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Abstract

SCHRAUWEN-HINDERLING, VERA B., MATTHIJS K.C. HESSELINK, PATRICK SCHRAUWEN, AND MARIANNE ELINE KOOI. Intramyocellular lipid content in human skeletal muscle. *Obesity.* 2006;14:357–367.

Fat can be stored not only in adipose tissue but also in other tissues such as skeletal muscle. Fat droplets accumulated in skeletal muscle [intramyocellular lipids (IMCLs)] can be quantified by different methods, all with advantages and drawbacks. Here, we briefly review IMCL quantification methods that use biopsy specimens (biochemical quantification, electron microscopy, and histochemistry) and noninvasive alternatives (magnetic resonance spectroscopy, magnetic resonance imaging, and computed tomography).

Regarding the physiological role, it has been suggested that IMCL serves as an intracellular source of energy during exercise. Indeed, IMCL content decreases during prolonged submaximal exercise, and analogously to glycogen, IMCL content is increased in the trained state. In addition, IMCL content is highest in oxidative, type 1 muscle fibers. Together, this, indeed, suggests that the IMCL content is increased in the trained state to optimally match fat oxidative capacity and that it serves as readily available fuel. However, elevation of plasma fatty acid levels or dietary fat content also increases IMCL content, suggesting that skeletal muscle also stores fat simply if the availability of fatty acids is high. Under these conditions, the uptake into skeletal muscle may have negative consequences on insulin sensitivity.

Besides the evaluation of the various methods to quantify IMCLs, this perspective describes IMCLs as valuable energy stores during prolonged exercise, which, however, in the absence of regular physical activity and with overconsumption of fat, can have detrimental effects on muscular insulin sensitivity.

Received for review March 18, 2005.

Accepted in final form December 23, 2005.

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Introduction

In 1913, Greene reported that the dark muscle of the King salmon is characterized by "the enormous loading of fat at all stages of the life cycle, but especially at the time the spawning migration begins" and that "the stored fat is gradually eroded during the migration" (1). These observations were followed by quantitative investigations of human muscle when the first intramyocellular lipid (IMCL)[1] data from biochemical analysis of biopsies from the human quadriceps were published in 1969 by Morgan et al. (2). Later, quantitative morphological determination of IMCLs was performed by electron microscopy (EM) in the beginning of the 1970s (3,4).

Using EM, individual lipid droplets in myocytes could be visualized in the sarcoplasma, usually in direct contact with mitochondria (5). This direct contact with mitochondria led to the hypothesis that the lipid droplets serve as a fuel for mitochondrial fat oxidation, in situations where rapid supply of fat is needed, such as during exercise. It is long recognized that during endurance exercise, fat oxidation contributes significantly to the energy requirements of skeletal muscle. While fatty acids (FAs) stored in adipose tissue as triglycerides first have to undergo lipolysis, be released in the blood, and be transported to the active muscle for oxidation, IMCL stores would be a readily available substrate source during endurance exercise. Indeed, tracer studies have reported that oxidation of the IMCL stores contribute to the energy used during exercise (6–8), although

[1] Nonstandard abbreviations: IMCL, intramyocellular lipid; EM, electron microscopy; FA, fatty acid; FFA, free FA; DAG, diacylglycerol; MRS, magnetic resonance spectroscopy; ^{1}H-MRS, proton MRS; EMCL, extramyocellular lipid; CT, computed tomography; MRI, magnetic resonance imaging; E% fat, percentage of energy as fat.

results are equivocal (9). Therefore, the question to what extent IMCL is used as a substrate source during exercise is still an ongoing debate.

Initially, the interest in the physiological role of IMCL came mainly from the field of exercise physiology. However, this changed dramatically after the finding that IMCL accumulation was associated with insulin resistance (10–12), an important risk factor for type 2 diabetes.

Recently, several reviews have been published that address the role of IMCLs during exercise (13,14) and in skeletal muscle insulin resistance (15–21). Here, we aim to provide an overview of the most important determinants of IMCL content along with possible physiological functions of IMCL stores. To this end, we will include discussion of the methodology presently available to examine IMCL content with consideration of the drawbacks and advantages of the distinct methods and their applicability in answering questions toward the physiological role of IMCLs. We hypothesize that the capability of skeletal muscle to store FAs as IMCLs has physiological relevance in providing rapidly available energy during physical activity. With the present lifestyle of low physical activity and overconsumption of fatty foods in westernized society, this capability may have a downside. Continuous supply of fat to the muscle without concomitant oxidation will have negative effects on insulin sensitivity.

