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Diabetes mellitus is associated with increased intramyocellular triglyceride, but not diglyceride, content in obese humans

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Abstract

It has been suggested that intramyocellular diglycerides may be associated with insulin resistance and thus may be linked to the pathophysiology of diabetes. We aimed to investigate intramyocellular diglyceride as well as triglyceride levels in diabetic subjects and to explore a possible association with glycemic control. The participants of the study were 30 obese subjects stratified according to the presence of diabetes into nondiabetic obese (n = 19) and diabetic obese (n = 11). Intramyocellular triglycerides and diglycerides were determined in biopsies from the vastus lateralis muscle under fasting conditions. Glycemic control and insulin resistance were assessed by an oral glucose tolerance test and the homeostatic model, respectively. Higher levels of intramyocellular triglycerides were observed in the diabetic obese group compared with the nondiabetic obese group (66.67 ± 23.75 vs 18.35 ± 4.42 nmol·mg^{-1} dry tissue, respectively; $P < .05$). Diglyceride levels were not significantly different between the study groups (1.65 ± 0.27 vs 1.94 ± 0.65 nmol·mg^{-1} dry tissue, respectively). Monounsaturated fatty acids represented the major constituent of intramyocellular triglycerides in both groups, whereas diglycerides contained mainly saturated fatty acids. A significant correlation was found between intramyocellular levels of triglycerides, but not diglycerides, and glycemic control, expressed as the area under the glucose curve ($r = 0.417$, $P < .05$). No correlations were found between intramyocellular levels of both lipid classes and insulin resistance. Our data support a relationship between glycemic control and intramyocellular triglycerides, but not diglycerides. The total flux of fatty acids toward esterification may be a much more important factor in the pathophysiology of diabetes.

1. Introduction

The etiology of insulin resistance and diabetes mellitus has been a subject of extensive research since the early 1960s. In 1963, Randle et al [1] were the first to link insulin sensitivity and the metabolic disturbances of diabetes with alterations in glucose and lipid kinetics at the cellular level of muscle. In vivo studies performed in the late 1990s demonstrated that insulin resistance is associated with an increased rate of intramyocellular lipid storage [2] and that insulin sensitivity is negatively correlated with intramyocellular lipid content [3].

Since then, many studies have investigated the association of intramyocellular lipid stores (ie, triglycerides) with insulin resistance, showing either a positive correlation [4] or no correlation at all [5]. In fact, no direct link has been established between insulin sensitivity and intramyocellular triglycerides. The available studies on the intramyocellular triglyceride accumulation under different insulin-resistance states (ie, obesity and diabetes) have produced controversial results. Van Loon et al [6] have found no difference in intramyocellular triglycerides between diabetic subjects and body mass index (BMI)–matched controls. On the contrary, Goodpaster et al [7] have reported almost 2-fold higher levels of intramyocellular triglycerides in diabetic obese subjects compared with nondiabetic obese subjects. There are some data indicating that intramyocellular lipids may be differentially regulated in diabetes and obesity. For example, diet-induced weight loss has resulted in a significant

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decrease in intramyocellular triglycerides in diabetic obese subjects, but not in obese normoglycemic subjects [8]. Consequently, the role of intramyocellular triglycerides in the pathophysiology of diabetes remains unclear.

On the other hand, many studies at the molecular level have shown that some intermediate molecules (diglycerides) or some derivatives (ceramides) of lipid metabolism may be directly linked to insulin resistance by inhibiting essential steps of insulin signaling. In particular, diglycerides have been found to activate some isoforms of protein kinase C (new and conventional forms), which block insulin receptor substrate I and phosphatidylinositol-3-kinase, whereas atypical forms of the enzyme have been associated with the activation of the insulin-stimulated glucose transport pathway [9]. Ceramides inhibit protein kinase B/Akt, a more downstream step of insulin signaling [10]. Regarding the relationship of intramyocellular ceramides with insulin resistance in humans, available studies have not provided consistent results, showing either increased levels in insulin-resistance states [11,12] or no differences compared with healthy controls, or states of increased insulin sensitivity, such as in endurance-trained athletes [13].

