Evaluation of intramyocellular lipid breakdown during exercise by biochemical assay, NMR spectroscopy, and Oil Red O staining

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METHODS

Nine healthy, physically active men (age 22.8 ± 0.4 yr, body wt 74.0 ± 2.3 kg) volunteered to participate in the study, which was set up to investigate the effects of exercise in the fasted state on IMCL breakdown. Partial results, including fiber-type-specific IMCL data obtained via ORO staining, and detailed methodology of this study have been published elsewhere (5). The study protocol was approved by the local Ethics Committee (K.U.Leuven, Faculty of Medicine). Subjects gave their written, informed consent after they were informed in detail of all experimental procedures and risks possibly associated with the experiments.

Protocol. Briefly, the study was designed as a balanced and randomized cross-over study in which the nine subjects participated in two experimental sessions with a 3-wk period in between. In one experimental session, they performed a 2-h constant-load exercise test in the fasted state, whereas in the other condition they ingested carbohydrates before and during exercise (5). However, for the single and specific purpose of this article, the data were analyzed independently of the experimental conditions, and the repeated measures in

INTEREST IN METABOLISM of intramyocellular lipids (IMCL) has increased over recent years. The role of IMCL in the disruption of intracellular fat homeostasis, which can lead to insulin resistance (2, 33, 36), has become an issue of primary interest. Furthermore, the role of IMCL as a substrate in endurance is even more relevant (25). This method allows for simultaneous visualization of IMCL and identification of muscle fiber type in muscle cross sections. 1H magnetic resonance spectroscopy (1H-MRS) offers a noninvasive approach to quantifying IMCL (4). 1H-MRS measures the resonances of methylene and methyl protons in triacylglycerols, which appear as multiple peaks on the proton spectrum of skeletal muscle tissue.

The majority of 1H-MRS (19, 35) and ORO studies (5, 42) have reported IMCL depots to significantly contribute to energy provision during prolonged moderate-intensity exercise. However, controversy as to what extent IMCL contributes as an energy substrate during exercise persists because most (1, 15, 21, 23, 37) but not all (17, 29) studies that used biochemical analyses on muscle biopsy samples did not find net IMCL breakdown during exercise of 1–2 h duration in males. Thus, different methods of measurement seem to have resulted in different conclusions with regard to the magnitude of IMCL in fueling muscle contractions. However, to date, no studies have directly compared exercise-induced IMCL breakdown in humans by the three methods currently available. Therefore, we integrated IMCL measurements by 1H-MRS and by biochemical assay in a previously published study (5), wherein we addressed the effect of carbohydrate intake on fiber type-specific IMCL breakdown during prolonged submaximal exercise. Thus, exercise-induced IMCL breakdown could be compared between the methods.

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the same subjects were treated as independent observations (n = 18). This strategy is justified by the fact that, as a rule, a valid method of measurement must specifically measure the variable of interest independently of the experimental condition. In addition, intra-individual variability for the two repeated measures per subject was enhanced by the dietary manipulation. Thus, if 1H-NMR, biochemical assay, and ORO staining measure the same aspect of IMCL, then the three measurements should yield similar results for the 18 observations.

On the morning of the experiments, the subjects arrived at the laboratory after an 11-h overnight fast. First, a 1H-MRS scan was performed. Two hours after arrival, a percutaneous needle biopsy sample was taken from the right vastus lateralis muscle under local anesthetic (2–3 ml of Lidocaine) through a 5-mm incision in the skin, and subjects immediately after started to cycle for 2 h (178 ± 8 W, ~75% VO₂_peak). At the end of the exercise bout, another muscle biopsy sample was taken. The muscle biopsy was taken through the same incision as the preexercise biopsy but with the needle pointing in another direction. Thereafter, they received 1 g/kg body wt maltodextrin to inhibit any further lipolysis, and a second 1H-MRS scan was performed between 30 min and 1 h postexercise.

