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Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes

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Van Loon, Luc J. C., René Koopman, Ralph Manders, Walter van der Weegen, Gerrit P. van Kranenburg, and Hans A. Keizer. Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *Am J Physiol Endocrinol Metab* 287: E558–E565, 2004. First published May 27, 2004; 10.1152/ajpendo.00464.2003.—Recent evidence suggests that intramyocellular lipid (IMCL) accretion is associated with obesity and the development of insulin resistance and/or type 2 diabetes. However, trained endurance athletes are markedly insulin sensitive, despite an elevated mixed muscle lipid content. In an effort to explain this metabolic paradox, we compared muscle fiber type-specific IMCL storage between populations known to have elevated IMCL deposits. Immunofluorescence microscopy was performed on muscle biopsies obtained from eight highly trained endurance athletes, eight type 2 diabetes patients, and eight overweight, sedentary men after an overnight fast. Mixed muscle lipid content was substantially greater in the endurance athletes (4.0 ± 0.4% area lipid stained) compared with the diabetes patients and the overweight men (2.3 ± 0.4 and 2.2 ± 0.5%, respectively). More than 40% of the greater mixed muscle lipid content was attributed to a higher proportion type I muscle fibers (62 ± 8 vs. 38 ± 3 and 33 ± 7%, respectively), which contained 2.8 ± 0.3-fold more lipid than the type II fibers. The remaining difference was explained by a significantly greater IMCL content in the type I muscle fibers of the trained athletes. Differences in IMCL content between groups or fiber types were accounted for by differences in lipid droplet density, not lipid droplet size. IMCL distribution showed an exponential increase in lipid content from the central region toward the sarcolemma, which was similar between groups and fiber types. In conclusion, IMCL contents can be substantially greater in trained endurance athletes compared with overweight and/or type 2 diabetes patients. Because structural characteristics and intramyocellular distribution of lipid aggregates seem to be similar between groups, we conclude that elevated IMCL deposits are unlikely to be directly responsible for inducing insulin resistance.

intramuscular triacylglycerol; intramuscular fat; endurance training; insulin resistance

THE CLOSE RELATIONSHIP between muscle lipid content and skeletal muscle insulin resistance has been reported for many years in both animal (32, 40, 41, 56) and human studies (42, 47, 55). In most of the earlier studies, muscle triglyceride extractions were performed to quantitate muscle lipid content. Subsequently, those studies were unable to differentiate between intramyocellular lipid (IMCL) and extramyocellular lipid deposits. With the introduction of ^{1}H magnetic resonance spectroscopy, a noninvasive means to quantitate both IMCL and extramyocellular lipid content in vivo has become available (9). Since then, numerous studies have provided evidence showing IMCL accretion to be associated with obesity (17, 45) and the development of insulin resistance and/or type 2 diabetes (26, 46). Direct visualization of IMCL aggregates by using histochemistry has confirmed the presence of greater IMCL content in obese (36) and/or type 2 diabetes patients (18, 19, 21) compared with healthy, lean controls.

Elevated IMCL stores are now regarded a risk factor for the development of skeletal muscle insulin resistance and type 2 diabetes. However, the reported correlations between IMCL content and insulin resistance (33, 42, 47) disappear with the inclusion of well-trained endurance athletes in such studies (18, 57). This is due to the fact that endurance-trained athletes are markedly insulin sensitive (15, 35, 51), despite their elevated IMCL storage (18, 25, 57). These findings make the general association between IMCL accretion and the development of skeletal muscle insulin resistance less obvious. To explain this apparent paradox, it has been suggested that the association between IMCL content and insulin resistance may be mediated by muscle oxidative capacity (18, 21, 57). Muscle lipid content and oxidative capacity are both strongly determined by muscle fiber type composition. Biochemical triglyceride extraction of dissected type I and II muscle fibers (16) as well as histochemical oil red O staining using either light (21, 36) or fluorescence microscopy (58, 59) have reported a two- to threefold greater lipid content in type I versus type II muscle fibers. Interestingly, there are reports suggesting type I muscle fibers to be more insulin sensitive than type II fibers (22, 27, 30). In accordance, a reduced proportion of type I muscle fibers has been reported in muscle from obese and/or type 2 diabetes patients (23, 39), whereas a higher proportion of type I muscle fibers is generally observed in highly trained endurance athletes (1, 3). Because both populations are known to have elevated IMCL deposits, it seems likely to assume that substantial differences in fiber type distribution and/or muscle fiber type-specific lipid content are present between overweight and/or type 2 diabetes patients and highly trained endurance athletes. However, a direct comparison of fiber type-specific IMCL content and/or distribution patterns between these groups has not yet been reported.

In this study, we aimed to investigate the differences and/or similarities in fiber type-specific IMCL storage characteristics between those populations associated with elevated muscle

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lipid storage. As such, this study could likely provide more insight in the proposed functional relationship between IMCL accumulation and the development of skeletal muscle insulin resistance. To enable such a direct and selective comparison of fiber type-specific IMCL content, we combined oil red O staining of muscle cross sections with immunofluorescence microscopy on muscle biopsies collected from type 2 diabetes patients, weight-matched sedentary subjects, and healthy, young, and highly trained endurance athletes.

