Cross contamination of intramyocellular lipid signals through loss of bulk magnetic susceptibility effect differences in human muscle using $^1$H-MRSI at 4 T

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Cui M-H, Hwang J-H, Tomuta V, Dong Z, Stein DT. Cross contamination of intramyocellular lipid signals through loss of bulk magnetic susceptibility effect differences in human muscle using $^1$H-MRSI at 4 T. J Appl Physiol 103: 1290–1298, 2007. First published August 2, 2007; doi:10.1152/japplphysiol.01088.2006.—Cross contamination of intramyocellular lipid (IMCL) signals through loss of bulk magnetic susceptibility (BMS) differences was detected in human muscles using proton magnetic resonance spectroscopic imaging ($^1$H-MRSI) at 4 T by varying nominal voxel sizes on healthy subjects. In soleus muscle the IMCL content estimated in 1.00-ml-sized voxels was 15% and 30% higher than that in 0.25-ml voxels for nonobese ($P < 0.05$) and obese ($P < 0.01$) subjects, respectively, whereas no effect was observed on IMCL estimation in tibialis posterior (TP) and tibialis anterior (TA) regions for different voxel sizes. The unbiased 0.25-ml voxel size $^1$H-MRSI method was applied to measure IMCL content in nonobese sedentary (NOB-Sed), moderately trained (Ath), sedentary obese (OB), and Type 2 diabetic mellitus (DM) subjects. IMCL content in soleus was greatest in OB, with decreasing content in DM, Ath, and NOB-Sed, respectively (12.6 ± 1.6, 9.7 ± 1.8, 7.4 ± 1.0, 4.9 ± 0.5 mmol/kg wet wt; $P < 0.05$ by ANOVA; $P < 0.05$ OB vs. NOB-Sed or Ath). In TA, IMCL was equivalently elevated in DM and OB, which was higher than in Ath or NOB-Sed, respectively (4.2 ± 0.4 and 4.2 ± 0.7 vs. 2.7 ± 0.5 and 1.5 ± 0.3 mmol/kg wet wt; ANOVA, $P < 0.05$; $P < 0.05$ DM or OB vs. NOB-Sed). We conclude that IMCL content is overestimated when voxel size exceeds 0.25 ml despite measurement by optimized high-resolution $^1$H-MRSI at high field. When IMCL is measured unbiased by concomitant obesity, we find that it is strongly influenced by muscle type, training status, and the presence of obesity and Type 2 diabetes.

intramyocellular lipid; signal cross contamination; bulk magnetic susceptibility; proton magnetic resonance spectroscopic imaging; higher magnetic field

PROTON MAGNETIC RESONANCE spectroscopy ($^1$H-MRS) has recently been developed as a powerful method to noninvasively assess muscular lipid stores and to specifically measure the intramyocellular lipid (IMCL) pool (1–3,5, 17, 20, 27, 29, 32, 41). Typical triglyceride concentrations detected by methylene resonances range from 1 to 20 mmol/kg wet weight (17, 26, 32). The possibility of using $^1$H-MRS for measurement of intramyocellular lipids arose out of the studies of Schick et al. (27), which suggested that the resonance frequency of triglycerides contained with spherical IMCL droplets is shifted by ~0.2 ppm from triglycerides within linear/asymmetrical adipocytes (extramyocellular lipid, or EMCL), thus allowing the two pools to be discriminated under high-resolution spectroscopy conditions (4, 17, 27, 32). The use of $^1$H-MRS method for measurement of IMCL was first reported at 1.5-T magnetic field strength (4, 18, 27, 31–32), and most studies have continued to be performed at this field strength due to the widespread availability of clinical systems at 1.5 T. Studies of the biophysics of the magnetic susceptibility effect have clarified that this measurement is prone to artifact, particularly at lower magnetic field strengths, unless extreme caution is taken for proper experimental setup (4, 32–33). The separation between the IMCL and EMCL methylene resonances are generally much better from voxels within tibialis anterior (TA) compared with soleus, with tibialis posterior (TP) being intermediate. This is due to greater alignment of muscle fibers in TA. This is crucially important because the difference in magnetic susceptibility effect is maintained only as long as muscle fibers maintain a roughly parallel orientation to the magnetic field. As fibers become more oblique, the magnetic susceptibility difference between lipids stored in IMCL and EMCL compartments decreases, resulting in overlap between EMCL and IMCL resonances (4, 33). As voxel size increases, it is more likely that large intermyofibril adipose deposits are contained within the voxel; thus the probability of spectral overlap between EMCL and IMCL becomes high (4). New postprocessing routines for lipid extrapolation (LE) (13, 38) can reduce the signal bleeding of EMCL from bulk lipid deposits outside of voxels of interest. Such postprocessing cannot, however, reduce the overlap between EMCL and IMCL caused by heterogeneously distributed myofibril adipose deposits in the voxels of interest. Additional approaches for enhancing data quality may be realized by moving to higher field strength, which improves overall spectral resolution and signal-to-noise ratio. Adequate signal-to-noise ratio is achievable from voxels much smaller than at lower field (e.g., 0.25 ml vs. 2 to 8 ml). This capability, coupled with a spectroscopic imaging approach, allows for the simultaneous acquisition of multiple small voxels. Only the best resolved spectra are chosen for unambiguous quantification of IMCL, which necessarily biases against voxels containing larger amounts of EMCL within the voxels of interest or due to signal bleeding from neighboring muscle voxels.