Quantification of IMCL Content

Biochemical Analysis of Biopsies

There are several ways to quantify IMCL content, all with their pros and cons. Biochemical analysis of biopsy data has been used extensively to quantify IMCL content. When using this method, muscle biopsy samples are freed from any visible fat, specimens are freeze-dried, and a chloroform-methanol extraction is used to isolate lipids. The triacylglycerols are hydrolyzed, and the final concentration is calculated from the amount of released glycerol or free FAs (FFAs) in solution. Although all visible adipose tissue is removed from the samples, small quantities of residual adipose tissue can have a large effect on the outcome, usually resulting in a high variability of the data; an interbiopsy variation of 23% has been reported (22), which renders this method insensitive to the detection of small changes. In well-trained subjects, who tend to have very little adipose tissue, the biochemical quantification is more reliable, and a coefficient of variation of 12.3% has been reported (23). The method can be refined by microdissection of the muscle specimens (24) or by mechanically isolating the individual muscle fibers from freeze-dried samples using a binocular.

EM

Another approach, in which contamination of adipose tissue is not a problem, is the morphometric analysis of muscle biopsy slices with EM. With a magnification of 20,000× to 30,000×, the volume density of lipid droplets inside myocytes can be analyzed in ultrathin slices (50 to 100 nm) of biopsy samples (3,4,25) (Figure 1). A disadvantage of this method is that it is limited to a very small muscle volume, and many measurements need to be done to obtain a reliable quantitative measure of whole-muscle IMCL content, making the analysis of data very elaborate. However, an advantage of EM is that it yields a lot of other morphological information such as the localization relative to mitochondria, the contact area between lipid droplets and mitochondria, and the intracellular localization of the droplets (subsarcolemmal vs. intramyofibrilar). Furthermore, differences in cytoarchitecture (z-line thickness) (26) can be used to identify the fiber type under examination.

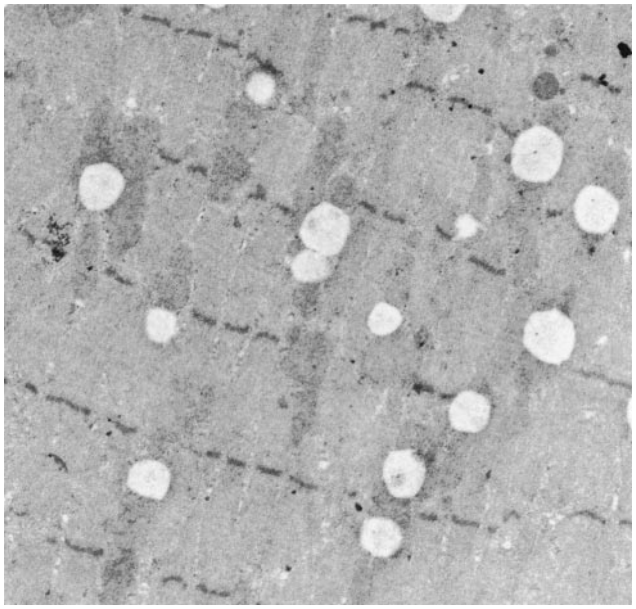

Figure 1: Quantification of IMCLs by EM. The figure shows an ultrathin section of rat tibialis anterior muscle. The white vacuoles are lipid droplets (IMCLs).

Histochemistry

Alternatively, cross-sectional, thin slices (±5 μm) of biopsy specimens can be treated with Oil Red O, which visualizes the lipid droplets with an orange-red tint under the light microscope. Oil Red O has long been used in light microscopy as a neutral lipid dye (27), but only recently has this dye been employed for truly quantitative analysis of IMCLs (28). Digital capturing of images and subsequent quantitative image analysis yield a percentage of the total cell area occupied by lipids. Interestingly, the Oil Red O dye is fluorescent; therefore, IMCLs can also be analyzed under a fluorescence microscope and quantified with the according software (29) (Figure 2). This also allows simultaneous performance of immunohistochemical stainings on the same