1H-MRS. Image-guided, localized, single-voxel 1H-MRS was performed in the left vastus lateralis. All measurements were performed on a 1.5-T whole body scanner (Sonata, Siemens, Erlangen) with a flexible surface coil wrapped around the upper leg with the leg placed in the parallel position. Care was taken that the positioning of the leg and coil were identical within (pre- and postexercise) and between the experimental conditions in the pre- and postexercise condition. Furthermore, by careful inspection of the images, care was taken to avoid visible vascular structures and adipose tissue within the voxel. To reproduce voxel position between measurements, the longitudinal distance from the voxel to the distal end of the medial femoral epicondyl was determined in a coronal image of the upper leg and used as a reference. Variability of the IMCL quantification was determined by measuring one subject five times. The subject left the imaging (IMCL-ORO) is shown in Table 1. Preexercise IMCL-BIO, IMCL-MRS, and IMCL-ORO, respectively, were applied. 1H-MRS showed a consistent decrease of IMCL with exercise IMCL values were lower than preexercise values, whereas an increase occurred in six. Finally, with IMCL-ORO, twelve postexercise values were lower than preexercise values, whereas an increase occurred in four. Accordingly, correlations between methods were low to absent. Absolute values obtained by IMCL-ORO and IMCL-MRS yielded a low correlation (r = 0.52, P = 0.002). No correlation existed between IMCL-BIO and MCIMCL.
and either IMCL-ORO (r = 0.31, P = 0.091) or IMCL-MRS (r = 0.19, P = 0.28). Furthermore, the exercise-induced IMCL breakdown tended to correlate between IMCL-MRS and IMCL-ORO (r = 0.48, P = 0.07) but not between IMCL-BIO and IMCL-MRS (r = 0.38, P = 0.13) or IMCL-ORO (r = 0.08, P = 0.78), respectively.

Fiber typing. Given the differential IMCL content between fiber types, fiber type distribution becomes an important feature of muscle metabolism. The differential IMCL content between fiber types, as determined by various methods, is presented in Table 1.

### Table 1. Effect of exercise on IMCL content as determined by ORO staining, 1H-MRS, and biochemical analysis

<table>
<thead>
<tr>
<th>Original Units</th>
<th>% Decline in IMCL Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>ORO staining, %Total fiber area covered</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>14.8</td>
</tr>
<tr>
<td>Post</td>
<td>9.0*</td>
</tr>
<tr>
<td>1H-MRS, Arbitrary units</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>276</td>
</tr>
<tr>
<td>Post</td>
<td>154*</td>
</tr>
<tr>
<td>Biochemical, mmol/kg dry wt</td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>33.2</td>
</tr>
<tr>
<td>Post</td>
<td>30.5</td>
</tr>
</tbody>
</table>

Data are expressed as original units, and values for mean percentage decline in IMCL by exercise are provided. Intramyocellular lipid content (IMCL) before (Pre) and after (Post) exercise as determined by Oil Red O (ORO) staining, 1H-MRS, and biochemical analysis (see METHODS for further details); n, no. of observations. *P < 0.05 vs. preexercise values.

Innovative Methodology

E430 EXERCISE AND IMCL

**Fig. 1.** Effect of exercise on intramyocellular lipid (IMCL) content as determined by biochemical analysis, 1H-MRS, and Oil Red O (ORO) staining: individual data. Pre- and postexercise IMCL content as determined by biochemical assay (A; n = 17), 1H-MRS (B; n = 17), and ORO (C; n = 16). Data are expressed as percentage of mean preexercise IMCL content, which was set equal to 100%. Dotted line, subject showing a decrease in IMCL content during exercise; solid line, subject showing an increase in IMCL content during exercise. Subjects are identified by the same symbol for the 3 methods.
In this study, we compared three conventional methods, notably an enzymatic fluorometric assay (IMCL-BIO), a histochemical ORO staining assay (IMCL-ORO) on muscle biopsies, and 1\textsuperscript{H}-MRS (IMCL-MRS) to evaluate the effect of a 2-h moderate-intensity cycling bout (~75% VO\textsubscript{2} peak) on IMCL content in vastus lateralis muscle. The data shown provide clear evidence that identical experimental conditions can result in totally different conclusions with regard to IMCL utilization during exercise depending on the assay used. IMCL-MRS and IMCL-ORO measurements showed substantial contribution of IMCL breakdown to energy provision in endurance exercise. In contrast, on the basis of IMCL-BIO it is concluded that during this type and duration of exercise IMCL do not contribute significantly to the energy pool fueling muscle contractions in young healthy males.