To date, in vivo $^1$H-MRS studies of muscles have been conducted largely using single-voxel techniques with voxel...
sizes ranging from 1.8 ml to 8 ml (1–2, 4, 18, 23, 26–27, 29, 32, 39) at lower magnetic field (e.g., 1.5 or 2.1 T) with a few exceptions using magnetic resonance spectroscopic imaging (MRSI) (16–17, 19, 28, 38). MRSI combined with stronger magnetic field strength (e.g., 4 T) has provided enhanced IMCL resolution, e.g., IMCL methylene resonance separation from EMCL methylene resonance (17, 19), which is critically important for determining unbiased IMCL content. Thus the first goal of this study was to define the critical conditions affecting measurement error of IMCL determination when voxel size was varied from 1.00 to 0.063 ml using an MRSI approach at 4 T.

Increased IMCL content is known to predict low insulin sensitivity in untrained individuals (9, 18, 20, 24, 25, 29, 31, 34, 39). Obese individuals (34) are reported to have higher IMCL content than lean subjects, as well as Type 2 diabetic patients (23) and their insulin-resistant offspring (18, 24). On the other hand, studies of athletes in general have determined that their IMCL levels are significantly elevated (11, 19, 34, 37), as is their insulin sensitivity. Some of these studies evaluated IMCL content either using muscle biopsy technique (11, 37) or via 1H-MRS at 1.5 T (26, 32) with a larger voxel size, which could potentially cause overestimation of IMCL value because of EMCL contamination (15). Therefore we sought to validate the optimum voxel size for unbiased IMCL measurements using our previously established MRSI method at 4 T (17). We applied this method to four groups of interest, e.g., nonobese sedentary, moderately trained athletic, sedentary obese, and Type 2 diabetic subjects, to provide normative data for this critical physiological measurement.

METHODS

Phantom 1

To measure the accuracy of quantification, spectra were acquired from a cylindrical phantom of known triglyceride concentration. The phantom was 12 cm in diameter, similar to the human calf dimensions and giving reasonable coil loading for the 1H transverse electromagnetic (TEM) coil used in our human studies. To a base solution consisting of 210 g of glycerol and 25 ml of H3PO4 in 50% glycerol in 1,890 g (105 mol) of water was dissolved 2.4 g (36.9 mmol) of sodium azide, 2.5 g (8.2 mmol) of sodium oleate, 6.5 g (49.7 mmol) of creatine, and 8.0 g (49.7 mmol) of carnitine. Then 42.4 g (45.5 mmol) of high oleic organic safflower oil (Spectrum Organic Products) suspended with egg yolk lecithin (100/6, wt/wt) was ultrasonically mixed with the above aqueous solution to make a lipid emulsion. Twenty-eight grams of Xanthan gum was then slowly added to the emulsion with continuous magnetic stirring. To the resulting thick emulsion was then added 125.1 g of Borax (sodium tetraborate) with slow stirring. The mixture provided a final safflower oil concentration of 21.3 mmol/l in triglyceride. The fatty acid content of the safflower oil according to the manufacturer was 75–80% oleic fatty acid, 2–16% linoleic acid, 4–9% palmitic, and <3% stearic and <1% linolenic fatty acids.

Phantom 2

Phantom 2 was designed to measure the signal contamination from pure fat voxel to neighboring muscle lipid voxels. A 2-liter storage box was filled with 0.5% Intralipid (IL) emulsion with 60 mmol/l KCl. Two screwcap tubes, 50 and 15 ml, filled with soybean oil (Soy), were placed on the bottom of the box (shown in Fig. 2A). IL emulsion of 0.5% has ~5.7 mM triglyceride. The signal intensity from one Soy voxel is ~100 times of that from one IL voxel.

