Use of intramuscular triacylglycerol as a substrate source during exercise in humans

Luc J. C. van Loon

Nutrition Research Institute Maastricht (NUTRIM), Departments of Movement Sciences and Human Biology, Maastricht University, 6200 MD Maastricht, The Netherlands

van Loon, Luc J. C. Use of intramuscular triacylglycerol as a substrate source during exercise in humans. *J Appl Physiol* 97: 1170–1187, 2004; 10.1152/japplphysiol. 00368.2004.—Fat and carbohydrate are the principal substrates that fuel aerobic ATP synthesis in skeletal muscle. Most endogenous fat is stored as triacylglycerol in subcutaneous and deep visceral adipose tissue. Smaller quantities of triacylglycerol are deposited as lipid droplets inside skeletal muscle fibers. The potential role of intramyocellular triacylglycerol (IMTG) as a substrate source during exercise in humans has recently regained much of its interest because of the proposed functional relationship between IMTG accumulation and the development of skeletal muscle insulin resistance. Exercise likely represents an effective means to prevent excess IMTG accretion by stimulating its rate of oxidation. However, there is much controversy on the actual contribution of the IMTG pool as a substrate source during exercise. The apparent discrepancy in the literature likely stems from methodological difficulties that have been associated with the methods used to estimate IMTG oxidation during exercise. However, recent studies using stable isotope methodology, ^{1}H-magnetic resonance spectroscopy, and electron and/or immunofluorescence microscopy all support the contention that the IMTG pool can function as an important substrate source during exercise. Although more research is warranted, IMTG mobilization and/or oxidation during exercise seem to be largely determined by exercise intensity, exercise duration, macronutrient composition of the diet, training status, gender, and/or age. In addition, indirect evidence suggests that the capacity to mobilize and/or oxidize IMTG is substantially impaired in an obese and/or Type 2 diabetic state. As we now become aware that skeletal muscle has an enormous capacity to oxidize IMTG stores during exercise, more research is warranted to develop combined exercise, nutritional, and/or pharmacological interventions to effectively stimulate IMTG oxidation in sedentary, obese, and/or Type 2 diabetes patients.

muscle metabolism; intramuscular triglycerides; intramyocellular lipid; insulin resistance; endurance training

INTRAMUSCULAR LIPID CONTENT

Fat and carbohydrate are the principal substrates that fuel aerobic ATP synthesis in skeletal muscle. Carbohydrates are mainly stored as glycogen in skeletal muscle and liver. Endogenous glycogen stores are relatively small and generally range between 0.46 and 0.52 kg, corresponding to a total energy storage of 7.5–8.4 MJ (1,785–2,000 kcal). Fat is mainly stored as triacylglycerol (TG) in subcutaneous and deep visceral adipose tissue. Smaller quantities of TG are present as lipid droplets inside muscle fibers (intramyocellular TG or IMTG). In addition, some fat is present in the circulation as nonesterified or free fatty acids (FFA) bound to albumin or as TG incorporated in circulating lipoprotein particles [chylomicrons and very-low- (VLDL), low-, intermediate-, and high-density lipoproteins]. In contrast to the endogenous carbohydrate deposits, endogenous fat stores are quite large and range between

9 and 15 kg in the average, nonobese male (body mass ~70 kg), corresponding to a total energy storage of 350–586 MJ (80,000–140,000 kcal).

Intramuscular TG contents have been shown to be quite variable and generally range between 2 and 10 mmol/kg wet wt (26, 29, 34, 36, 63, 64, 89, 108, 149, 160, 183, 200, 203, 206), which would correspond with an estimated total mixed muscle IMTG content of ~0.2 kg (7.8 MJ or 1,850 kcal). The physiological function of this IMTG pool has regained much of its interest over the past few years, because various studies have reported a strong association between elevated plasma FFA availability, IMTG accumulation, and the development of insulin resistance and/or Type 2 diabetes (17, 94, 116, 136, 137, 143, 145, 146, 148, 158, 172, 173, 177, 184). Initially, the Randle [glucose-fatty acid (FA)] cycle was used to explain the mechanism behind FA-induced skeletal muscle insulin resistance. It was proposed that an increase in plasma FFA availability stimulates fat oxidation and decreases carbohydrate oxidation by suppressing pyruvate dehydrogenase complex activation (via a rise in the ratio of mitochondrial acetyl-CoA to CoA) and reducing glycolytic flux (via the inhibitory effect of high citrate concentrations on phosphofructokinase activity).