The extent to which IMCL is degraded during exercise has been a controversial issue for quite some time (for reviews see Refs. 20, 34, 40, 41, 43). Early isotope studies showed that not all of the fat oxidation during exercise could be accounted for by plasma free fatty acid oxidation (18), leading to the suggestion that, besides circulating lipoproteins (14), also lipids stored inside muscle cells as well as between muscle cells (21) could serve as a fuel during exercise. However, studies using biochemical assay showed that exercise did not decrease IMCL content in males (15, 21, 37) unless it was extremely prolonged and strenuous (13, 27). More recent data generated by IMCL-ORO or IMCL-MRS showing substantial IMCL degradation during exercise have lead to a consensus that there is indeed net IMCL breakdown during endurance exercise (20). However, the magnitude of its contribution to energy production is still in question. The present article, in fact, for the first time directly demonstrates that the methods used to quantify IMCL hydrolysis during exercise in healthy young males result in quite different conclusions as to what extent IMCL contributes to energy provision during exercise. The present study highlights the differential output from conventional methods to assay IMCL in human muscle during exercise. However, because of the absence of a well-validated reference measurement, it is difficult to select the method of choice. Still, a rational line of reasoning can be developed to balance one assay method against the other.

Based on available literature with regard to regulation of triglyceride breakdown and synthesis, it is probably reasonable to state that prolonged exercise does not provide a condition to facilitate net IMCL synthesis. IMCL concentration reflects a dynamic balance between IMCL synthesis and breakdown. During muscle contractions, the balance shifts toward IMCL breakdown due to inhibition of glycerol reesterification (9, 10) as well as stimulation of hormone-sensitive lipase activity in electrically stimulated rat muscle (6–8, 27) and during exercise in humans (31, 32, 44). Figure 1 shows that in IMCL-BIO and in IMCL-ORO, respectively six and four of the measurements yielded net IMCL synthesis during exercise, which is unlikely to reflect a true metabolic event, although it cannot be excluded that net IMCL synthesis takes place in the fibers that are not recruited during submaximal exercise. In contrast, with IMCL-MRS, IMCL was consistently lower at the end of exercise. It has been argued that failure to detect an exercise-induced IMCL decrease by biochemical assay is caused by large (~20–25%) between- (47) and even within- (39) biopsy assay variability with regard to extramyocellular fat content, contamination with subdermal adipocytes, and/or fiber type distribution. In the current study, to minimize random variations in both fiber type composition and extramyocellular fat content, much care was taken to free all muscle material from any visible fat tissue, and measurements were performed on pooled muscle fibers from a large muscle sample (wet weight 51 ± 7 mg). Careful visual inspection of ORO images also showed that, in the current sample of young lean males, the presence of extramyocellular fat was negligible. Accordingly, the fact that perilipin at the protein level (20) and adipin at the mRNA level (26) are undetectable in dissected muscle fibers from young lean subjects also indicates the absence of a significant amount of either extracellular lipid droplets or adipocytes interspersing the muscle cells. The presence of a clear EMCL peak with MRS proves that EMCL is certainly present, but this signal does not indicate the presence of discrete adipose cells interspersed between muscle cells but could just as well be confined to larger fat streaks or contamination from subcutaneous fat. Despite the fact that needle biopsies were taken by a most experienced physician and through the same incision, variation of fiber type distribution between pre- and postexercise biopsies was substantial (see Fig. 2). Studies in young healthy subjects with IMCL-BIO or electron microscopy in pools of dissected human single muscle fibers or IMCL-ORO in combination with MHC immunofluorescence have shown that human type I fibers contain two- to threefold more IMCL than type II fibers (11, 28, 38, 42). Moreover, using the present study protocol (5), we and others (42) already showed that the exercise-induced IMCL decrease is confined to type I fibers, which are highly activated during
prolonged endurance exercise. In fact, in at least one of the present observations, exercise-induced increase in IMCL as measured by ORO can be easily explained by substantially higher fraction of type I fibers in the postexercise biopsy. Although fiber type analysis was not performed on the exact same biopsy material that was used for biochemical analysis, fiber type differences in the pre- and post-muscle biopsy likely also played a significant role for the IMCL content obtained with biochemical measurement. Therefore, a fiber-specific method is advantageous to use for evaluation of exercise-induced IMCL breakdown, which leaves us with either ORO or biochemical assay on pools of dissected single muscle fibers. The latter method is extremely labor intensive and time consuming and therefore rather inappropriate as a routine laboratory method. We (5) previously reported that IMCL content in type I muscle fibers is on average about twofold higher than in type II fibers. Reinspection of the latter data for the purpose of this article (Fig. 3) shows that the higher IMCL content in type I vs. type II fibers was consistent for all measurements, which in fact supports the validity of the ORO method and is consistent with electron microscopy studies (38) and results from fluorometric assay on single muscle fiber pools (11). The reproducibility of IMCL-ORO remains a point of concern. We (5) have previously reported a coefficient of variation of 14% in the current samples, which is better than IMCL-BIO, but still too high if one wants to detect small effects. However, some minor technical adaptations in the ORO procedure in our hands recently have cut down the variability of the procedure from ∼14 to ∼7% (De Bock et al., unpublished observations). Furthermore, it must be emphasized that ORO is a semiquantitative method, which by definition generates results with arbitrary units. Thus, values typically presented as “percent surface area covered with lipids” do not represent true muscle IMCL contents. Therefore, by analogy with other semiquantitative methods, it may be a better option to present ORO results as relative values relative to a reference value that is set equal to 1 or 100. Such a strategy would certainly facilitate comparison between studies.

