (CH_2) (ms)
T_1	1,295 ± 25	342 ± 12	341 ± 40
T_2	31.7 ± 0.8	76.6 ± 2.1	81.3 ± 1.5

Data are means ± SE. T_1 and T_2, spin–spin relaxation time.

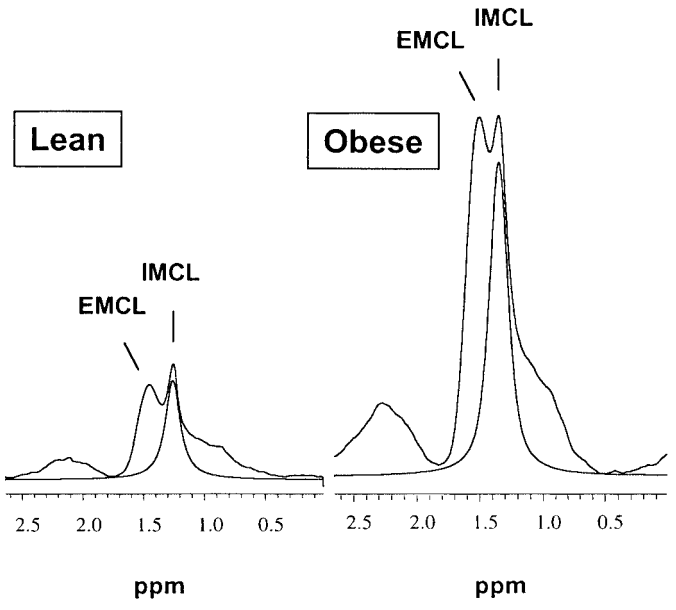

FIG. 1. Typical localized ^{1}H-NMR spectra of the soleus muscle in a lean and obese adolescent. IMCL denotes the (CH_2) resonance of the intramyocellular lipids (1.30 ppm) and EMCL denotes the (CH_2) resonance (1.5 ppm) of the EMCL lipids.

eride concentrations were determined by a commercially available assay (C336-10; Sigma, St. Louis, MO). Plasma nonesterified fatty acid concentrations were measured by a microfluorimetrical method.

The glucose infusion rates (*M* value) were calculated from 90 to 120 min, corrected for glucose space, and expressed in milligrams per meter squared per minute. During the insulin clamp study, the amount of glucose required to maintain euglycemia provides an index of total glucose disposal. The lack of use of glucose tracer in the present study limits determination of hepatic glucose production rates. As a result, the calculated glucose infusion rates are not a true absolute measure of total body glucose utilization rates.

Statistical analysis. All data are presented as means ± SE. Comparison between the two groups was performed using analysis of variance and by a two-tailed unpaired *t* test. Spearman's correlation coefficients were calculated to assess the degree of association between variables. Partial correlation analyses were performed to determine the independent effects of IMCL and EMCL lipid content on insulin sensitivity. Differences were regarded as statistically significant if corresponding *P* values were ≤0.05. All statistical analyses were performed using the SAS computer analysis program (Version 6; SAS Institute, Cary, NC).

RESULTS

^{1}H-NMR spectroscopy. Figure 1 depicts typical localized proton NMR spectra of the soleus muscle from a lean and an obese adolescent male. Both the IMCL and EMCL lipid content are considerably greater in the obese subject than in the lean subject. The quantitative difference between lean and obese adolescents in IMCL and EMCL lipid content of the soleus muscle is summarized in Fig. 2. In obese adolescents, IMCL ranged from 0.85 to 3.76% of water resonance peak area (mean 2.15 ± 0.22%), whereas in lean adolescents, IMCL ranged from 0.46 to 1.22% (mean 0.69 ± 0.07%, $P < 0.01$). Likewise, EMCL ranged from 1.11 to 6.2% (mean 2.47 ± 0.36%) in the obese adolescents. In contrast, it ranged from 0.36 to 1.28% (mean 0.72 ± 0.13%, $P < 0.002$) in the lean control subjects.