Address for reprint requests and other correspondence: L. J. C. van Loon, Nutrition Research Institute Maastricht (NUTRIM), Dept. of Human Biology, Maastricht Univ., PO Box 616, 6200 MD Maastricht, The Netherlands (E-mail: l.vanloon@hb.unimaas.nl).

This would lead to the accumulation of glucose-6-phosphate, which inhibits hexokinase activity, causing intracellular glucose concentrations to rise, thereby downregulating skeletal muscle glucose uptake (152). More recent insights from various lipid infusion studies have led to an alternative mechanism (17, 18, 40, 62, 158, 173, 212), suggesting that elevated plasma FFA delivery and/or impaired FA oxidation result in intramyocellular accumulation of TG and FA metabolites (such as fatty acyl-CoA, ceramides, and diacylglycerol), which have been suggested to induce defects in the insulin-signaling cascade causing skeletal muscle insulin resistance (1, 18, 40, 45, 92, 158, 164, 173, 212).

The proposed relationship between elevated IMTG content and skeletal muscle insulin resistance is not entirely unambiguous because correlations between IMTG content and insulin resistance (117, 143, 148) would simply disappear with the inclusion of well-trained endurance athletes (59, 187), owing to the fact that trained athletes are markedly insulin sensitive (44, 127, 159) despite an elevated IMTG storage (59, 81, 187). Moreover, Thamer et al. (187) reported insulin sensitivity to be negatively correlated with IMTG content in untrained subjects, whereas in endurance-trained subjects elevated IMTG contents actually predicted high insulin sensitivity. These findings make the general association between IMTG accretion and the development of skeletal muscle insulin resistance less obvious. To explain the apparent metabolic paradox, it has been suggested that the association between IMTG content and insulin resistance may be mediated by muscle oxidative capacity (59, 66, 187). Subsequently, it seems likely that the capacity to mobilize and/or oxidize IMTG stores is one of the main factors that modulates the association between IMTG accretion and the development of skeletal muscle insulin resistance. The present paper provides an overview on the role of the IMTG pool as a substrate source during exercise in humans.

ROLE OF IMTG AS A SUBSTRATE SOURCE DURING EXERCISE

Electron microscopic analysis of skeletal muscle tissue has shown that intramyocellular lipid (IMCL) droplets are located adjacent to the muscle mitochondria (81), implying that IMTG stores function as a readily available pool of FA for oxidative purposes (Fig. 1). Furthermore, several studies have reported an approximately threefold greater lipid content in type I vs. type II muscle fibers (47, 128, 196, 197). The latter finding indicates that the greater fat oxidative capacity in the type I fibers is also associated with greater IMTG storage. Furthermore, the presence of hormone-sensitive lipase (HSL) in skeletal muscle tissue (78, 120) and its reported activation by catecholamines (113, 122, 205) and muscle contraction (113, 119, 121, 202) strongly suggest that the IMTG pool can function as a dynamic fuel store during physical activity. This is further supported by the observation that HSL content correlates with IMTG content and oxidative capacity of the different muscle fiber types (47). As total fat oxidation rates can increase >10-fold during the transition from rest to moderate-intensity exercise, it seems likely that the IMTG pool offers a readily available pool of FA during exercise conditions. However, in the literature, there has been much controversy on the contribution of IMTG oxidation to total energy expenditure during exercise (203). Numerous studies have
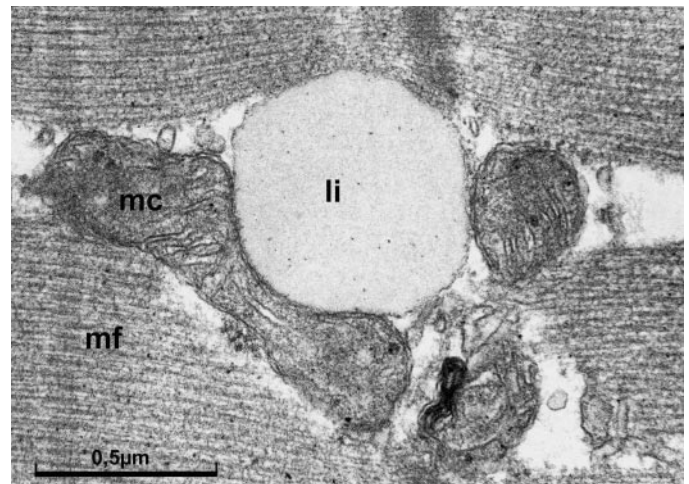