METHODS

Subjects. Eight highly trained male endurance cyclists and eight male overweight type 2 diabetes patients as well as eight weight-matched sedentary males were selected to participate in this study. Subject characteristics are shown in Table 1. All subjects were informed about the nature and risks of the experimental procedure before their written informed consent to participate was obtained. This study was approved by the local Medical Ethics Committee.

Pretesting. The trained athletes had a training history of >5 yr and participated in regular endurance exercise training (≥5 days/wk), with a reported training time of 9 ± 3 h/wk. In the trained athletes, maximal workload capacity (W_{max}) and maximal oxygen uptake ($\dot{V}O_{2\,max}$) were measured on an electronically braked cycle ergometer (Lode Excalibur; Groningen, The Netherlands) during an incremental exhaustive exercise test (34) at least 1 wk before muscle biopsy collection. The eight athletes with the highest scores on the incremental exercise test were then selected. The selected athletes were significantly younger compared with the type 2 diabetes patients and their weight-matched controls (Table 1).

The type 2 diabetes patients were all sedentary and overweight/obese (body mass index: 25–35 kg/m^2) and had been diagnosed with type 2 diabetes for over 5 yr. Exclusion criteria were impaired renal or liver function, cardiac disease, hypertension, other diabetic complications, and (exogenous) insulin therapy. All patients were using oral blood glucose-lowering medication [metformin with (n = 6) or without (n = 2) the combined use of a sulfonylurea derivative]. Fasting plasma glucose concentrations as well as blood Hb A_{1c} contents were elevated in all diabetes patients (fasting plasma glucose: >7.0 mmol/l, Hb A_{1c}: >6.0%; Table 1). The weight-matched, sedentary males were younger, were normoglycemic, had normal blood Hb A_{1c} content, and were not under any medical treatment (Table 1). Nonetheless, they were at an increased risk of developing insulin resistance because of their body composition and the fact that they had not participated in any regular exercise or (work related) physical activity regimen for over 10 yr. Insulin resistance was estimated by the homeostasis model assessment or homeostasis model assessment of insulin resistance (HOMA-IR) index (38), which was calculated by dividing the product of fasting plasma glucose (in mmol/l) and insulin concentrations (mU/l) by 22.5. Insulin resistance was significantly greater in the type 2 diabetes patients compared with the trained athletes, with the overweight, sedentary males at an intermediate value (Table 1).

Table 1. *Subject characteristics*

	Trained	Sedentary	Diabetes
Age, yr	23.1±0.5	45.5±2.5*	54.3±2.5*†
Height, m	1.84±0.03	1.81±0.02	1.78±0.02
Body mass, kg	74.5±4.2	93.9±8.9	94.1±4.1*
BMI, kg/m^2	22.0±0.7	28.8±2.9*	29.9±1.2*
Basal plasma glucose, mmol/l	5.2±0.2	5.5±0.4	7.9±0.5*†
Basal plasma insulin, mU/l	5.6±0.6	8.6±2.2	11.1±1.5*
HOMA-IR	1.3±0.1	2.6±1.0	4.2±0.6*
Hb A_{1C}, %	5.2±0.1	5.4±0.2	7.4±0.4*†
Endurance training, h/wk	9±3		
$\dot{V}O_{2\,max}$, l/min	4.7±0.1	NA	NA
W_{max}, W	400±13	NA	NA

Data are means ± SE. Body mass index (BMI) was calculated by dividing body weight by height^2; insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated by dividing the product of basal plasma glucose and insulin concentrations by 22.5 (38). $\dot{V}O_{2\,max}$, maximum oxygen uptake; W_{max}, maximum workload capacity. *Significantly different from the trained group; †significantly different from the weight-matched sedentary controls ($P < 0.05$).

All subjects were instructed to refrain from strenuous physical activity and/or exercise training for 3 days before muscle biopsy collection and to maintain their normal habitual dietary intake. Food intake diaries were filled in for 24 h preliminary to muscle biopsy collection. Average energy intake and macronutrient composition of the diet were calculated using the Eet-meter (2001) software package (Dutch Nutrition Centre; The Hague, The Netherlands). Energy intake was highest in the trained athletes with 14 ± 0.1 MJ/day [with 13 ± 1, 56 ± 1, and 29 ± 1% of total energy intake (En%) being derived from protein, carbohydrate, and fat, respectively]. In the type 2 diabetes patients and weight-matched sedentary subjects, En% averaged 9 ± 1.1 MJ/day (with 18 ± 1, 57 ± 2, and 25 ± 4 En% from protein, carbohydrate, and fat, respectively) and 9 ± 1.6 MJ/day (with 18 ± 1, 57 ± 2, and 25 ± 4 En%, respectively). From 2100 on the day before the muscle biopsy collection, subjects remained fasted until blood and muscle biopsy collection.