Subjects

Ten nonobese healthy subjects [NOB; 9 men/1 woman; body mass index (BMI) = 24.9 ± 3.8 kg/m², age = 36.0 ± 11.7 yr (mean ± SD)] and 12 obese subjects (OB; 3 men/9 women; BMI = 32.0 ± 1.7 kg/m², age = 46.2 ± 10.4 yr) were studied to compare the effect of MRSI voxel size on the quantification of IMCL content in calf muscle. Not all subjects participated in both comparisons of voxel size (1.00 vs. 0.25 and 0.25 vs. 0.063 ml). IMCL content was compared in four different groups of subjects: seven nonobese sedentary (NOB-Sed; 6 men/1 woman; BMI = 25.4 ± 2.1 kg/m², age = 37.9 ± 9.0 yr), nine moderately trained athletic (Ath; 7 men/2 women; BMI = 24.3 ± 2.7 kg/m², age = 33.9 ± 8.1 yr), nine obese (OB; 3 men/6 women; BMI = 32.3 ± 1.5 kg/m², age = 46.0 ± 8.4 yr), and seven Type 2 diabetic mellitus subjects (DM; 4 men/3 women; BMI = 31.4 ± 5.8 kg/m², age = 44.7 ± 5.8 yr). IMCL content was quantified in soleus and TA muscles in the right calf. Informed written consent was obtained from all subjects, and the study was approved by the Committee on Clinical Investigations (Institutional Review Board) of the Albert Einstein College of Medicine.

1H-MRSI

Data were acquired with a 4-T whole body NMR system using a Varian INOVA console and a volume 1H TEM coil (17). The phantom or the right calf of the human subject was positioned in the coil, and the left calf was enclosed in a radio-frequency shield for human studies. 1H images were acquired to determine the location of the right calf. The axial slice of interest was positioned at the insertion point of the gastrocnemius. Shimming over the entire slice was performed manually, resulting in a water line width of ~20 Hz for human studies. 1H-MRSI, 16 × 16, 32 × 32, or 64 × 64 phase encodings (PEs), were acquired using a rectangular sampling scheme and a field of view of 160 × 160 mm². A slice thickness of 10 mm was employed, resulting in a nominal voxel size of 1.00, 0.25, and 0.063 ml, respectively. The spectral width was 1,600 Hz. Localization was achieved using a slice-selective excitation (10 mm) pulse with a single spin echo without any additional prelocalization scheme. Water suppression was achieved by using an optimized semiselective refocusing pulse (14). Additional water suppression was achieved using a shaped pulse (14). Additional water suppression was achieved using a shaped
delays alternating with nutations for tailored excitation (DANTE) pulse train before the spin echo (17). For the water-suppressed (metabolite) spectroscopic imaging (SI), the acquisition time was 4.5 and 17 min for $16 \times 16$ and $32 \times 32$ PEs [time to repetition (TR)/echo time (TE) = 1,000/24 ms] and 43 min for $64 \times 64$ PEs (TR/TE = 600/24 ms). To provide an internal concentration reference, a nonsuppressed water SI (TR/TE = 5,000/24 ms) was acquired from the same slice using $16 \times 16$ PEs (acquisition time = 21 min).

Data Processing

Data postprocessing was described in detail in our previous work (17). In summary, the data were processed using a 10 Hz Lorentzian-to-Gaussian transformation and 250-Hz convolution difference. A Hanning filter was applied in both spatial domains. After Fourier transformation, the spectroscopic data were corrected for main magnetic field strength ($B_0$) shift by referencing the total creatine (TCr) resonance at 3.0 ppm. For each muscle group (soleus, TA, and TP), three voxels from each muscle were selected using the anatomic images as a guide. The selection criteria for the three voxels in each muscle were as follows: 1) the voxel was completely within the muscle; 2) the voxel was distinctly separated from bone, blood vessels, subcutaneous fat (SF) layers, bone marrow (BM), or intrafascial fat deposits; 3) the best resolved voxels in terms of methylene peak separation between 1.3 and 1.5 ppm were chosen; 4) the voxels selected in soleus muscle were at least two voxels away from SF while those in TA were at least one voxel away from BM or SF. The spectra were phased and baseline corrected using a cubic spline. The corresponding water spectra were used to provide an internal reference for quantification.