The available research in intramyocellular diglycerides in relation to insulin resistance is scarce. Intramyocellular diglycerides in obese rats have been found to be 40% to 136% higher, depending on the oxidative capacity of the muscle examined, compared with those in lean controls, while denervation also increased intramyocellular diglyceride accumulation [14]. In healthy nonobese humans, it has been shown that insulin resistance caused by lipid infusion during euglycemic-hyperinsulinemic clamping causes accumulation of diglycerides in the skeletal muscle [15]. However, to our best knowledge, no studies in humans have investigated intramyocellular diglyceride levels in various levels of insulin resistance, especially diabetes mellitus.

In vitro studies in myocytes have provided evidence that the channeling of fatty acid substrates into triglyceride or diglyceride pools may be a more essential factor in the development of insulin resistance. Liu et al [16] have shown that overexpression of diacylglycerol acyltransferase (the enzyme that catalyzes the conversion of diglycerides to triglycerides) protects from fat-induced insulin resistance, with a parallel increase in the triglyceride pool and a decrease in the diglyceride pool. However, not all fatty acids are incorporated with the same efficiency into triglyceride pool. Saturated fatty acids, for example, are purely incorporated into intracellular triglycerides, causing an increase in diglyceride levels, whereas the opposite effect has been observed for monounsaturated fatty acids [17]. Thus, the quality of fatty acids contained in both lipid pools may be linked to insulin resistance.

The aim of the present study was to compare intramyocellular diglyceride and triglyceride levels in human obesity and diabetes mellitus. We hypothesized that diabetic subjects would illustrate more profound levels of intramyocellular lipids that would account for differences in glycemic control and insulin sensitivity. Comparisons and potential associations were tested not only for the total amounts of both lipid classes, but also for the quality of fatty acids contained on them.

2. Methods

2.1. Subjects

A total of 30 subjects participated in the study. Participants were stratified according to the presence of type 2 diabetes mellitus and obesity status into nondiabetic obese (nDiab-Ob, n = 19) and diabetic obese (Diab-Ob, n = 11) subjects. The physiologic characteristics of the study participants are presented in Table 1. All diabetic subjects were treated with oral hypoglycemic medications. Eligibility criteria included the presence of obesity and weight stability for a minimum of 1 year (no more or less than 3 kg, based on self-reports), the absence of any disease and use of medication in the nDiab-Ob group, and the absence of medically diagnosed macro- or microvascular disease in the Diab-Ob group. From the diabetic subjects, 9 were on metformin therapy, 5 subjects were taking sulfonylureas, 2 subjects were taking a combination of both, 2 subjects were prescribed rosiglitazone, and 1 subject was on a combination of metformin and rosiglitazone. Three subjects in the Diab-Ob group were also taking hypertensive medication. Insulin therapy was an exclusion criterion for the diabetic group. *Diabetes* was defined according to the criteria of World Health Organization [18]. *Obesity* was defined as BMI greater than 30 $kg \cdot m^{-2}$ [19]. Subjects were given detailed information about the study procedures and potential hazards, and a written consent was obtained. The study was conducted according to the standards set by the latest version of the Declaration of Helsinki and was approved by the University Review Board.

2.2. Testings

Subjects were asked to visit the laboratory on 2 separate occasions after an overnight fast (at least 10 hours), separated by 3 to 5 days. On day 1, a medical history and basic demographic characteristics were obtained; and a medical examination was performed, including measurements of blood pressure and pulse rate and auscultation of the heart and lungs. Body composition was also assessed by dual-

Table 1
Physiologic characteristics of the study participants

	nDiab-Ob	Diab-Ob
Sex, male/female	9/10	2/9
Weight, kg	111.6 ± 4.5	114.0 ± 7.9
Age, y	40 ± 3	47 ± 3
BMI, $kg \cdot m^{-2}$	39.7 ± 1.5	40.7 ± 1.8
Lean body mass, kg	57.0 ± 2.7	52.4 ± 3.9
Body fat, %	42.3 ± 2.5	45.8 ± 1.5