Muscle biopsy collection. After an overnight fast, subjects arrived at the laboratory in the morning by car or public transportation. Height was determined, and body weight was measured with a digital balance with an accuracy of 0.001 kg (E1200, August Sauter; Albstadt, Germany). Thereafter, a fasting blood sample was collected from an antecubital vein. After 30 min of supine rest, an anesthetic (1% xylocaine) was injected locally in skin, soft tissue below, and in the muscle fascia in the middle region of the vastus lateralis muscle. Thereafter, a small incision (6 mm) was made through the skin and the fascia at ~15 cm above the patella. A Bergström needle was inserted to a depth of ~2–3 cm below the entry of the fascia, and a muscle sample (~60 mg) was obtained by suction (4).

Blood and muscle tissue analysis. Blood samples (3 ml) were collected in EDTA-containing tubes and centrifuged at 1,000 *g* at 4°C for 10 min. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at −80°C. Plasma glucose (Uni Kit III, Roche; Basel, Switzerland) concentrations were analyzed with a COBAS FARA semiautomatic analyzer (Roche). Plasma insulin was measured by radioimmunoassay (HI-14K, Linco Research; St. Charles, MO). To determine basal fasting blood Hb A_{1c} content, a 3-ml blood sample was collected in EDTA-containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat; Munich, Germany). Muscle samples were dissected carefully, freed from any visible nonmuscle material, rapidly frozen in liquid nitrogen cooled isopentane, and embedded in Tissue-Tek (Sakura Finetek; Zoeterwoude, The Netherlands). Multiple serial cryostat sections (5 μm) from biopsy samples of one subject from each group were thaw mounted together on uncoated, precleaned glass slides. To permit quantification of IMCL stained by oil red O together with immunolabeled cellular constituents, we used the protocol described by Koopman et al. (31), as has been applied before (58, 59). Briefly, cryosections were fixed in 3.7% formaldehyde for 1 h. Slides were then rinsed with deionized water, treated with 0.5% Triton X-100 in PBS, and washed with PBS. Thereafter, sections were incubated with antibodies against human laminin (polyclonal rabbit antibody, Sigma Diagnostics; Steinheim, Germany) and human myosin heavy chain (A4.840), developed by Dr. H. M. Blau (12), enabling us to visualize individual cell membranes and to determine muscle fiber type (I or II), respectively. Incubation was followed by washes in PBS, after which the appropriate conjugated antibodies GARIgGAlexa350 and GAMIgMAlexa488 (Molecular Probes; Leiden, The Netherlands) were applied. After several washes with PBS, glass slides were

immersed in the oil red O working solution. Oil red O stock solution was prepared by adding 500 mg Oil Red O (Fluka Chemie; Buchs, Switzerland) to 100 ml of 60% triethylphosphate. Before the staining, a 36% triethylphosphate working solution containing 12 ml oil red O stock solution and 8 ml deionized water was prepared and filtered to remove crystallized oil red O. After 30 min of oil red O immersion, slides were rinsed with deionized water followed by a 10-min wash with tap water. Stained sections were embedded in Mowiol and covered with a coverslip. All muscle cross sections were stained and prepared within a single batch using the same dye preparation to minimize variability in oil red O staining efficiency.

Data analysis. After 24 h, glass slides were examined using a Nikon E800 fluorescence microscope (Uvikon; Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan color charge-coupled device camera, with a Bayer color filter. The epifluorescence signal was recorded using a Texas red excitation filter (540–580 nm) for oil red O, a FITC excitation filter (465–495 nm) for muscle fiber type, and a DAPI UV excitation filter (340–380 nm) for laminin (Fig. 1). Digitally captured images (×240 magnification), at least five fields of view/muscle cross section (average 11 ± 0.8 fibers/field of view), were processed and analyzed using Lucia 4.81 software (Nikon; Düsseldorf, Germany). The oil red O epifluorescence signal was quantified for each muscle fiber, resulting in a total of 54 ± 5 muscle fibers analyzed for each muscle cross section (24 ± 3 and 29 ± 4 type I and II muscle fibers, respectively). An intensity threshold representing minimal intensity values corresponding to lipid droplets was set manually and applied uniformly in all samples. Total area measured and the area as well as the number of objects emitting oil red O epifluorescence signal were recorded with the integration time set at 300 ms. Identical settings were used for all muscle cross sections, because the recorded oil red O signal depends on staining efficiency as well as on the applied image-acquisition settings (intensity treshold and integration time). As such, the applied epifluorescence technique represents a semiquantitative method to compare fiber type-specific IMCL content between muscle cross sections. Fiber type-specific IMCL content was expressed as the fraction of the measured area that was stained with oil red O. Average lipid droplet size was calculated by dividing total area lipid stained by total number of lipid aggregates. Lipid droplet density was calculated by dividing total number of lipid aggregates by total area measured. Mixed muscle lipid content, lipid droplet size, and lipid droplet density were determined by calculating the average value in the type I and type II muscle fibers, with a correction for the relative area occupied by each fiber type within each field of view of each muscle cross section, within each individual subject. In a preliminary study (58, 59), repeated biopsies were collected from two subjects after which different muscle samples were collected and analyzed separately; the coefficient of variance averaged 10.5% and 38.6% for the type I and II fibers, respectively.