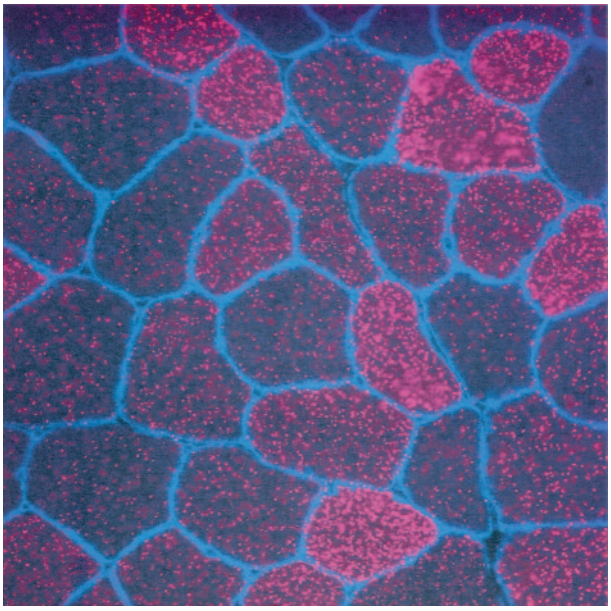

Figure 2: Quantification of IMCLs by histochemistry. The figure shows a stained section of rat tibialis anterior muscle. The red fluorescent signal originates from Oil Red O, showing IMCLs; the blue fluorescent signal originates from a laminin staining, showing the cell membranes.

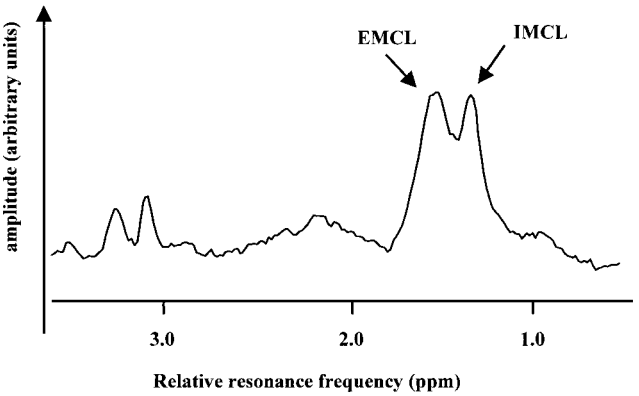

Figure 3: Quantification of IMCLs by MRS. The figure shows an ^{1}H-MRS spectrum from a human vastus lateralis muscle. The arrows point out the peaks originating from the methylene protons of EMCLs and IMCLs, respectively.

slices (29), e.g., to determine fiber type or proteins involved in FA handling. A disadvantage of this methodology, however, is that Oil Red O stains all neutral lipids and, therefore, most likely also stains FA metabolites, such as diacylglycerol (DAG).

Magnetic Resonance Spectroscopy (MRS)

With the demonstration that IMCLs can be quantified by proton MRS (^{1}H-MRS) with a standard clinical whole-body scanner (30,31), it became possible to determine the IMCL content non-invasively, and repeated measurements in the same muscle volume could be done. In contrast to EM or the Oil Red O method, where lipids can be visualized within single muscle fibers, ^{1}H-MRS measures lipid content over a larger muscle area. Because the resonance frequency of small lipid droplets surrounded by an aqueous phase (sarcoplasma) is different from that of lipids surrounded by bulk lipid (as in adipose tissue) and because additionally the resonance frequency of extramyocellular lipids (EMCLs; lipid layers between muscle bundles) depends on the orientation of the layer relative to the main magnetic field, ^{1}H-MRS can differentiate between IMCLs and adipose tissue between muscle bundles (31,32) (Figure 3). The coefficients of variation of repeated quantification of IMCLs by MRS measurements typically are ~6% to 14% (31,33–37). Importantly, ^{1}H-MRS can be used to investigate all muscles of the upper or lower leg and/or arm, and the volume of investigated muscle tissue is relatively big (2 to 3 cm^{3} for single voxel spectroscopy). Either a single volume of interest (voxel) or a matrix of many voxels (if magnetic resonance spectroscopic imaging is used) can be investigated. Therefore, by using magnetic resonance spectroscopic imaging, all of the muscles of, e.g., the lower leg can be investigated in one measurement (38).