In vivo data with $16 \times 16$ and $32 \times 32$ PEs were also preprocessed with LE through following three steps. 1) The LE procedure was employed to extrapolate SF and BM signals in k-space, from the original $16 \times 16$ or $32 \times 32$ to $64 \times 64$ (13), to suppress the bleeding of signals from SF or BM to muscles. The mask defining the bulk lipid regions was created on the extended MRSI with a threshold of 20% of maximum lipid signal. 2) After the LE procedure, bulk lipid signals within the mask were extracted in the image space and the resultant signals were transformed back to k-space. 3) The “fat-free” k-space was chopped back to its original $16 \times 16$ or $32 \times 32$ matrix format for further postprocessing as described above. The rationale of retain-
Table 1. IMCL content obtained from 1.00-ml voxels and 0.25-ml voxels via 16×16 and 32×32 PE MRSI at 4 T

<table>
<thead>
<tr>
<th>Subject Type</th>
<th>1.00 ml</th>
<th>0.25 ml</th>
<th>1.00 ml</th>
<th>0.25 ml</th>
<th>1.00 ml</th>
<th>0.25 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOB (n = 7)</td>
<td>7.6±1.0</td>
<td>6.7±1.2*</td>
<td>2.9±0.4</td>
<td>2.6±0.4</td>
<td>4.1±0.5</td>
<td>4.4±0.5</td>
</tr>
<tr>
<td>OB (n = 8)</td>
<td>14.4±2.5</td>
<td>11.1±2.2†</td>
<td>4.1±1.4</td>
<td>4.1±1.6</td>
<td>7.9±1.3</td>
<td>7.7±1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE of intramyocellular lipid (IMCL) content in units of mmol/kg wet wt. Different subjects participated in the 0.25- vs. 1.00-ml and the 0.25- vs. 0.063-ml voxel experiments. NOB, nonobese subjects; OB, obese subjects; S, soleus; TA, tibialis anterior; TP, tibialis posterior; PE, phase encoding; MRSI, magnetic resonance spectroscopy imaging. 0.25 ml vs. 1.00 ml: *P < 0.05, †P < 0.01.

RESULTS

Phantom Studies

 Phantom 1. The T1 and T2 values of safflower oil methylene protons in the phantom mixture measured at 4 T in our whole body human NMR system were 0.44 s and 56 ms, respectively, which were close to the reported T1 and T2 values of IMCL methylene proton in human soleus muscle, e.g., 0.39 s and 75 ms (17). Water proton T1, 1.47 s, and T2, 35 ms, were measured in a 200-MHz (4.7 T) high-resolution vertical Varian INOVA spectrometer and were similar to those of water proton in muscle at 4 T (170 MHz), e.g., 1.9 s and 26 ms. The safflower oil concentration was calculated according to methylene-to-water proton ratio provided that the average proton density of (CH2)n in monounsaturated enriched safflower oil is 59 mmol/ml. A T1 relaxation correction was applied to the CH2/H2O signal calculation since different TRs were applied in the SI acquisitions of metabolite and water. The measured value, 20.8 mmol/l, was very close to the theoretical value, 21.3 mmol/l. The phantom was then studied under different PE MRSI at 4 T (16 × 16, 32 × 32, and 64 × 64, respectively). The 1H-NMR spectrum with nominal voxel size of 0.25 ml is shown in Fig. 1. The signal intensity was then compared for different voxel sizes, e.g., 1.00, 0.25, and 0.063 ml. The ratio from one 0.25-ml voxel to the sum of four 0.25-ml voxels falling into the same region gave a mean value of 1.00 ± 0.01 for four different regions. Similar results were also obtained for one 0.25-ml voxel and four 0.063-ml voxels after the T1 correction. These results confirm that the signal intensity is proportional to the voxel volume when the proton signal from lipid methylene is well resolved from other resonances and the sample is mostly uniform in the region of interest under relatively homogeneous field strength.