Within each separate muscle cross section, 20 ± 2 muscle fibers (9 ± 1 and 11 ± 1 type I and II fibers, respectively) were selected to further investigate IMCL distribution patterns. To determine IMCL distribution, we recorded the location of lipid droplets within eight successive bands of 2 μm in width from the sarcolemma toward the central region of each muscle fiber. Lipid content in these bands and in the central region were recorded both as area fraction (area lipid stained divided by total area measured in each band/central region) and percentage of total IMCL content (area lipid stained in each band divided by total area lipid stained in the entire muscle fiber). In addition, digitally captured images (×60 magnification), at least one large overview/muscle cross-section, were also processed and analyzed to add to the accuracy of the fiber type distribution and average muscle fiber surface area measurements, resulting in an additional 77 ± 13 muscle fibers analyzed for each muscle cross section (32 ± 5 and 45 ± 9 type I and II muscle fibers, respectively).

Statistics. All data are expressed as means ± SE. To compare IMCL characteristics, fiber type distribution, and muscle fiber surface area between all groups, a one-factor ANOVA was applied. A repeated-measures ANOVA was applied to compare differences in IMCL distribution between the different bands in all groups. Scheffé's post hoc test was applied in case of a significant *F*-ratio

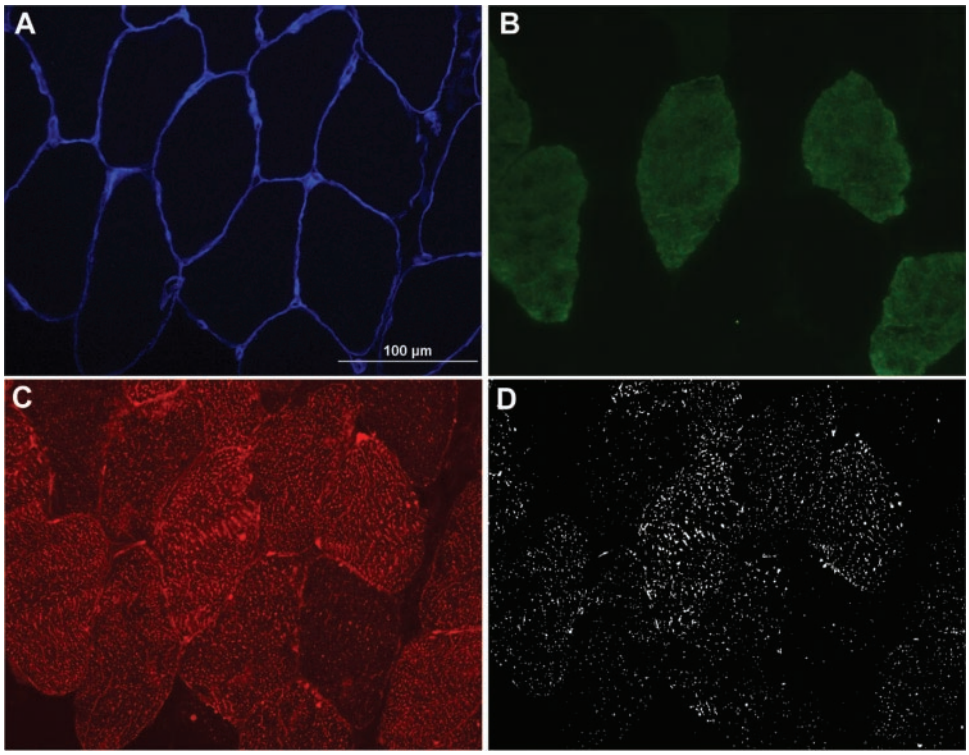

Fig. 1. Digitally captured images of one single field of view (×240 magnification) taken from a muscle cross section obtained from a muscle biopsy sample of a type 2 diabetes patient. Images show the epifluorescence signal as recorded using a DAPI UV excitation filter for laminin (showing the cell membranes in blue; *A*), an FITC excitation filter for myosin heavy chains (showing the type I muscle fibers in green; *B*), and a Texas red excitation filter for the oil red O signal [showing the intramyocellular lipid (IMCL) droplets in red; *C*]. *D*: oil red O signal obtained in *C* after the intensity threshold was applied (showing the lipid droplets in white), which was used for data processing and quantitative analysis.

to locate specific differences. Significance was set at the 0.05 level of confidence.

RESULTS

Fiber type distribution and surface area. Type I and type II muscle fiber content (in %) and average muscle fiber surface area (in μm^2) are shown in Table 2. A greater proportion of type I muscle fibers was observed in the endurance athletes (61.9 ± 8.2%) compared with the type 2 diabetes patients (38.4 ± 2.6%) and healthy, weight-matched, sedentary males (32.9 ± 7.4%, $P < 0.01$). Fiber type composition was similar in the diabetes patients and the sendentary males. No differences were observed in muscle fiber surface area between groups or fiber types.