Euglycemic-hyperinsulinemic clamp. During the insulin clamp, plasma glucose levels were kept at comparable baseline levels in both groups, and plasma insulin levels reached a steady-state level during the last 30 min of the clamp, which was similar in the two groups (300 ± 20 pmol, nonobese adolescents; 330 ± 18 pmol, obese adolescents). Likewise, plasma C-peptide levels fell by 58% in both groups during the clamp. Rates of whole-body glucose uptake (*M* value), as measured by the glucose infusion rate, were significantly lower in the obese adolescents (102 ± 20 mg · m^{-2} · min^{-1}) than in the lean adolescents (187 ± 30 mg · m^{-2} · min^{-1}, $P < 0.01$).

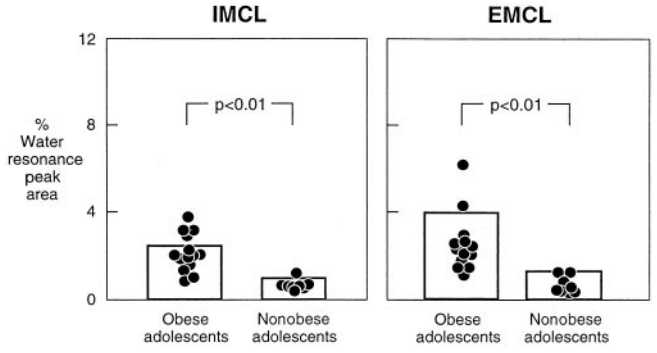

FIG. 2. IMCL and EMCL lipid content of the soleus in lean and obese adolescent participants.

Relationship between leg lipid content, percent total body fat, central adiposity, and insulin sensitivity (*M*). Correlative analyses were performed by combining both lean and obese adolescents together. As indicated in Table 3, IMCL leg lipid content was found to be positively correlated with indexes of overall obesity, such as BMI ($r = 0.665$, $P < 0.001$) and percent total body fat ($r = 0.69$, $P < 0.0001$). Likewise, positive associations were found between EMCL and these measures of adiposity. Both the subcutaneous abdominal and visceral fat depots were found to be significantly associated with IMCL and EMCL. Plasma triglyceride and free FA concentrations were found to be positively correlated with IMCL but not with EMCL lipid content.

As shown in Fig. 3, IMCL had the strongest inverse correlation with insulin sensitivity (Pearson's correlation $r = -0.59$, $P < 0.02$; Spearman's rank correlation $r = -0.689$, $P < 0.001$), followed by a weaker correlation between EMCL lipid content and insulin sensitivity ($r = -0.53$, $P < 0.05$). Partial correlation analysis was performed in an attempt to better quantify the independent contribution of IMCL to insulin sensitivity. We found that the inverse relationship between IMCL and insulin sensitivity persisted and became even stronger after controlling for percent total body fat and abdominal subcutaneous fat

TABLE 3
Matrix of the Spearman's rank correlation analyses between IMCL and EMCL lipid content and variables of body composition of interest in lean and obese adolescents

	IMCL	EMCL
% Total body fat	0.689*	0.661†
BMI	0.665*	0.682*
EMCL lipid content	0.684*	1.00
Visceral fat	0.729*	0.861*
Subcutaneous abdominal fat	0.539	0.795
Triglycerides	0.50*	0.13
Free FAs	0.429†	0.231
Insulin-to-glucose ratio	0.72*	0.68†

*$P < 0.001$, †$P < 0.04$.