The main drawback of ^{1}H-MRS is that although two separated peaks are detectable, the IMCL and EMCL peaks do partially overlap, and sophisticated peak-fitting software is needed to quantify the two peaks separately. Especially in obese subjects, the high number of EMCLs makes it difficult to position the voxel in an area with few EMCLs, and a large EMCL peak will overlap more severely with the IMCL peak. The true separation of IMCLs and EMCLs and the non-invasiveness of the method make MRS very often the method of choice. Because the measurement can ensure the repeated investigation of identical muscle volumes in a patient-friendly, non-invasive way, the method is especially interesting to monitor the effect of interventions.

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI)

Other non-invasive techniques, namely CT and MRI (39,40), are also used to quantify fat content in skeletal muscle. With these imaging techniques, the fat content of muscle is determined based on different signal intensities between fat and lean muscle tissue. A drawback of these imaging techniques is, however, that they cannot distinguish between a pixel containing muscle tissue with a high IMCL content and a pixel with muscle tissue with low IMCL content but partially filled with adipose tissue (EMCLs) because both pixels can have the same signal intensity (this phenomenon is called partial volume effect). The reproducibility of CT measurements is very high, and coefficients of variation of 0.51% to 0.85% have been reported (41).

Table 1. Comparison of different methods to quantify IMCL content

Method	Advantage	Disadvantage
Electron microscopy	1. Depicting additional structures (e.g. mitochondria) 2. Not biased by EMCL	1. Invasive (biopsy specimen) 2. Very small area investigated 3. Laborious
Histochemistry (Oil red O)	1. Possibility of other (immunofluorescent) stainings 2. Not biased by EMCL	1. Invasive (biopsy specimen)
MRS	1. Non-invasive, patient friendly 2. Repeated measurements in identical volume 3. Relatively large volume investigated	1. Partial overlap of IMCL and EMCL peak 2. Absolute quantification is laborious, usually relative measures presented
Biochemical analysis	1. Extensively used in literature (lots of reference data)	1. Invasive (biopsy specimen) 2. Large variation, contamination by adipose tissue likely
CT	1. Non-invasive, patient friendly 2. Repeated measurements in identical volume 3. Large volume investigated	1. No true separation of IMCL and EMCL 2. Exposure to ionizing radiation
MRI	1. Non-invasive, patient friendly 2. Repeated measurements in identical volume 3. Large volume investigated	1. No true separation of IMCL and EMCL

EMCL, extramyocellular lipid; IMCL, intramyocellular lipid; MRS, magnetic resonance spectroscopy; CT, computed tomography; MRI, magnetic resonance imaging.

When comparing the different methods, it has been shown that ^{1}H-MRS data correlate closely with EM data (25), and a good correlation between ^{1}H-MRS data and Oil Red O staining was reported as well (42). However, the correlation of chemical analysis with ^{1}H-MRS and EM was poor (25), probably due to the high variability of the chemical analysis, as discussed above.

Therefore, ^{1}H-MRS, EM, and Oil Red O stainings are all valuable methods to quantify IMCL content, whereas biochemical methods should only be used if refinements to diminish contamination of adipose tissue are applied. ^{1}H-MRS has the advantage of being non-invasive. Other non-invasive methods, namely CT and MRI, cannot truly separate IMCLs and EMCLs. For an overview of the different methods, see Table 1.

IMCL as an Intracellular Source of Fuel

Early isotope studies (8) indicated that during exercise not all of the oxidized fat could be accounted for by oxidation of plasma FFA, leading to the suggestion that the lipid droplets inside the muscle cells serve as important fuel during exercise. Especially during prolonged exercise, IMCL is thought to be an important fuel source, and the oxidation of IMCLs might have a sparing effect on glycogen oxidation. Data from biochemical analysis are equivocal on the effect of acute exercise, with some laboratories reporting a decrease in IMCL content (43–49), whereas others did not (50–54). This discrepancy may be due to the high variability of the biochemical IMCL determination, especially in untrained subjects, where more adipose tissue can contaminate the IMCL determination (14). In contrast to biochemical analysis, EM data showed that IMCLs decreased by 42% in the gastrocnemius muscle after completion of a marathon race (55), and IMCLs were nearly depleted after a 100-km run in seven well-trained subjects (56). These results are in line with more recent ^{1}H-MRS results, showing a consistent decrease in IMCL content after acute exercise (31,33,57–64). Only a high-intensity interval protocol reported no decrease of IMCL content after exercise when ^{1}H-MRS was employed (34). The latter is not surprising because during high-intensity interval protocols, carbohydrate is the main fuel oxidized. In summary, there is little doubt that IMCLs are, indeed, oxidized during endurance exercise and can serve as a readily available energy source during exercise.