IMCL content. Fiber type-specific and mixed muscle IMCL content (expressed as area fraction lipid stained) in the trained endurance athletes, type 2 diabetes patients, and normoglycemic sedentary males are illustrated in Fig. 2. Type I muscle fiber lipid content was significantly greater in the endurance-trained athletes (5.4 ± 0.5% area lipid stained) compared with the type 2 diabetes patients (3.3 ± 0.5%, $P < 0.05$) and tended to be greater compared with the overweight, sedentary males (3.5 ± 0.8%, $P = 0.09$). No significant differences in type II muscle fiber lipid content were observed between groups. Mixed muscle lipid content was significantly greater in the endurance-trained athletes (4.0 ± 0.4%) compared with the other two groups (2.3 ± 0.4 and 2.2 ± 0.5%, respectively, $P < 0.01$). In all groups, lipid content was significantly higher in the type I vs. the type II muscle fibers (2.8 ± 0.3-fold difference, $P < 0.01$).

Mixed muscle lipid content in the endurance-trained athletes was on average 77.5% greater than mixed muscle lipid content observed in the type 2 diabetes patients. This value for mixed muscle lipid content includes average muscle lipid content in the type I and II muscle fibers corrected for both fiber type composition and surface area. Because type I muscle fiber content is substantially increased in the endurance-trained athletes, we also calculated mixed muscle lipid content in the endurance-trained athletes based on the fiber type distribution as observed in the diabetes group (38% type I and 62% type II). This calculation revealed that 43% of the difference in mixed muscle lipid content between groups could be explained by the apparent difference in muscle fiber type distribution, leaving the remaining difference accounted for by a greater (type I) IMCL content in the endurance-trained athletes.

Lipid droplet size and density. Fiber type-specific and mixed muscle IMCL droplet size (in μm^2) and density (in droplets/μm^2) in the endurance-trained athletes, type 2 diabetes patients, and weight-matched sedentary males are illustrated in Figs. 3 and 4, respectively. There were no differences in lipid aggregate size or aggregate size distribution between groups or fiber types (average size 0.83 ± 0.03 μm^2 with a variance between 0.15 and 6.64 μm^2). As such, differences in intramyocellular lipid content between groups and/or fiber types were fully attributed to differences in lipid droplet density. Lipid droplet density was significantly greater in both the type I fibers and mixed muscle in the trained athletes (0.06 ± 0.004 and 0.05 ± 0.004 droplets/μm^2, respectively) compared with the type 2 diabetes patients and the sedentary males (0.04 ± 0.005 and 0.03 ± 0.004, and 0.03 ± 0.006 and 0.02 ± 0.003 droplets/μm^2, respectively, $P < 0.01$). No significant differences in lipid droplet density in the type II muscle fibers were observed between groups. In all groups, lipid droplet density was 2.4 ± 0.2-fold higher in the type I vs. type II muscle fibers ($P < 0.01$).

IMCL distribution. The IMCL distribution of lipid droplets is illustrated in Fig. 5. Lipid distribution is presented as lipid

Table 2. *Muscle fiber type distribution and surface area in vastus lateralis muscle*

	Trained	Sedentary	Diabetes
Type I fiber content, %	61.9±8.2	32.9±7.4*	38.4±2.6*
Type II fiber content, %	38.1±8.2	67.1±7.4*	61.6±2.6*
Type I muscle fiber area, μm^2	5,873±836	6,525±470	5,832±883
Type II muscle fiber area, μm^2	6,644±641	5,905±466	5,044±768

Data are means ± SE. Muscle fiber type content is expressed as the percentage of type I and II fibers (based on an average count of 77 ± 13 fibers/subject). Muscle fiber type size is expressed as surface area (μm^2). *Significantly different from the endurance-trained subjects ($P < 0.01$).

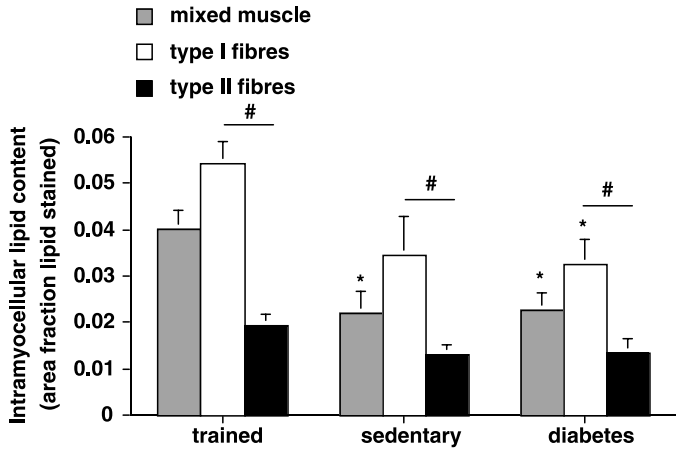

Fig. 2. Fiber type-specific IMCL content (expressed as %area lipid stained) in type I, type II, and mixed (average) muscle fibers in endurance-trained athletes, type 2 diabetes patients, and weight-matched, sedentary men. Data were obtained by using fluorescence microscopy on oil red O-stained muscle cross sections. Data provided are means + SE. *Significantly lower compared with values observed in the trained athletes; #significant difference between type I and II muscle fibers ($P < 0.05$).