Values are means ± SE.

energy-x-ray absorptiometry (model DPX-MD; Lunar, Madison, WI). Afterward, a venous catheter was placed into a forearm vein; and a 75-g oral glucose tolerance test (OGTT) was performed. On day 2, a muscle biopsy was obtained (~80 mg) from the middle portion of the vastus lateralis muscle by percutaneous needle biopsy with suction [20], under local anaesthesia (0.1% lidocaine on the skin and fascia). Nonmuscle tissue (blood or visible fat) was discarded, and specimens were frozen immediately in liquid nitrogen until analysis. Subjects were instructed to abstain from any vigorous physical activity and alcohol consumption the 2 days before each testing day and to consume an unrestricted, high-carbohydrate diet. Diabetic medication was not discontinued the previous days of the experimental procedure in the diabetic subjects; however, subjects were asked to refrain from any medication until the completion of all examinations on the days of the testings.

2.3. Blood analysis and insulin sensitivity assessment

Aliquots of plasma from fasting samples were used for the measurement of glucose, triglycerides, free fatty acids, as well as total, high-density lipoprotein, and low-density lipoprotein cholesterol. Glucose was also measured in samples taken during the OGTT (30, 60, 90, and 120 minutes). Aliquots of serum from fasting samples were used for the measurement of baseline insulin levels. Biochemical analyses were performed in duplicate in an automated analyzer using standard reagents (ACE Schiapparelli Biosystems, Fairfield, NJ). Insulin was measured in triplicate by an enzyme-linked immunoassay (Insulin; DakoCytomation, Glostrup, Denmark). The area under the glucose curve during the OGTT was calculated by the trapezoidal rule and was used as an index of glycemic control. Insulin resistance was assessed by the homeostasis model assessment (HOMA) [21].

2.4. Muscle biopsies analysis

To reduce the possibility of contamination of muscle tissue with other tissues, samples were frieze-dried (−50°C for 24 hours) and visualized in a stereoscope; and nonmuscle material (connective or fat tissue) was discarded. Net dry muscle tissue was weighted, and total lipids were extracted according to the method of Folch et al [22] for 12 hours at room temperature under shaking. Butylated hydroxytoluene was added as an antioxidant. The chloroform phase containing total lipids was dried in a Speed Vac Model AES 1010, SpeedVac Systems, Thermosavant, Holbrook, NY at room temperature, and lipids were redissolved in a small volume of chloroform. Lipid classes were separated with solid phase extraction by the use of aminopropyl-bounded silica gel cartridges [23]. Eluates containing triglycerides and diglycerides were dried, and fatty acids of each lipid class were transmethylated in an appropriate mixture of boron trifluoride in methanol at boiling temperature [24]. Methyl esters were extracted in pentane, dried, redissolved in a small volume of hexane, and quantified by gas-liquid chromatography equipped with a flame ionization detector. A 60-m capillary column was used (DB-23; Agilent Technologies, Wilmington, DE), calibrated against a standard containing 37 fatty acids methyl esters, ranging in chain length from 4 to 24 carbon atoms (Supelco 37 Component Fame Mix; Supelco, Bellefonte, PA). Total triglycerides and diglycerides were calculated by dividing the sum of the molar concentrations of methyl esters by 3 and 2, respectively. Fatty acids contained in each lipid class were further categorized on the basis of the degree of saturation (saturated, monounsaturated, and polyunsaturated) and the omega carbon atom in polyunsaturated fatty acids (n-3 and n-6).