Fat Content of Different Muscles

Most studies examining IMCL content have been limited to a few muscle groups. With ^{1}H-MRS especially, the muscles of the calf have been extensively studied. In the calf, the highest fat contents have been found in the medial part of the soleus muscle and lower values (by a factor of 2

to 3) in the tibialis anterior and posterior (65) and the gastrocnemius muscle (38). This matches the different fiber-type distribution of these muscles and their substrate use. The soleus muscle is a more oxidative muscle relying more on fat oxidation than the tibialis and gastrocnemius muscles. Likewise, the soleus muscle has a high percentage (~88%) (66), whereas the gastrocnemius muscle has a lower percentage (~50%) of type 1 fibers (66). The fiber composition of the tibialis anterior muscle lies in between (~70% of type 1 fibers). EM (67) and histochemistry (Oil Red O) (42,68), indeed, showed that oxidative type 1 fibers are characterized by a higher fat content than glycolytic type 2 fibers. In addition, oxidative type 1 fibers contain more mitochondria, suggesting that the IMCL droplets can serve as a rapid available source of energy for mitochondrial oxidation.

In summary, consistent with the observation that IMCLs can serve as an energy source during exercise, IMCL content is dependant on the fiber-type composition of muscles, with oxidative muscle groups being characterized by a high IMCL content.

Replenishment after Exercise

For IMCLs to be used during repeated exercise bouts, they need to be replenished in the post-exercise state. Analogous to a high-carbohydrate diet speeding up the replenishment of glycogen stores, investigators examined whether the replenishment of IMCL stores is faster on a high-fat diet.

IMCL content assessed with biochemical methods during the 24 hours after an exercise bout on either a high-fat [68% of the energy as fat (E% fat)] or a low-fat (5 E% fat) diet showed an increase on the high-fat diet, whereas it remained unchanged on the low-fat diet (54). The investigation with ^{1}H-MRS of the time course of IMCL recovery after an exercise-induced decrease in IMCLs revealed that the IMCL content returned to a level higher than baseline (supercompensation of 30% to 45%) after 30 hours on a high-fat diet (55 E% fat), whereas on a low-fat diet (15 E% fat), the IMCL content did not recover within 30 hours (69). Also, in female runners, IMCL content was restored to baseline 22 hours after running exercise on a moderate-fat diet (35 E% fat), with a supercompensation of IMCL content on the moderate-fat diet after 70 hours. In contrast, on a low-fat diet (10 E% fat), IMCL content did not recover in the 70 hours post-exercise (62). Similarly, we reported that in healthy male athletes, the replenishment of IMCL content on a moderate-fat diet (39 E% fat) 48 hours after exercise was complete, whereas IMCL content on a typical athletes' diet (24 E% fat) did not recover in 48 hours (42). Interestingly, the difference in IMCL replenishment between the low- and moderate-fat diets was only visible in type 1 (oxidative) muscle fibers (42).

Given that IMCL is used during prolonged exercise, replenishment of IMCL content post-exercise could potentially improve performance. However, when a 120-minute time trial was used to deplete IMCL stores and was followed by 24 hours with either a high-fat or a high-carbohydrate diet, the performance on a subsequent self-paced cycling time trial was lower with the high-fat diet (54). Also, in studies focusing on performance after long-term adaptation to a high-fat diet, results are equivocal with studies reporting a positive effect (70), a negative effect (71), or no change in performance (72,73), and one study showing no effect on high-intensity exercise but a positive effect on submaximal exercise (74).

Therefore, although IMCL is used during exercise, no strong evidence is available yet that IMCL content is limiting athletic performance (for recent review, see Ref. 75).