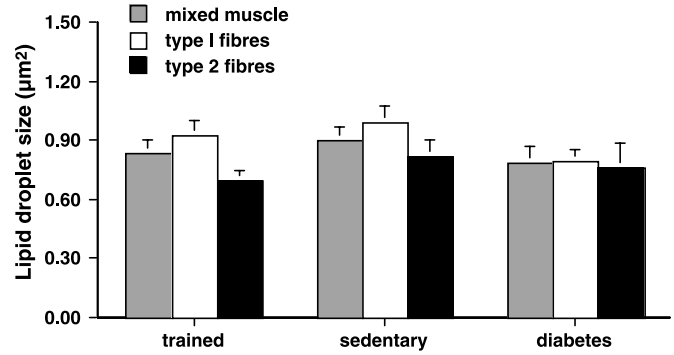

Fig. 3. IMCL droplet size (in μm^2) in type I, type II, and mixed (average) muscle fibers in endurance-trained athletes, type 2 diabetes patients, and sedentary weight-matched, sedentary men. Data were obtained by using fluorescence microscopy on oil red O-stained muscle cross sections. Data provided are means + SE. There were no significant differences between groups or muscle fiber types ($P < 0.05$).

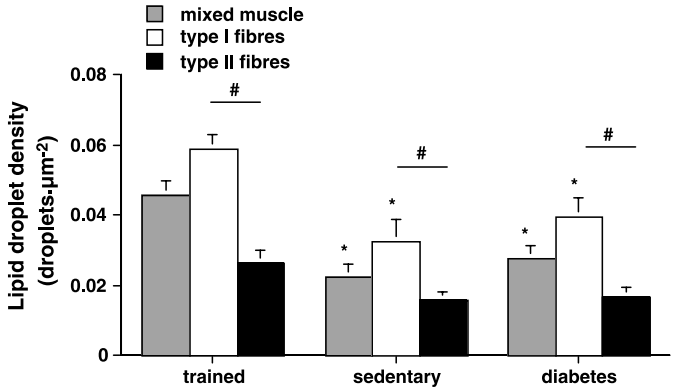

Fig. 4. IMCL droplet density (expressed as droplets/μm) in type I, type II, and mixed (average) muscle fibers in endurance-trained athletes, type 2 diabetes patients, and weight-matched, sedentary men. Data were obtained by using fluorescence microscopy on oil red O-stained muscle cross sections. Data provided are means + SE. *Significantly lower compared with values observed in the trained athletes; #significant difference between type I and II muscle fibers ($P < 0.05$).

content in 2-μm-wide bands from the sarcolemma toward the onset of the central region of the fiber. Figure 5*A* shows the IMCL content in these separate bands expressed as area fraction lipid stained. We observed significant differences in lipid content within each muscle fiber (between consecutive bands) as well as between groups ($P < 0.001$). Lipid content, expressed as area fraction lipid stained, decreased exponentially from immediately below the sarcolemma (2-μm band) toward the central region of the muscle fiber in all groups ($P < 0.001$). Between groups, lipid content was substantially greater in the highly trained endurance athletes compared with the type 2 diabetes patients and the sedentary males. Toward the central region of the muscle fibers these differences were no longer apparent.

Figure 5*B* shows IMCL distribution, with the amount of lipid in each consecutive 2-μm band expressed relative to total lipid content (in %). The relative distribution of lipid was very similar between groups, and only differences between the different bands were observed ($P < 0.001$). Most lipid was shown to be present in the first 2–4 μm compared with the other bands. In addition, the central region ("to center") also showed a high relative lipid content. However, the high lipid content in this section is entirely accounted for by the greater average size of this region (~6-fold greater area compared with the 14- to 16-μm band), which is accordance with the observation that lipid content expressed as area fraction is low (see Fig. 5*A*). There were no differences in IMCL distribution between muscle fiber types (data not shown). Therefore, only mixed muscle IMCL distribution data are presented.

DISCUSSION

In the present study, we compared fiber type-specific IMCL storage characteristics between populations known to have elevated IMCL deposits. Our data show that healthy, young and highly trained endurance athletes can have substantially greater IMCL contents than type 2 diabetes patients and/or overweight, sedentary males. This greater mixed muscle lipid content is shown to be largely attributed to a higher proportion type I muscle fibers, which contain two- to threefold more lipid than the type II fibers. The remaining difference is shown to be attributed to significantly greater IMCL content in the type I muscle fibers in the endurance athletes. Aside from these