2.5. Statistical analysis

Data are presented as means ± 1 standard error of the mean. Comparisons between groups were performed by Student unpaired t tests for normally distributed variables and by the nonparametric Mann-Whitney test for skewed variables. Comparisons within groups were performed by analysis of variance for repeated measures (for normally distributed data) and the Wilcoxon or the Friedman test (for nonnormally distributed data). The Shapiro-Wilk test was applied to assess normality. Potential associations between the continuous variables were tested with single regression analysis. Interactions between categorical variables were evaluated with the Pearson χ^2 test. Significance was set at $P < .05$.

3. Results

Baseline glucose, insulin, and lipid profiles of the study participants are presented in Table 2. The 2 groups did not differ in regard to plasma triglycerides, free fatty acids, and cholesterol levels. The Diab-Ob subjects had higher levels of baseline glucose and insulin levels, although the difference in insulin levels was not statistically significant. Plasma glucose levels at 120 minutes during the OGTT (Fig. 1) as well as the total area under the curve in the Diab-Ob group were approximately 2 times the values of the nDiab-Ob group ($P < .005$). The 2 obese groups had also significantly different values of the HOMA index ($P < .005$).

Table 2
Fasting blood glucose, insulin, and lipid profile of the study participants

	nDiab-Ob	Diab-Ob
Glucose, mmol·L^{-1}	5.81 ± 0.14	7.56 ± 0.40*
Insulin, μIU·L^{-1}	13.21 ± 1.35	18.67 ± 2.59
Triglycerides, mmol·L^{-1}	1.37 ± 0.15	1.61 ± 0.21
Free fatty acids, mmol·L^{-1}	0.72 ± 0.05	0.98 ± 0.11
Total cholesterol, μmol·L^{-1}	5.49 ± 0.21	5.41 ± 0.33
HDL cholesterol, μmol·L^{-1}	1.03 ± 0.05	1.08 ± 0.08
LDL cholesterol, μmol·L^{-1}	3.83 ± 0.16	3.60 ± 0.28

Values are means ± SE. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein.

* Statistically significant difference; $P < .05$.

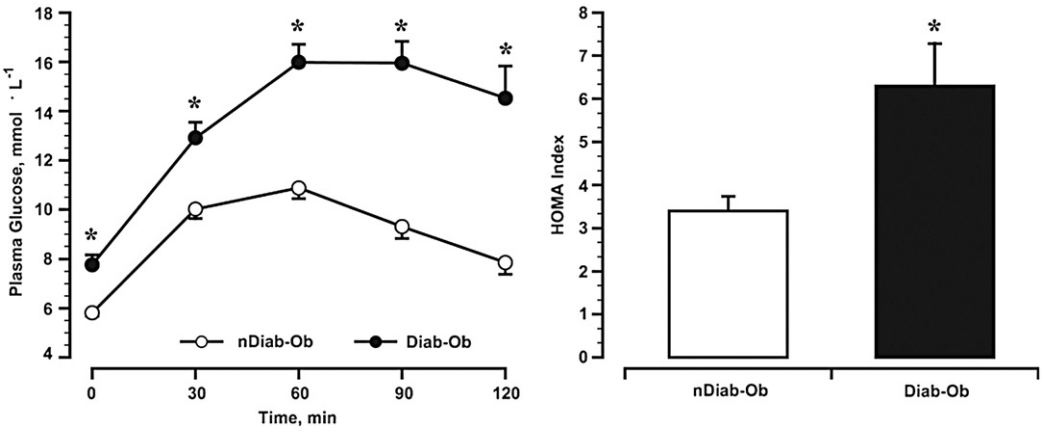

Fig. 1. Plasma glucose during the OGTT and HOMA index in the 2 study groups: nDiab-Ob (n = 19) and Diab-Ob (n = 11). Values are means ± SE. *Statistically significant difference for Diab-Ob compared with nDiab-Ob; $P < .05$.