 Phantom 2. Figure 2 illustrated the spectra from Soy and some IL voxels. Because of the inevitable truncation artifacts in 1H-MRSI, IL methylene (IH-CH2) from voxel IL-1 adjacent to the Soy voxel is severely obscured by the strong Soy methylene (Soy-CH2) signal. When the IL voxel was one voxel

Table 2. IMCL content obtained from 0.25-ml voxels and 0.063-ml voxels via 32×32 and 64×64 PE MRSI at 4 T

<table>
<thead>
<tr>
<th>Subject Type</th>
<th>0.25 ml</th>
<th>0.063 ml</th>
<th>0.25 ml</th>
<th>0.063 ml</th>
<th>0.25 ml</th>
<th>0.063 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOB (n = 8)</td>
<td>6.7±0.8</td>
<td>6.1±0.7</td>
<td>2.1±0.4</td>
<td>2.1±0.3</td>
<td>4.0±1.0</td>
<td>3.8±0.8</td>
</tr>
<tr>
<td>OB (n = 7)</td>
<td>8.2±1.5</td>
<td>8.3±1.5</td>
<td>3.5±0.5</td>
<td>3.4±0.4</td>
<td>6.1±1.1</td>
<td>5.4±1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of IMCL content in units of mmol/kg wet wt. Different subjects participated in the 0.25- vs. 1.0-ml and the 0.25- vs. 0.063-ml voxel experiments.
away from the Soy voxel (e.g., IL-2), IL-CH$_2$ is well resolved from Soy-CH$_2$ because of the signal bleeding from the Soy voxel. When two voxels away from the Soy voxel, the Soy-CH$_2$ signal intensity is relatively smaller than that from IL-CH$_2$ in voxel IL-3. It is also shown from this experiment that the signal bleed from the Soy voxel does not overlap with IL-CH$_2$ when the selected IL voxel is at least one voxel away from the Soy voxel for both 16$^/$H$_{11003}$16 and 32$^/$H$_{11003}$32 PEs under the current magnet field, e.g., 4 T.

**IMCL Measurements in Human Subjects**

Displayed in Fig. 3 are spectra from soleus, TA, and TP muscles of a marathon runner with a nominal voxel size of 0.25 ml. The spectra in soleus, TA, and TP all show IMCL methylene resonances at 1.3 ppm fully resolved from EMCL at 1.5 ppm.

The IMCL content for three different regions (soleus, TA, and TP) acquired via 16$^/$H$_{11003}$16, 32$^/$H$_{11003}$32, and 64$^/$H$_{11003}$64 PEs are listed in Tables 1 and 2. The voxels with smaller nominal size, e.g., 0.25 and 0.063 ml, were chosen to fall into the voxels with bigger size, e.g., 1.00 and 0.25 ml, for appropriate comparison. When the voxel size is increased to 1.00 ml from 0.25 ml, IMCL content in TA and TP muscles did not change for both NOB and OB subjects (Table 1). IMCL resolution was equivalently well resolved for 1.00-ml MRSI compared with 0.25-ml MRSI (Fig. 4) in these two regions. In the soleus region, IMCL resolution is decreased (Fig. 5) probably because of more varied fiber orientation when the voxel size is increased to 1.00 ml. As a result, IMCL obtained from 1.00-ml MRSI in soleus is nearly 15% higher for NOB subjects (7.6 ± 1.0 vs. 6.7 ± 1.2 mmol/kg wet wt; $P < 0.05$), whereas it is 30% higher for OB subjects (14.4 ± 2.5 vs. 11.1 ± 2.2 mmol/kg wet wt; $P < 0.01$, Table 1).

The IMCL resonance was equivalently resolved within 0.25-ml voxels compared with 0.063-ml voxels in soleus, TA, or TP (Figs. 4 and 5). Consistent with the equivalent degree of resolution, the IMCL content was unchanged when measured with a 0.25-ml MRSI compared with 0.063-ml MRSI for both NOB and OB subjects (Table 2).

**IMCL Content With and Without LE**

IMCL contents estimated with or without LE procedure are listed in Table 3 for 16$^/$H$_{11003}$16 and 32$^/$H$_{11003}$32 PEs. The IMCL contents stay unchanged after LE compared with those estimated without LE employed for both 1.00-ml and 0.25-ml sized voxels in soleus and TA muscles.

**IMCL Content in Four Different Groups of Subjects**

Four different groups of subjects were studied via 0.25-ml MRSI at 4 T to provide normative data on skeletal muscle IMCL content. The results are plotted in Fig. 6. In soleus, OB subjects (12.6 ± 1.6 mmol/kg wet wt; $P = 0.002$ vs. NOB-Sed, $P = 0.020$ vs. Ath; $P = 0.003$, 1-way ANOVA for 4 groups of subjects) had the highest IMCL content, followed by DM subjects (9.7 ± 1.8 mmol/kg wet wt), Ath (7.7 ± 1.0 mmol/kg wet wt), and NOB-Sed subjects (4.9 ± 0.5 mmol/kg wet wt).