Fig. 1. Electron micrograph of a longitudinal section of skeletal muscle tissue. In the center, on the level of the z-line, we find interfibrillar mitochondria with a lipid droplet immediately adjacent. li, Lipid droplet; mc, central mitochondria; mf, myofilaments; marker indicates 0.5 μm. Micrograph from Hoppeler (Ref. 79). Copyright permission kindly provided by the Nature Publishing Group (http://www.nature.com/).

addressed this question using various different approaches. Some methods estimate IMTG use on a whole body and/or limb level using indirect flux measurements, whereas others have addressed this topic by measuring net changes in muscle lipid content before and after exercise. To correctly interpret published data on the contribution of the IMTG pool as a substrate source during exercise, it is crucial to know the advantages and disadvantages of the different methods that have been used.

Muscle biopsy and chemical TG extraction. Since the introduction of the percutaneous needle biopsy technique in physiological and clinical metabolic research (8), numerous studies have investigated skeletal muscle fuel use by determining net changes in skeletal muscle substrate content from muscle samples collected before and after exercise. This approach has proven quite effective as it formed the basis on which our present insight in the role of muscle glycogen as a substrate source was obtained (9, 10, 12, 13). The same approach has also been applied to study the role of IMTG as a substrate source during exercise. After collection of muscle biopsy samples from the vastus lateralis before and after prolonged moderate-intensity exercise, most studies have applied chemical TG extraction analyses (51) on homogenized (mixed) muscle. Whereas several studies have reported a substantial net decrease in muscle TG content after prolonged exercise (13, 26, 29, 46, 48, 56, 89, 150, 165, 200), many others have failed to observe any significant decline (7, 64, 107, 111, 180, 206). In addition, it has also been reported that IMTG stores do not play a significant role during moderate-intensity exercise in endurance-trained men as opposed to trained women (160, 183) but rather form an important substrate source during postexercise recovery (111). These contradictory findings on the role of the IMTG pool as a substrate source during exercise has since been a topic of many discussions (203).

A more detailed evaluation of the different studies that have applied the chemical TG extraction method indicates that studies in endurance-trained subjects generally report a de-

crease in IMTG content following exercise (203). The latter seems to be time dependent, with studies reporting as much as a 50% decline in IMTG content after more prolonged (2–7 h) moderate- to high-intensity exercise (13, 35, 56), whereas smaller, nonsignificant decreases (~10–20%) have been reported after only 1–2 h of exercise (160, 180, 200). Less consistent and more variable findings have been reported in studies in which untrained, healthy subjects were studied. Nonetheless, the majority of these studies also report a minor decline in IMTG content after exercise (26, 29, 46, 150, 165, 206). However, these minor decreases generally do not reach statistical significance and appear to be of little quantitative importance in the scope of total energy expenditure. Furthermore, there are even reports showing a significant increase in IMTG content after 1 h of low- to moderate-intensity exercise (7). It could be argued that the discrepancy in the literature is related to the heterogeneity in the research protocols used (mode, duration, and/or intensity of the implemented exercise protocol) and/or the selected subject population (age, body composition, gender, insulin sensitivity, training status, and/or nutritional status). Although such factors are likely responsible for some of the variance in the contribution of IMTG as a substrate source during exercise (see FACTORS THAT DETERMINE IMTG USE DURING EXERCISE), they do not explain these contradictory findings.

According to Watt et al. (203), the apparent discrepancies on the proposed role of IMTG as a substrate source during exercise are largely explained by methodological difficulties associated with the TG extraction method. Although the within-biopsy variability of the IMTG measurement in freeze-dried muscle samples is small (6%), the between-biopsy variability has been reported to be quite large, with a coefficient of variation of 20–26% (206). The latter fact implies that net decreases in IMTG content smaller than 20–26% generally do not reach statistical significance in such studies (206). This large between-biopsy variability is most likely due to differences in the presence of extramyocellular fat and/or the fiber-type composition of the muscle samples (63, 200, 206). As described earlier, type I muscle fibers have been reported to contain about threefold more lipid than the type II muscle fibers. The latter has been confirmed in several studies applying different (semi)quantification techniques, like biochemical TG extraction analyses on individually dissected muscle fibers (47) as well as histochemical oil red O staining using conventional light (128) or fluorescence microscopy (196, 197). In addition, ^{1}H-magnetic resonance spectroscopy (MRS) studies have also clearly shown large differences in IMTG content between muscle groups containing either predominantly type I (soleus) or type II (tibialis anterior) muscle fibers (90). Although the large difference in muscle fiber type lipid content could form an important confounding factor, it is unlikely that muscle fiber-type composition is substantially different between relatively large muscle biopsies (20–100 mg) taken from the same muscle at a similar location (126). In accordance, Guo (63) reported that muscle histological heterogeneity does not appear to be the major source for the high variability in IMTG content. Rather, the procedures that are commonly used to process muscle samples before TG extraction analyses appear inadequate to completely remove extramyocellular lipid (EMCL) deposits (63).