Fig. 2 presents total intramyocellular triglycerides in the 2 study groups as well as their fatty acid content according to the degree of saturation and the omega carbon atom in the unsaturated fatty acids. The Diab-Ob subjects had significantly higher total triglycerides compared with the nDiab-Ob group ($P < .05$). Similar patterns with total triglycerides were observed for the categories of fatty acids examined. The most abundant fatty acids within the categories examined were the monounsaturated (overall effect for the degree of saturation, $P < .001$) and the n-6 fatty acids ($P < .001$ vs n-3 fatty acids). Little abundance was observed for n-3 fatty acids.

Intramyocellular diglycerides levels and their fatty acid content are depicted in Fig. 3. No significant differences between the study groups were observed for total diglycerides or the categories of their fatty acid content. The most abundant fatty acids in the diglyceride fraction were the saturated fatty acids (overall effect for the degree of saturation, $P < .001$).

Regression analysis between intramyocellular lipids and the area under the glucose curve during the OGTT, as an index of glycemic control, revealed a significant correlation between total triglycerides ($F = 5.461$, $b = 2.498$, $r = 0.417$, $P = .027$). Insulin resistance assessed by the HOMA index was not significantly correlated with triglycerides or their fatty acid content. Similarly, no significant associations were observed between intramyocellular diglycerides and insulin resistance or glycemic control.

To investigate a possible impact of gender on our comparisons, we examined the association between study groups and sex (male/female). The P value for Pearson χ^2 correlation coefficient for the interaction between sex and study group was equal to 0.101, indicating no significant interaction.

4. Discussion

In the present study, we examined the effect of type 2 diabetes mellitus on intramyocellular accumulation of 2 major classes of lipid metabolism—triglycerides and diglycerides—that have been linked to insulin resistance. Our novel finding is that intramyocellular diglycerides did not differ in diabetic subjects compared with obese nondiabetic controls. On the other hand, the presence of diabetes was associated with increased intramyocellular triglyceride accumulation.

Diglycerides are intermediate products of triglyceride biosynthesis and/or degradation that may alter insulin signaling through activation of protein kinase C [9]. Our results do not support a major role of fasting intramyocellular diglyceride levels on diabetes pathophysiology because no difference was observed between the 2 study groups. Furthermore, no correlation was found between diglycerides and insulin sensitivity or glycemic control indices. To our best knowledge, we are not aware of any studies in humans exploring directly this relationship in insulin-resistance states. In rodents, lipid infusion has been found to activate protein kinase–θ and –δ, 2 diglyceride-sensitive isoforms [25,26]. In humans, lipid infusion during hyperinsulinemia-euglycemia increased total activity of the enzyme in both cytosolic and plasma membrane fractions, in parallel with an increase in intramyocellular diglycerides, compared with lipid infusion alone [15]. However, these observations do not establish a cause and effect relationship between intramyocellular diglyceride levels and protein kinase C activation. In addition, in vitro studies in L6 myotubes have shown that the ζ isoform of the enzyme is involved in insulin stimulated glucose uptake, whereas blocking the diglyceride-sensitive isoforms had no effect, providing evidence that muscle protein kinase C activity and diglyceride levels may be dissociated [17].

In the present study, intramyocellular triglycerides levels were higher in obese diabetic subjects compared with nondiabetic obese subjects. An increase in intramyocellular triglyceride in obese compared with lean subjects is well documented [27,28]. However, studies comparing diabetic obese subjects with obese nondiabetic subjects have produced mixed results, showing increased levels in the