In TA, DM and OB subjects had the highest IMCL content (4.2 ± 0.4 and 4.2 ± 0.7 mmol/kg wet wt respectively; $P = 0.010$, DM vs. NOB-Sed; $P = 0.004$, OB vs. NOB-Sed; $P = 0.003$, one-way ANOVA for 4 groups of subjects), followed by Ath (2.7 ± 0.5 mmol/kg wet wt) and NOB-Sed subjects (1.5 ± 0.3 mmol/kg wet wt).

**DISCUSSION**

With the increased availability of high-strength magnetic field as well as MRSI acquisition sequences in research and clinic MR systems, it is important to understand the effect of voxel size on the estimation of IMCL content. By using 0.25-ml MRSI at 4 T, IMCL resolution from EMCL was dramatically improved, particularly in soleus where muscle fiber orientation is more variable. IMCL resolution and pre-

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**Fig. 4.** $^1$H-NMR spectra in TA obtained from nonobese (left) and obese (right) subjects with different nominal voxel sizes: 1.00, 0.25, and 0.063 ml from top to bottom, respectively. The spectra were scaled identically.

**Fig. 5.** $^1$H-NMR spectra in soleus obtained from nonobese (left) and obese (right) subjects with different nominal voxel sizes: 1.00, 0.25, and 0.063 ml from top to bottom, respectively. The spectra were scaled identically.
sumably fiber orientation was improved in trained subjects compared with sedentary controls (Fig. 3 vs. Fig. 5). When voxel size was decreased to 0.063 ml, improved IMCL resolution is also observed in obese subjects as shown in Fig. 5, where IMCL is nearly baseline resolved from EMCL. Higher magnetic field strength, smaller voxel size, and MRSI technique all contribute to detecting and quantifying the best-resolved spectra for unbiased IMCL quantification.

This is the first study to compare the effect of reducing nominal voxel size on IMCL content, e.g., 1.00 to 0.063 ml in human subjects at higher magnetic field, e.g., 4 T, using a spectroscopic imaging technique. The result from the phantom study comparing lipid content via different PEs, e.g., 16 × 16, 32 × 32, or 64 × 64, supports the a priori proposition that different voxel sizes will not result in different lipid concentrations since there is no overlapping resonance to obscure the methylene resonance in a relatively homogeneous sample. In contrast, in human muscle, due to bulk magnetic susceptibility effects, IMCL content is significantly higher when measured with larger nominal voxel size, e.g., 1.00 ml in our study, in soleus containing muscle fibers oriented obliquely to the external magnetic field for both nonobese and obese healthy subjects. The overestimation of IMCL in soleus via 1.00-ml MRSI is not caused by the contaminating lipid signals from SF or BM in our present study since the voxels chosen in soleus muscle are at least two voxels away from SF and BM. The phantom study has shown that contaminating signal from SF or BM region is negligible if the voxels of interest are two voxels away from SF or BM. The unchanged IMCL content estimated with or without the LE procedure also indicates that the contaminating signal from SF or BM has no effect on IMCL peak when the voxels of interest in soleus are at least two voxels away from SF or BM. Even though some voxels in TA are only one voxel away from BM or SF, the better muscle fiber orientation in TA retains a good IMCL separation from EMCL. However, the signal bleeding originating from adjoining muscle voxels can lead to unpredictable overlap with IMCL resonances in the voxel of interest. The signal bleeding from different EMCL layers outside the voxel with a different BMS may impose on the original EMCL resonance in the voxel of interest, resulting in decreased IMCL separation from EMCL. In the mean time, the larger voxel, e.g., 1.00 ml, will lead to increased EMCL contribution from inside the voxel and thus complicate IMCL estimation. However, the overestimation of IMCL can be minimized by applying smaller voxel sized MRSI for both nonobese and obese subjects. This may explain, at least in part, why the IMCL in soleus estimated by 0.25-ml MRSI methods for these nonobese subjects (4.9 mmol/kg wet wt in Fig. 6) (17) is significantly lower than values previously reported, e.g., ~10 mmol/kg wet wt or higher, obtained at 1.5 T from single-voxel (~2.4 or 8 ml) studies in similar populations (5, 9–10, 23, 26, 30, 32, 34–35). The use of an MRSI approach allowed us to analyze the best-resolved IMCL/EMCL resonances by inspecting all MRSI spectra. By design, this approach biased voxels to minimize EMCL and potentially could cause some user-dependent bias, since the voxels were selected manually based on best IMCL-
EMCL peak resolution. The IMCL content, however, estimated from the three-voxel method, is strongly correlated with that from nine-voxel method for both nonobese and obese subjects ($R = 0.901, P < 0.01$) and therefore was bias independent. Analysis of only three voxels with the best IMCL resolution was chosen to give an averaged IMCL content, which saved data analysis time compared with nine voxels.