The precise reason that IMCL-BIO does not show net IMCL degradation in males during exercise is at present unclear but fiber type variation in pre- and postexercise biopsies likely plays a significant role as discussed above. However, the lack of decrease in IMCL-BIO may indicate that the true breakdown of IMCL may be smaller than that found with the other two methods. Certainly, IMCL-BIO has previously been able to demonstrate high-fat diet-induced increments in IMCL (22, 37), IMCL changes occurring during recovery after exercise (23), and exercise-induced IMCL breakdown in women (30, 31, 39).

A high correlation (r = 0.93) between electron morphometric (EM) IMCL data and IMCL-MRS, but not between IMCL-BIO and IMCL-MRS or EM, has previously been shown (16). Unfortunately, no ORO experiments were included in the latter study, and measurements were performed on a very heterogeneous sample of subjects, which probably has facilitated higher correlation coefficients. van Loon et al. (43) found a correlation of 0.6 between ORO and MRS, which is in close agreement with the present study (r = 0.52, P = 0.002). However, because the two methods are in fact supposed to measure the same parameter, such a correlation can be termed poor. Despite possible differences in fiber type composition (see paragraph above) between sites of biopsy sampling and placing of the voxel for MRS measurements, this is probably at least partly due to the use of a small sample of a selected population of lean, well-trained subjects, resulting in a narrow range of IMCL contents. Furthermore, muscle biopsies were taken immediately postexercise, whereas a considerable delay occurred between the termination of exercise and the actual 5-min MRS acquisition window. In fact, immediate postexercise measurement of IMCL is not possible, because reproducible positioning of the subject in the magnet plus reproducible anatomic localization of the voxel followed by tuning of the signal (suppression of the water signal), takes ∼30–35 min. We have previously demonstrated (5, 23) that, despite the high rate of carbohydrate intake to blunt IMCL hydrolysis by virtue of inhibition of hormone-sensitive lipase (46), substantial net IMCL degradation can occur during the early recovery period after an endurance exercise bout. This may explain the presence of net IMCL degradation in all subjects as measured by 1H-MRS, whereas with ORO, 3 of 16 subjects still exhibited a small increase in IMCL after exercise. Such a mechanism is also compatible with the finding that the IMCL-ORO and IMCL-MRS values were more closely correlated before (r = 0.46, P = 0.09) than after exercise (r = 0.27, P = 0.33). Thus, the time delay between the end of exercise and the MRS acquisition must be as short as possible. Furthermore, it must also be noted that reliable measurement of IMCL with MRS in vastus lateralis muscle is possible almost exclusively in lean subjects, in whom peaks of IMCL and EMCL in the acquired spectrum can be clearly isolated. For this reason, preexperimental screening eliminated several subjects from further participation in the study because of inability to separate IMCL and EMCL peaks. Such a procedure for selection of subjects obviously may introduce a bias in any study using MRS spectroscopy to assess IMCL content. Along this issue, a pilot experiment in a female subject with normal body fat resulted in complete fusion of the MRS IMCL and EMCL peaks sampled in vastus lateralis muscle.

In summary, this study clearly demonstrates that different assay methods to measure IMCL in muscles from young healthy males can result in totally different data on IMCL utilization during exercise. In fact, none of the available methods is tightly validated, which means that any conclusion with regard to the quantity of IMCL used during exercise must be
taken with care. MRS has the advantage of being noninvasive, but it is not fiber type specific and is hampered by at least a 30-min delay in measurements after exercise completion. Furthermore, in individuals with high extramyocellular fat content, determination of IMCL content is difficult. The biochemical method is the only quantitative method but is subject to large variation when pre- and postexercise biopsies have different fiber type composition. ORO has the advantage of allowing a fiber type-specific though semiquantitative analysis of muscle IMCL content. Therefore, balancing the limitations of the available assays against each other, it would probably be premature at present to recommend one method as the “standard.” However, because ORO is fiber type specific, it provides information that is not available with the other methods.

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