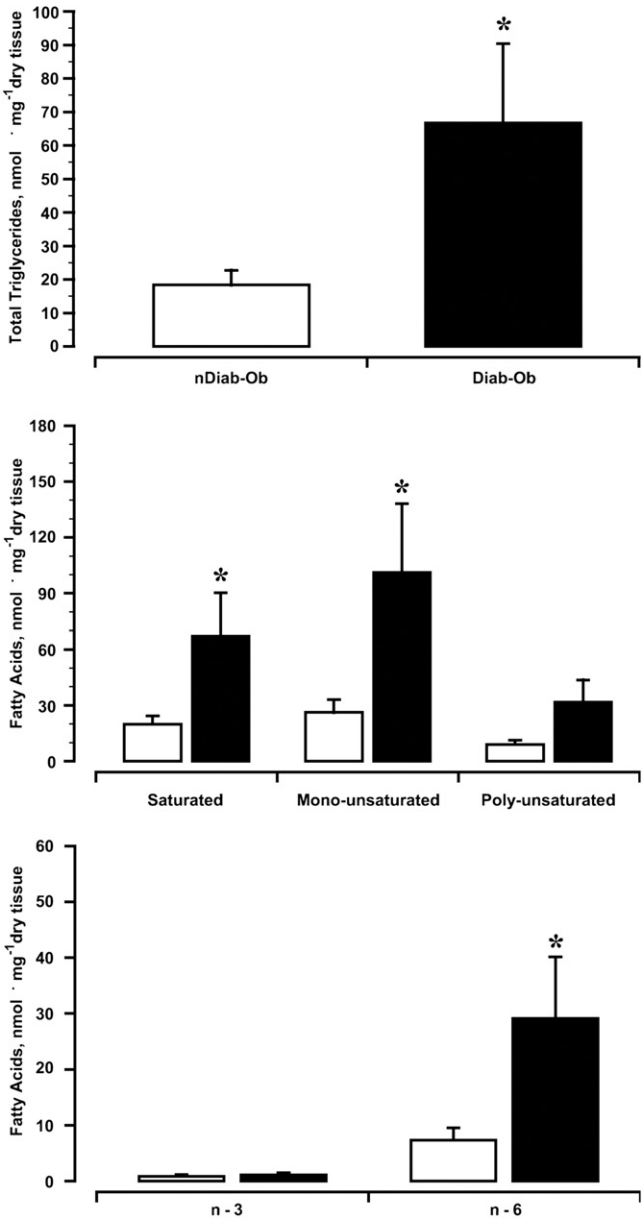

Fig. 2. Intramyocellular total triglycerides and their fatty acid content in the 2 study groups: nDiab-Ob (n = 19) and Diab-Ob (n = 11). Values are means ± SE. Scaling in the y-axis may be different in each graph. *Statistically significant difference for Diab-Ob compared with nDiab-Ob; $P < .05$.

diabetic population [7], no difference [27,28], or an intermediate value for obese subjects between diabetic obese and nondiabetic lean counterparts [8]. Our results suggest that diabetes mellitus combined with obesity results in a higher accumulation of intramyocellular triglycerides compared with obesity alone.

Although a direct relationship between intramyocellular lipid storage composition and nutritional intake has not been firmly established in humans, we observed a higher accumulation of monounsaturated fatty acids in the triglyceride fraction, reflecting possibly the high consumption of olive oil in our Greek subjects. However, monounsaturated fatty acids were not the primary component of diglycerides. Instead, saturated fatty acids were the major component of diglycerides. In vitro studies in human muscle cells have shown that incubation of cells with oleic acid (monounsaturated fatty acid) or linoleic acid (polyunsaturated fatty acid) results in an almost full incorporation of these fatty acids into the intracellular triglyceride pool, whereas incubation with palmitic or stearic acid (saturated fatty acids) resulted in the accumulation of these fatty acids into diglycerides [29]. In the same study, insulin-stimulated glucose uptake was impaired in cells incubated with saturated fatty acids, while being unaltered in cells incubated with oleic or linoleic acid. In rodents, high saturated fat–diet