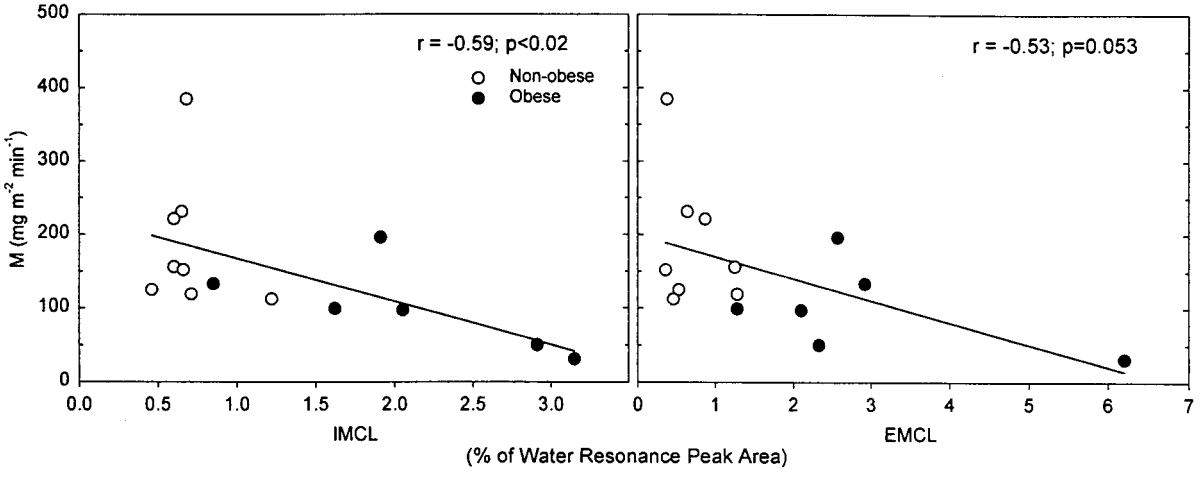

FIG. 3. Relationships between IMCL and EMCL and whole-body insulin sensitivity in lean and obese adolescent participants.

mass ($r = -0.73$, $P < 0.01$), but not when adjusting for visceral fat mass ($r = -0.54$, $P < 0.08$). When performing the correlation analysis in the two groups separately, we found in the obese group that IMCL tended to be inversely related to M ($r = -0.68$, $P < 0.10$). On the other hand, in the nonobese group, there was no relationship between these two variables ($r = -0.21$, $P < 0.62$).

The ratio between insulin and glucose (an excellent marker of insulin resistance in nondiabetic subjects) was found to be strongly and positively correlated with IMCL (Table 3, $r = 0.72$, $P < 0.0001$, Spearman's rank correlation; $r = 0.53$, $P < 0.02$, Pearson's correlation). This relationship became even stronger after adjusting for percent total fat and body distribution (partial correlation $r = 0.82$, $P < 0.0002$). In contrast, a much weaker relationship was found between the insulin-to-glucose ratio and EMCL ($r = 0.53$, $P < 0.01$, Spearman's rank correlation). No relationships were found between these variables when using Pearson's simple correlation analysis.

DISCUSSION

In the present study, ^{1}H-NMR spectroscopy was used to noninvasively quantitate the IMCL and EMCL triglyceride content in lean and obese adolescents. We then explored the relative contribution of IMCL and EMCL, independently of the percentage of total body fat and measures of central adiposity, to differences in whole-body insulin sensitivity, as assessed by the glucose clamp technique, in a pediatric population. Our study indicates that, in obese adolescents, both the IMCL and EMCL lipid stores of the soleus muscle are significantly increased compared with those in nonobese adolescents. Of particular interest, we found that whole-body insulin sensitivity varied as a function of IMCL stores (Pearson's correlation $r = -0.59$, $P < 0.02$). It is noteworthy that these relationships were independent of percent total body fat and subcutaneous abdominal fat but not of visceral fat mass. Thus, our data suggest that IMCL may play an important role in modulating insulin sensitivity, particularly in obese adolescents. The striking relationship between IMCL and insulin sensitivity in such a young population suggests that these findings are not a consequence of aging but are actually expressed early in the natural course of obesity. To further explore these relationships, we calculated the insulin-to-glucose ratio and used it as a biochemical marker of insulin sensitivity. The strong relationship between a biochemical marker (insulin-to-glucose ratio) and a tissue marker (IMCL) of insulin resistance is important because it justifies the rationale for using this ratio as a good, inexpensive, and easily measurable biomarker of insulin sensitivity. As indicated in RESEARCH DESIGN AND METHODS, we calculated glucose infusion rates, which are a measure of whole-body glucose disposal and not of glucose utilization rates, because we do not have any information on hepatic glucose production rates. At the insulin infusion rates used in the present study, it is conceivable that hepatic glucose production was not fully suppressed. As a result, we may have underestimated glucose uptake in the obese adolescents, weakening the association between IMCL and insulin sensitivity.