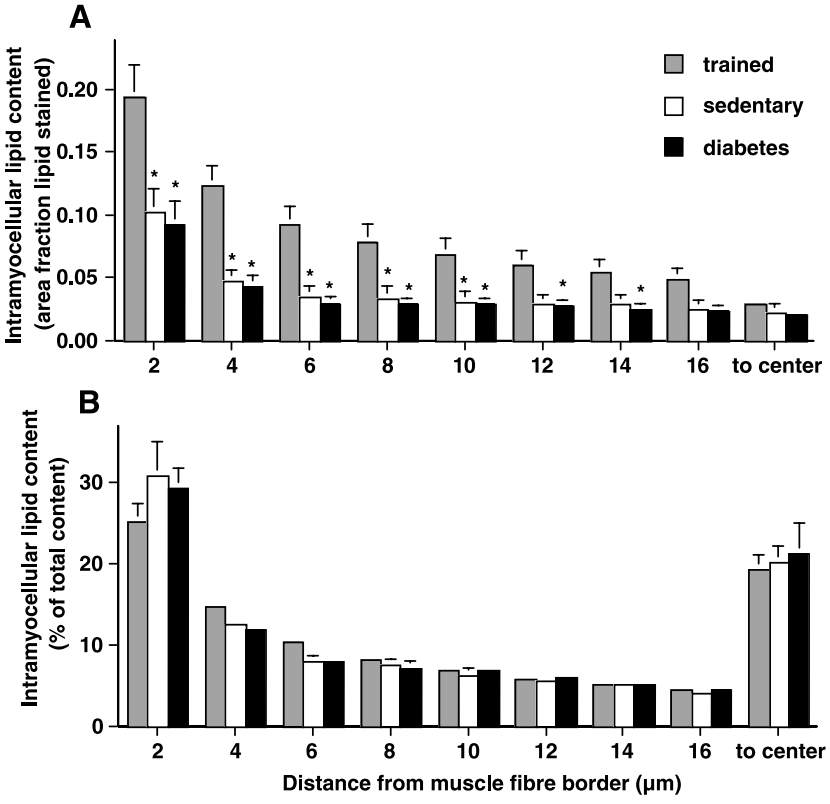

Fig. 5. IMCL distribution presented as lipid content in 2-μm-wide bands from the sarcolemma toward the onset of the central region of the fiber. *A*: lipid content in these separate bands expressed as area fraction lipid stained. *Significantly lower lipid content compared with the endurance-trained group ($P < 0.05$). Lipid content shows a significant increase from the central region toward the periphery in all groups ($P < 0.001$), with lipid content in the 0- to 2-μm band being significantly greater than in the other 7 bands ($P < 0.01$). *B*: lipid content in these separate bands expressed as a percentage of total lipid content in each muscle fiber. There were no differences in lipid distribution between groups when expressed relative to total lipid content. Lipid content shows a significant increase from the central region toward the periphery in all groups ($P < 0.001$), with lipid content in the 0- to 2-μm band being significantly greater than in the other 7 bands ($P < 0.01$). Data provided are means + SE. $P < 0.05$.

observations, no structural differences in intramyocellular lipid aggregate size and/or spatial distribution were observed between groups.

Numerous reports have shown a strong relationship between elevated plasma free fatty acid (FFA) levels, IMCL accumulation, and the development of skeletal muscle insulin resistance (6, 11, 17, 26, 33, 42, 44, 46). Consequently, elevated muscle lipid content is now regarded a risk factor for the development of insulin resistance and/or type 2 diabetes. However, the positive correlations between IMCL content and insulin resistance (33, 42, 47) would entirely disappear with the inclusion of trained endurance athletes in such correlation studies (18, 57). Goodpaster et al. (18) quantified IMCL content by histochemical oil red O staining of muscle cross sections and reported elevated IMCL content in both type 2 diabetes patients and endurance-trained athletes. Because insulin sensitivity was impaired in the diabetes patients only, no significant correlation could be observed between IMCL content and insulin sensitivity when the endurance-trained athletes were included in the regression analyses (18). More recently, Thamer et al. (57) reported insulin sensitivity to be negatively correlated with IMCL content in untrained subjects, whereas in endurance-trained subjects elevated IMCL contents actually predicted high insulin sensitivity.

Various studies have reported high IMCL contents in both overweight and/or type 2 diabetes patients and endurance-trained athletes (18, 19, 36, 37). We extended on these earlier findings by comparing fiber type-specific IMCL storage characteristics between these groups. To enable a selective quantification of type I and II muscle fiber lipid content, we combined the use of conventional oil red O staining of muscle cross sections with immunofluorescence microscopy (31, 59). Mixed muscle lipid content was shown to be ~75% greater in the healthy, young, and highly trained endurance athletes compared with the type 2 diabetes patients and/or weight-matched, sedentary males (Fig. 2). The higher mixed muscle lipid content in the trained athletes was largely attributed to a greater proportion of type I muscle fibers (61.9 ± 8.2%) compared with the type 2 diabetes patients (38.4 ± 2.6%) and the sedentary, overweight males (32.9 ± 7.4%; Table 2). Type I muscle fibers contained 2.8 ± 0.3-fold more lipid than the type II fibers, which is in accord with earlier observations (16, 21, 36, 58, 59). The greater proportion of type I muscle fibers in the trained athletes vs. the type 2 diabetes patients accounted for over 40% of the difference in mixed muscle lipid content, with the remaining difference being attributed to a significantly greater IMCL content in the type I muscle fibers (Fig. 2). Differences in type II muscle fiber lipid content between groups were small and did not reach statistical significance ($P = 0.1$). The absence of a significant difference was likely due to the large variance in type II muscle fiber lipid content, as the applied methodology does not allow to distinguish between muscle fiber subtypes (IIa, -b, and -x). In comparison, various MRS studies have reported similar relationships between IMCL content and insulin sensitivity in various populations after measuring mixed muscle IMCL content in both soleus (predominantly type I fibers) and tibialis anterior (with predominantly type II fibers) muscle (2, 8, 26, 28, 57).