In summary, analogous to glycogen levels being rapidly replenished when glucose is ingested after exercise, high-fat diets accelerate the replenishment of IMCL stores after exercise. Although for glycogen it is generally accepted that such repletion is essential for subsequent performance, such evidence is not available in regard to the high-fat-induced replenishment of IMCLs. This does not imply that high IMCL content is not essential to athletic performance but might be explained by the lack of studies examining the impact of IMCL content, while keeping glycogen levels constant, on endurance performances that lead to a substantial depletion of IMCLs (as, for example, a 100-km run) (56).

Endurance Training

As discussed above, during exercise, both intramyocellular glycogen and lipid stores are used as energy source, dependent on the exercise intensity, and both substrate stores are replenished in the recovery phase post-exercise. With endurance training, glycogen levels are elevated, which promotes fatigue resistance. Analogously to glycogen, it might be expected that IMCL content is increased in the endurance-trained state, too. With biochemical analysis of IMCLs, some authors reported increased IMCL content after 4 to 6 weeks of training (2,76), whereas others reported no change after 12 weeks of training at high intensity (51) or even a decrease in IMCL content (77). These equivocal results are probably again due to the high variability of the biochemical method.

EM revealed 2.5 times higher IMCL content in the vastus lateralis of well-trained orienteers (3) and 2.5 times higher IMCL content in the gastrocnemius muscle of elite rowers compared with controls (78). With 6 to 8 weeks of endurance training, IMCL content increased up to 3.4 times in the vastus lateralis muscle (79,80). Although almost all studies using EM and involving endurance training reported increased IMCL content after training, the change did not always reach statistical significance (81,82), and one study reported no difference in IMCL content of the gastrocnemius of endurance-trained athletes and untrained subjects

(83). An increased IMCL content in trained subjects limited to type 1 fibers has been reported in some studies (84,85), whereas a fiber-type-specific increase in IMCL content in types 2a and 2b fibers was reported by others (67), and a higher IMCL content in trained cross-country runners was limited to type 2a fibers (86). Similarly to the results from EM, Oil Red O stainings report large differences (50% to 70%) between trained and untrained subjects (11,68) and increased IMCL content (+12%) after a 12-week training period in older subjects (87). The EM and the histological data are in line with ^{1}H-MRS data, which report higher IMCL content in trained subjects, compared with untrained (61,88). We recently showed with ^{1}H-MRS that a 2-week training program in young sedentary male subjects resulted in a 42% increase in IMCL content (33), thereby confirming results obtained with EM and Oil Red O (87).

Taken together, independent of the methodology used, there is unequivocal evidence that IMCL content is increased on endurance training, again consistent with IMCL being an energy source for physical activity. Because endurance training also increases the relative amount of type 1, oxidative muscle fibers, this may partly explain the increase in IMCLs (11,68).

High-Fat Diets

The results discussed so far suggest that IMCL functions as a rapidly available energy source to deliver fuel for the mitochondrial ATP formation necessary for muscle contraction. As with glycogen, the levels of these intramuscular substrate stores are influenced by the diet, at least in the recovery phase after exercise. Many investigators have also examined the effect of high-fat diets per se, and not limited to athletes, on IMCL content. With biochemical methods, triglyceride content in skeletal muscle has been reported to increase by 36% to 90% after high-fat feeding periods ranging from 24 hours to 7 weeks (54,89–93). Similarly, data from EM showed a 130% increase in IMCL content after 5 weeks of a high-fat diet (94). Using ^{1}H-MRS, the effect of high-fat diets (55 to 60 E% fat) has been investigated after 2 to 3 days (95,96), and after 1 week (97); in all three studies, an increase (between 48% and 56%) in IMCL content was reported.

The reason for this increase in IMCLs on a high-fat diet is not yet clear. We and others have shown that a high-fat diet increases fat oxidation, which is not due to an increased oxidation of FFA (91,98). This suggests that an increased IMCL (and/or very low-density lipoprotein) oxidation occurs after a high-fat diet, suggesting that the increase in IMCLs drives increased IMCL oxidation. Alternatively, high-fat feeding promotes overfeeding and a positive fat balance. The surplus of fat is mainly stored in adipose tissue but also in non-adipose tissue such as skeletal muscle. In this respect, the increase in IMCLs on a high-fat diet could also simply be seen as an excessive storage of a surplus of circulating FAs. In that respect, it is interesting to note that obese subjects are characterized not only by increased adipose tissue but generally also by increased plasma lipid concentrations and by high IMCL content and low fat oxidative capacity (28). Additionally, in obese subjects with normal fat oxidation, IMCL content was normal too (99). This further suggests that the increase in IMCLs may be simply due to a mismatch between delivery to and oxidative capacity of skeletal muscle.