In most of our human studies, 0.25-ml MRSI was sufficient to give reasonable IMCL resolution from EMCL in soleus muscle as well as in TA and TP. In some extreme cases, especially for some obese subjects, the 0.063-ml MRSI method is a good alternative when IMCL was inadequately resolved from EMCL even in the case of 0.25-ml voxel size. This situation occurred only rarely (i.e., 2/100). We interpret this finding to indicate that myofibril microheterogeneity is increased in at least the soleus muscle of obese subjects. The acquisition time to acquire 0.063-ml MRSI is $\approx 43$ min, which can be uncomfortable for subjects because of the requirement to remain motionless. The data should be analyzed with caution for 0.063-ml MRSI since IMCL content varies from voxel to voxel, unlike that for 0.25-ml voxel size. This may have reflected the inherent variability of measurement due to the relatively low signal-to-noise ratio. We suggest analyzing more than three voxels when applying a 0.063-ml MRSI method to obtain an average that is less susceptible to bias caused by manual voxel selection.

Various studies have reported IMCL content differences in subjects subjected to different diets (low fat vs. high fat), levels of physical activity, obesity, and history of diabetes by biochemical Oil Red O staining of muscle cross-sections (11, 37) and by $^1$H-MRS (18, 23, 26). IMCL content assessed from $^1$H-MRS studies have been mainly acquired at lower magnetic field using larger voxels. As discussed above, the cross-contamination caused by BMS effects can potentially affect the accuracy of IMCL quantification and lead to overestimation of IMCL content when EMCL overlaps onto IMCL, especially with larger voxel sizes. To our knowledge, no study has reported IMCL content from nonobese sedentary, athletic, obese, and Type 2 diabetic subjects using the MRSI method with a smaller voxel size of 0.25 ml at high magnetic field. The 0.25-ml MRSI method at 4 T minimized the cross-contamination effect on IMCL quantification, giving a more accurate quantification of IMCL content.

Goodpaster et al. (11) quantified IMCL content in percutaneous vastus lateralis biopsy specimens by quantitative image analysis of Oil Red O staining and reported significantly elevated IMCL content in obese subjects with Type 2 diabetes mellitus, as well as endurance-trained athletes compared with sedentary lean subjects. Misra et al. (23) studied IMCL of nonobese healthy and Type 2 diabetic Asian Northern Indian males by MRS using a single-voxel method (8 ml) at 1.5 T and reported that IMCL content in soleus was approximately two times higher in lean Type 2 diabetic males compared with healthy nondiabetic subjects. Our results are qualitatively similar, in that IMCL content in soleus and TA among obese and obese diabetic subjects was higher than in normal sedentary controls. In contrast, we did not observe higher IMCL content in athletes compared with nonobese sedentary subjects, while Goodpaster et al. (11) reported significantly elevated IMCL content histochemically in their athletes compared with lean subjects. These differences may have been due to the degree of fitness training. In addition we cannot exclude a type 2 error as our numbers were not very large (and the trend was in the same direction). The athletes we studied were only moderately trained, exercising three to five times per week, while Goodpaster et al. reported on endurance-trained subjects with a relatively high aerobic capacity as evidenced by their maximal oxygen uptake. High maximal oxygen uptake in endurance-trained subjects has been associated with significantly elevated IMCL content (34). It should be acknowledged that obese and nonobese subjects were not equivalently matched for age, and sex, and that these variables may have impacted independently on concentrations of IMCL.

To date, most studies on IMCL concentration have only reported the relative IMCL content in terms of percentage of muscular water or creatine signal intensity in $^1$H-MRS or percentage of area lipid stained with microscopy methods. It is therefore difficult to compare our IMCL values with other data in the literature. Howald et al. (15) reported a mean IMCL content in TA of 2.4 mmol/kg wet wt from three untrained and seven regular endurance-trained subjects using a $^1$H-MRS single-voxel method with voxel size of 2.4 ml at 1.5 T. This is in line with our results from normal sedentary subjects and moderately trained subjects (1.5 ± 0.3 and 2.7 ± 0.5 mmol/kg wet wt, respectively). The good consistency of IMCL content between our data estimated from 0.25-ml MRSI at 4 T with their data from 2.4-ml single-voxel method at 1.5 T is likely due to the high degree of uniformity of muscle fiber alignment in TA. This enables consistent orientation of TA muscle fibers parallel to the external magnetic field, ensuring ideal IMCL resolution in TA even at lower magnetic field. Szczepaniak et al. (32) reported an IMCL content in midsoleus muscle of 10.7 mmol/kg wet wt from nine normal control subjects at 1.5 T with a single-voxel volume of $\approx 2.3$ ml. Rico-Sanz et al. (26) reported IMCL content from sedentary to highly trained male subjects was 10.3 mmol/kg wet wt in soleus and 5.6 mmol/kg