The IMCL lipid content measured in the lean adolescents is lower than that reported by Krssak et al. (15) in a group of lean adults with normal sensitivity. Differences in the methodology used in these two studies may account for the lower values of IMCL reported in the adolescents. In the present study, we measured the peak area rather than the intensity of the peak. Therefore, a true comparison between our study in children and that of Krssak et al. (15) in adults is not possible. Nevertheless, it is conceivable that the age difference may account for the 50% lower triglyceride content because we have focused on a pediatric group, whereas in the group studied by Krssak et al. (15), the age of the subjects varied widely from 19 to 70 years. It is likely that aging might be associated with an increase in muscle triglyceride content, just as it is associated with an increased amount of visceral fat mass, which together may contribute to the decline in whole-body insulin sensitivity that normally accompanies aging (24).

Although adipose tissue is the major depot of body fat and the main source of FAs delivered to the circulation for use at distant sites (e.g., skeletal muscle, heart), increased IMCL lipid content is likely to result in an accumulation of intracellular fatty acyl CoAs or other FA metabolites, which are

capable of modulating local glucose metabolism (11,12,25). Consistent with this hypothesis are studies that have found high triglyceride stores in patients with type 2 diabetes and in obese adults (7,16). In these studies, fat accumulation was assessed by muscle biopsies or computerized tomography of the vastus lateralis muscle (7). Because of methodological limitations, neither procedure allowed the distinction between IMCL and EMCL lipid accumulation. Moreover, the invasiveness of these methods limited the determination of muscle lipid stores in children. ^{1}H-NMR spectroscopy has been validated in humans and was found to be capable of differentiating between IMCL and EMCL lipid content (18,22) of the soleus muscle. Using this approach, we (15) and others (16,17,19,22) have found a relationship between IMCL and whole-body insulin sensitivity, as measured by the euglycemic-hyperinsulinemic clamp technique. Using similar methodologies, these associations were also reported in offspring of type 2 diabetic patients (16,17). Although most studies have found IMCL to be related to insulin sensitivity, independently of BMI and other measures of obesity, these relationships were not found in South Asian men (26), implying that other mechanisms may contribute to the insulin resistance found in other ethnic groups.

The mechanism by which increased muscle lipid content, particularly IMCL, might be affecting insulin action is not clear. High-fat diets have been found to increase muscle lipid content and to induce insulin resistance in animals (9,27). Recent studies by Dresner et al. (13) indicate that increased concentrations of plasma FAs in healthy nonobese humans induce insulin resistance through a reduction in insulin stimulation of glucose transport activity. During these studies, they found in the vastus lateralis a reduction in insulin receptor substrate (IRS)-1 tyrosine phosphorylation and IRS-1–associated phosphatidyliinositol-3 kinase activity after acute elevations in FA levels (13). Similar findings were also found by Griffin et al. (14) in rats after a 5-h infusion of lipids to raise plasma FA levels. It is conceivable that the hydrolysis of triglyceride located within the myocyte will supply an increase in long-chain acyl CoA levels (25,28), which might lead to an increase in fatty acyl CoA diacylglycerol, which, in turn, can activate a protein kinase C, thereby altering insulin signaling (25,28).

In summary, the results of this work demonstrate that obese adolescents have increased intramuscular triglyceride storage. A significant association was found between both EMCL and IMCL and visceral fat mass, implying that the visceral fat depot and intramuscular leg lipid content might not be independent from each other. Furthermore, the relationship between insulin sensitivity and IMCL is independent of percent total body fat and subcutaneous abdominal fat but not of visceral fat mass. It is intriguing to speculate that the intracellular lipid accumulation might be a result of an increased flux of free FAs into muscle from the enlarged visceral adipose depot.

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