In summary, a high-fat diet increases IMCL stores, which may simply be due to a positive fat balance when changing to a high-fat diet. In physically inactive humans consuming a high-energy, high-fat diet, a positive energy and fat balance may occur chronically, resulting in fat accumulation in adipose tissue and probably also in skeletal muscle.

High FFA Plasma Concentrations

In accordance with the suggestion above, other conditions with high FA availability have also led to increases in IMCL content. The acute elevation of plasma FFA by infusions has resulted in increased IMCL content (95,100). Also, during fasting, lipolysis is stimulated, and plasma FFA concentrations are elevated. Interestingly, it has been reported that 72 hours of fasting increased IMCL content (101).

In line with an elevation of IMCL in the presence of high FFA plasma concentrations, we found that exercise that was accompanied by increased plasma FFA resulted in a decrease in IMCLs in the exercising legs but an increase of IMCL content in the inactive, non-exercising muscle (63). The increase in IMCLs under conditions of high FA availability can simply be the due to a higher supply of fat to the muscle. Alternatively, non-active muscle could act as a buffer for elevated plasma FFA by taking them up from the circulation. High plasma concentrations of FA can harm the endothelial wall; therefore, their removal from the circulation is important. Although adipose tissue is readily taking up and storing FA, muscle tissue also seems to fulfill this function if FA availability is high. In this way, both the white adipose tissue and the muscle help to prevent or limit elevation of circulating plasma FA levels, and the stored IMCLs could serve as an energy source when lipid supply is decreasing. However, as we are all aware, in our westernized society with a surplus of dietary energy available, periods of low lipid supply are scarce, resulting in continued high levels of IMCLs in non-active muscle tissues.

In summary, an elevation of plasma FFAs results in increased IMCL content. It is yet unclear whether this is a passive mechanism or whether skeletal muscle actively buffers FFA plasma concentrations.

Association with Insulin Resistance

The prevalence of insulin resistance and type 2 diabetes is increasing rapidly, and high-energy intake and lack of phys-

ical activity in westernized societies are considered major risk factors. The development of insulin resistance coincides with the accumulation of IMCLs. With biochemical methods, a correlation of triglyceride content with insulin resistance in Pima Indians was reported (12), and a similar correlation was shown in sedentary subjects with histochemical methods (11) and with ^{1}H-MRS (10). Interestingly, IMCL content has been described as an early marker of the development of insulin resistance. Philipps et al. (102) investigated normoglycemic women and found a negative relation between increased triglyceride content (assessed by histological and biochemical methods) and decreased glycogen synthase activity. Furthermore, histochemically determined high IMCL contents were related to high waist-to-hip ratios and high FFA plasma concentrations. These data suggest that high IMCL content, perhaps due to high plasma FA levels, can lead to the development of insulin resistance, although the possibility cannot be excluded that insulin resistance leads to the accumulation of IMCLs. To investigate early aberrations in metabolism in the pathogenesis of type 2 diabetes, a valuable approach is the investigation of healthy subjects with a high risk of developing diabetes in later life, such as family members of patients with type 2 diabetes. With ^{1}H-MRS, Perseghin et al. (103) showed that first-degree relatives of type 2 diabetic patients had higher IMCL content (and were less insulin sensitive) than matched controls. Similarly, the offspring of type 2 diabetic patients were investigated (104) and divided into insulin-sensitive and -resistant subjects. The results showed that the insulin-resistant subjects had a higher IMCL content than the insulin-sensitive counterparts. The increased IMCL content in insulin-resistant but otherwise healthy subjects suggests that the accumulation of IMCLs is an early step in the development of type 2 diabetes. Also, in overt diabetes, IMCL content is severely increased. Early data from biochemical analysis of biopsy samples revealed a 6-fold excess of triglyceride content in subjects with type 2 diabetes (105). This was confirmed by histochemical methods revealing higher IMCL content in type 2 diabetic subjects, compared with obese or lean controls (11). In accordance, obese subjects who had normal insulin sensitivity were also characterized by normal IMCL content (99).