![Fig. 7. $^1$H-NMR spectra from TA of an obese male. A: 1.5-T single-voxel method (1.2 ml). B: 4-T magnetic resonance spectroscopy imaging, voxel size of 0.25 ml. Calculated IMCL content = 4.8 vs. 3.7 mmol/kg wet wt, A vs. B.](Image)
wet wt in TA, which are approximately double our IMCL values (4.9 and 1.5 mmol/kg wet wt in soleus and TA, respectively). The higher IMCL value in these studies (26, 32) compared with ours cannot only be attributed to differences in aerobic fitness, as healthy sedentary subjects were studied in all the studies. The other potential explanation is their use of larger voxel sizes (2.3 or 8 ml) acquired at lower magnetic field, e.g., 1.5 T. IMCL spectra from TA can still exhibit overlap of the EMCL resonance at 1.5 ppm onto the 1.3-ppm IMCL resonance (4, 26). We studied one obese subject both at 1.5 T via a single-voxel method and at 4 T via 0.25-ml MRSI to compare the spectra quality in terms of IMCL methylene resonance resolution in TA (Fig. 7). Although both spectra were acquired from the TA region where myofibrils are better oriented, the spectrum acquired using the larger single-voxel method at 1.5 T (GE Signa extremity coil, single voxel size = 1.2 ml) demonstrated obvious overlap of EMCL onto IMCL. In contrast, the spectrum from the same individual with a 0.25-ml MRSI method at 4 T clearly demonstrated that the IMCL resonance was well resolved from EMCL. The calculated IMCL content was 4.8 vs. 3.7 mmol/kg wet wt for 1.5-T single-voxel method and 4-T 0.25-ml MRSI, respectively. This clearly demonstrates that decreased IMCL resolution can arise when using a larger voxel size at lower magnetic field strength, even in TA where parallelism of muscle fibers can be expected to be maximum. Muscle biopsy samples (15, 21, 40) are also prone to overestimation of intramyocellular triglyceride content unless extreme precautions to eliminate adipocyte contamination are performed (12). In fact, the amount of the potential contaminating triglycerides from EMCL in soleus is almost as much as IMCL itself.

In summary, IMCL content in soleus muscle estimated from 1.00-ml voxel MRSI is significantly higher than that estimated from 0.25-ml and 0.063-ml methods for both nonobese and obese healthy subjects at 4 T. This overestimation via 1.00-ml voxel MRSI was caused by overlap of the EMCL methylene resonance onto IMCL, arising from the oblique orientation of muscle fibers close to the magic angle. This adverse effect can be identified and minimized by applying smaller-size voxel MRI to isolate voxels within uniform fibers orientated parallel to B0. The 0.2-ppm BMS effect between IMCL and EMCL is thus reliably maintained and ensures appropriate separation of IMCL from EMCL. The 0.25-ml voxel MRSI, which lasts 17 min, can give relatively high-quality spectra with good IMCL resolution from EMCL for most lean and obese subjects. Rare obese subjects from our other human studies demonstrated poor IMCL resolution even at 0.25-ml voxel MRSI. For these subjects 0.063-ml voxel MRSI was used for the estimation of IMCL content. Obese subjects and individuals with Type 2 diabetes mellitus demonstrate higher IMCL content in both soleus and TA muscle compared with nonobese sedentary subjects, while moderately trained athletes have intermediate IMCL values.

In conclusion we show that despite optimized high-resolution 1H-MRSI at high field, IMCL is overestimated by 15–30% in soleus when voxel size exceeds 0.25 ml. It can be predicted that this overestimation of IMCL in soleus will be proportionally worse under low-resolution conditions at 1.5 T. Studies at lower field strength can be predicted to achieve the best possible accuracy when limited to the smallest achievable voxel sizes given the limitations on gradient strengths and coil sensitivities and when restricted to muscles with the most uniform fiber orientation, e.g., TA or rectus femoris.

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