There is ample evidence to support that elevated FFA levels, obesity, inactivity, and/or an age-related decline in mitochondrial function are associated with increased IMCL storage and the development of skeletal muscle insulin resistance (6, 11, 17, 26, 33, 42, 44, 46). However, the presence of such high IMCL contents in healthy, young, and highly trained cyclists as reported in the present study clearly shows that merely elevated IMCL concentrations are unlikely to be responsible for the development of insulin resistance. Furthermore, in accord with He et al. (21), we did not observe any difference in fiber type-specific IMCL content between overweight type 2 diabetes patients and weight-matched, sedentary males. However, insulin resistance as estimated using the HOMA-IR index was also not significantly different between these groups. The relationship among IMCL content, insulin resistance, and/or type 2 diabetes is clearly not unambiguous. Therefore, we speculated on whether structural differences in IMCL storage characteristics and/or distribution patterns between groups could possibly explain the presence or absence of a functional relationship among elevated IMCL contents, insulin resistance, and/or type 2 diabetes.

It has been hypothesized that elevated muscle lipid content in obese and/or type 2 diabetes patients could be associated with a disproportionate increase in IMCL aggregate size. Such a structural difference in lipid aggregate size could likely contribute to the reduced capacity to oxidize muscle lipid stores in the obese and/or type 2 diabetic state (5, 10), because it would lead to a less efficient surface-to-volume ratio of the lipid droplets. Others have speculated that large lipid aggregates could potentially form structural barriers that would hinder GLUT4 translocation, docking, and/or fusing with the sarcolemma. However, the average IMCL aggregate size (0.83 ± 0.03 μm^2; Fig. 3) as well as aggregate size distribution were observed to be quite similar between groups as well as fiber types. Therefore, differences in fiber type-specific IMCL content were entirely accounted for by differences in lipid droplet density (Fig. 4).

Malenfant et al. (36) reported a greater central distribution of IMCL aggregates in obese vs. lean subjects. This greater central distribution was suggested to be indicative for the associated defects in lipid metabolism and potentially relevant to the proposed relationship between IMCL accretion and the development of insulin resistance. In accord with their data, we observed most lipid aggregates to be located near the sarcolemma (Fig. 5), most likely associated with the large amount of mitochondria present in the immediate subsarcolemmal region (13). The amount of lipid was shown to decrease exponentially from the periphery toward the central region of the myofibers. Between groups, large differences in lipid content (area fraction) were observed in each 2-μm band, with a significantly greater lipid content in the trained athletes (Fig. 5*A*). However, when lipid content in each band was expressed relative to total IMCL storage, these differences were no longer apparent (Fig. 5*B*). In other words, lipid storage distribution patterns are quite similar among highly trained endurance athletes, type 2 diabetes patients, and their sedentary controls. Therefore, our findings do not provide any evidence to suggest that a more centralized compartmentalization of IMCL deposits could be associated with defects in lipid metabolism or with the presence or development of insulin resistance and/or type 2 diabetes.

The present study reports no structural differences in IMCL storage characteristics or distribution patterns between overweight and/or type 2 diabetes patients and highly trained

endurance athletes. As such, structural lipid storage properties seem to be unrelated to the presence or absence of an association between elevated IMCL content and reduced insulin sensitivity. In the endurance-trained state, IMCL storage is likely upregulated to compensate for the increased oxidative capacity (24, 25) and the concomitant regular depletion and repletion of the IMCL stores (58, 59) and would be in line with studies reporting an increase in IMCL storage (53) and utilization (48, 52) after 1–3 mo of endurance training. In contrast, in the obese and/or type 2 diabetes patients, it seems likely that elevated plasma FFA concentrations (43, 49) and/or impaired skeletal muscle fat oxidation (29) contribute to excess IMCL accretion. Novel insights from various lipid infusion studies (6, 7, 14, 20, 50, 54, 60) suggest that elevated FFA delivery and/or impaired fatty acid (FA) oxidation result in intramyocellular accumulation of lipid and FA metabolites, of which the latter (such as fatty acyl-CoA and/or diacylglycerol) are thought to be responsible for inducing defects in the insulin-signaling cascade. More research is warranted to determine whether the increase in IMCL content is merely a response to an imbalance between FA uptake and oxidation or a regulatory factor in the mechanisms responsible for the development of skeletal muscle insulin resistance and/or type 2 diabetes.

In conclusion, the present study indicates that healthy, young, and highly trained endurance athletes can show substantially greater IMCL contents than type 2 diabetes patients and/or overweight, sedentary males. The greater mixed muscle lipid content is attributed to an increased proportion of type I muscle fibers as well as a greater type I muscle fiber lipid content. Because there are no structural differences in intramyocellular lipid aggregate size and/or distribution patterns between groups, we should be more careful when assuming elevated IMCL deposits to represent a risk factor for the development of skeletal muscle insulin resistance and/or type 2 diabetes.

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