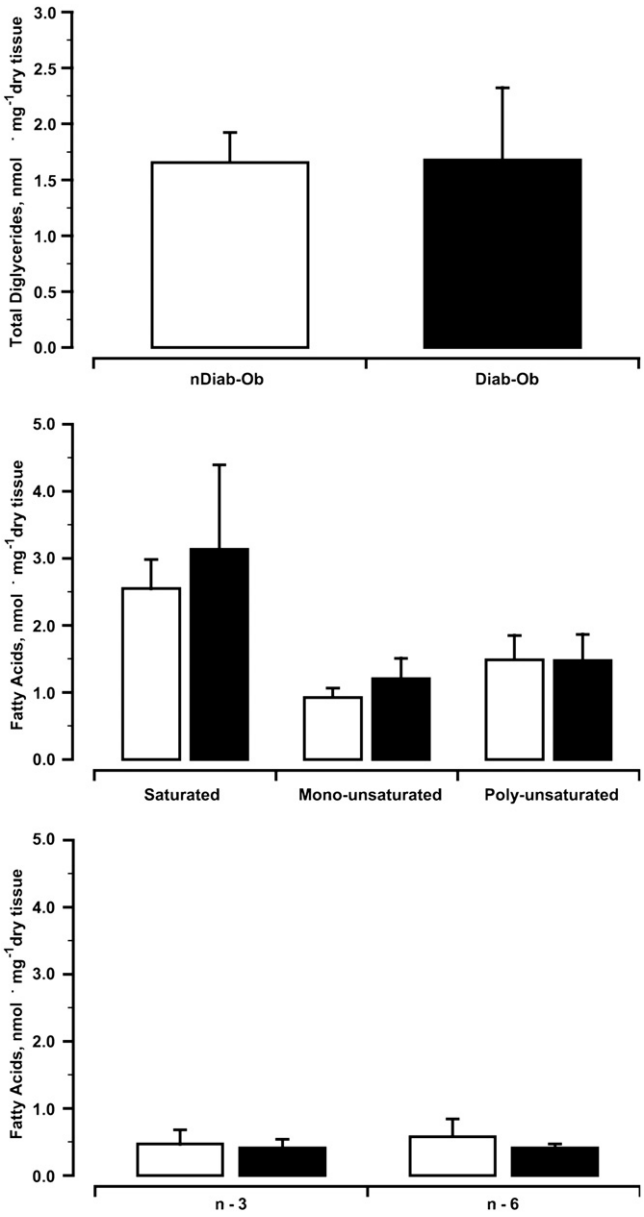

Fig. 3. Intramyocellular total diglycerides and their fatty acid content in the 2 study groups: nDiab-Ob (n = 19) and Diab-Ob (n = 11). Values are means ± SE. Scaling in the y-axis may be different in each graph.

increased mainly the intramyocellular diglycerides pool, whereas high polyunsaturated fat directed the excess fatty acids in triglyceride storage [30]. The results of the above-mentioned studies suggest an inability of saturated fatty acids to incorporate into the intramyocellular triglyceride pool, causing an increase of saturated fatty acids in the diglycerides, as observed in the present study. On the contrary, monounsaturated fatty acids and polyunsaturated fatty acids are easily incorporated into triglycerides. In this regard, it could be speculated that the Mediterranean diet of our study participants may have protected them from a substantial expansion of diglyceride levels; and thus, a main effect of these lipids on insulin sensitivity and glycemic control could not be detected. However, because dietary intake was not controlled, such a conclusion cannot be drawn.

Our study has many strengths. It is the first study reporting intramyocellular diglyceride levels in diabetes and obesity, 2 insulin-resistance states that may not necessarily be regulated by common mechanisms. Second, the parallel measurement of intramyocellular triglycerides and the fatty acid composition of both lipid classes allowed interesting observations to be made. Overall, it should be noted that this is a cross-sectional study that cannot provide definite conclusions, but only state hypothesis for future research.

In conclusion, in the present study, we did not find differences in intramyocellular diglyceride accumulation between 2 different states of insulin resistance: diabetic obese and nondiabetic obese subjects. On the contrary, the presence of diabetes exacerbated intramyocellular triglyceride accumulation. According to our results, intramyocellular diglyceride pool is unlikely to play a significant role in the pathophysiology of diabetes, at least under baseline conditions. It is probably the overall flux of the fatty acids of the peripheral circulation toward esterification and storage in muscle that may account for glycemic control and insulin resistance and not intermediary molecules of this process, such as diglycerides.

Acknowledgment

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