The data above show clearly that an increased IMCL content is associated with insulin resistance if training status is not confounding. Weight loss is well known to improve insulin sensitivity in overweight, insulin-resistant subjects, and this is accompanied by a decreased IMCL content. Histochemically determined IMCL content decreased by 30% after bariatric surgery and extreme weight loss (106,107).

To summarize, IMCL content is a marker of insulin resistance in diabetic and healthy physically non-active subjects, and the accumulation of IMCLs has been shown to be an early phenomenon in the development of diabetes.

Mechanisms of Decreased Insulin Sensitivity

High IMCL content, thus, seems to be associated with the development of insulin resistance, which is in contrast to the observations that endurance training leads to an increase in IMCL content, too. This has been described as the training paradox in the literature (11). However, as reviewed above, IMCLs can be increased for two different reasons: a functional increase, whereby IMCL serves as a rapidly available energy source; and a pathophysiological increase, whereby increased IMCL is merely due to a continuous oversupply of fat. In the former condition, the increase in IMCLs can only be functional if at the same time the capacity to liberate these IMCLs and rapidly divert them to oxidation is also increased. Indeed, gene expression of a key component of FA transport (carnitine palmitoyltransferase) and the fat oxidative capacity are also increased in the trained state (108). In the second condition, however, the increased IMCL content most likely is not accompanied by such a strongly increased fat oxidative capacity. Indeed, Goodpaster et al. (11) suggested that fat oxidative capacity might be more important than IMCL content in determining insulin sensitivity, and Petersen et al. (109) reported decreased mitochondrial function (oxidative capacity) in the insulin-resistant offspring of type 2 diabetes patients, compared with insulin-sensitive controls. In the search for a functional explanation for the relation between IMCL and insulin sensitivity, it has been suggested that lipid metabolites such as fatty acyl-CoA or DAG are more likely to intervene with insulin signaling than IMCLs per se. DAG is the intermediate between fatty acyl-CoA that can enter the mitochondria for oxidation and triacylglycerol, the form in which lipids are stored in muscle. As outlined above, a functional increase in IMCLs is accompanied by an increased capacity to divert IMCLs to oxidation; therefore, the level of the lipid intermediates will stay low. However, in the condition of continuous fat oversupply, the increased IMCL is not accompanied by a sufficiently increased fat oxidative capacity; therefore, not only IMCLs but also DAG and fatty acyl-CoA will increase. As mentioned, the latter two have been shown to be able to interfere with insulin signaling (for review, see ref. 110).

In summary, although the accumulation of IMCLs coincides with the development of insulin resistance, the relationship is most likely indirect. Various intermediates of fat metabolism have been named as candidates to be the culprits of decreasing insulin action. As the intermediates accumulate in situations with high fat availability and low fat oxidation, the balance between availability and oxidation may be crucial.

Concluding Remarks and Future Perspective

The physiological function of fat stores in the muscle is to serve as a readily available energy source during exercise.

Although physical activity levels in our westernized society are generally very low, this capacity to store fat inside muscle may have conferred an evolutionary advantage to permit physical activity during cycles of feast and famine. In that respect, it was recently suggested that humans' endurance-running capacity may have been instrumental in the evolution of *Homo sapiens* (111). Alternatively, it has been suggested that temporal insulin resistance that accompanies the fasting-induced accumulation of IMCLs may have been instrumental in evolution to spare glucose for the brain during periods of famine (15). Nowadays, however, in westernized society, the importance of having high IMCL levels may have faded due to the low levels of physical activity and the continuous availability of food. However, the capability to store IMCLs is still preserved; as a consequence, in conditions of high circulating FA levels or high dietary lipid supply, muscle may act as a sink for circulating FAs. In these conditions in which IMCLs are not being used for oxidation, IMCLs and their intermediates have a negative impact on insulin signaling and induce insulin resistance. Therefore, the preserved capacity to store fat in muscle may nowadays have detrimental effects on insulin sensitivity, especially when IMCLs are not being used as substrate. In that respect, the capacity to use IMCLs may be more important than the magnitude of IMCL levels per se in determining the negative effects on insulin sensitivity.

Acknowledgment

This study was supported by VIDI Research Grant 917.66.359 for innovative research from the Netherlands Organization for Scientific Research (to M.H.) and a fellowship of the Royal Netherlands Academy of Arts and Sciences (to P.S.).

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