There have been many speculations about the presence of EMCL in muscle tissue samples. Histological and biochemical analyses of rat skeletal muscle by Guo (63) showed significant amounts of extramyocellular adipocytes on the exterior surface of gastrocnemius, soleus, and soleus muscle. TG content of these exterior adipocytes was reported to be two- to threefold greater than the IMTG pool. This clearly indicates that these adipocytes form a major source of contamination when not carefully and completely removed before chemical TG extraction analysis is performed. Howald et al. (86) have shown electron microscopy (EM) photographs of adipocytes located in small clusters between muscle fiber bundles. In addition, adipocytes have also been located near small blood vessels in muscle samples (Ref. 63 and personal observations). However, after structural analyses of large numbers of muscle cross sections by various microscopy techniques, my coworkers and I as well as others (63, 125) have failed to provide evidence for the presence of any substantial EMCL depots and/or large numbers of adipocytes situated in the inter- and/or intrafascicular space. Although proper removal of extramyocellular adipocytes from skeletal muscle samples by precise microdissection with a stereomicroscope has been proven effective (63), even the presence of a few adipocytes can already result in a substantial overestimation of IMTG content. When a normal muscle biopsy sample for TG analyses (20 mg wet wt) with an IMTG content of ~3–6 μmol/g is considered, the presence of as little as ~10–20 adipocytes in the entire sample (representing only 0.01–0.02% of total sample weight) would already result in a ~5% overestimation of total IMTG content.

Another way to reduce the variability in IMTG content when using the chemical TG extraction approach is to select only well-trained endurance athletes because, in endurance-trained athletes, skeletal muscle has been shown to contain considerably less EMCL (19, 185, 206). In accordance, a much lower between-biopsy variability (<12%) has been reported when chemical TG extraction analyses were performed on muscle samples taken from endurance-trained athletes (200). The latter may partly explain why most studies that report significant net reductions in muscle TG content after exercise were performed in endurance-trained athletes. However, the fact that trained subjects generally perform exercise at a much higher absolute exercise intensity over longer time periods compared with untrained subjects is also likely to contribute to this observation. In contrast to the athletes, the between-biopsy variability in IMTG content when using the chemical TG extraction approach in muscle obtained from sedentary, obese, and/or Type 2 diabetes patients will likely be even greater (>20–26%) because of the large subcutaneous fat layers and the suggested increase in EMCL deposits in these subjects, which are likely to result in substantial contamination of muscle biopsy samples. Clearly, additional techniques need to be applied to elucidate the role of the IMTG pool as a substrate source during exercise in various populations.

In short, much of the controversy concerning the importance of IMTG as a substrate source during exercise is based on the presence or absence of net changes in muscle TG content and determined from chemical TG extraction analyses of muscle biopsies collected before and after exercise. In general, these studies report small, but statistically insignificant, reductions in muscle TG content. The latter seems to be associated with the high between-biopsy variability in IMTG content when using

this methodology. The high between-biopsy variability is likely due to contamination of muscle biopsy samples with EMCL. Although precise microdissection of muscle biopsy samples to remove EMCL before chemical TG extraction analyses will improve the validity of the measurements, additional techniques are warranted to elucidate the role of the IMTG pool as a substrate source during exercise.

Stable isotopes. Another approach to quantitate substrate source oxidation rates during exercise is to combine indirect calorimetry with the use of stable and/or radioactive isotope tracers (155). Numerous studies have applied continuous intravenous infusions of labeled FA to determine plasma FFA rate of appearance (R_a), rate of disappearance (R_d), and/or their rate of oxidation (R_{ox}). After total fat and plasma FFA oxidation rates are determined, the contribution of other fat sources can be calculated. These other fat sources have often been suggested to represent the IMTG pool. However, the applied methodology does not differentiate between muscle- and plasma-derived TG. It is generally accepted that both muscle- as well as lipoprotein-derived TG (mainly VLDL) contribute to total fat oxidation (50, 65, 68, 142). However, the contribution of lipoprotein-derived TG oxidation to total energy turnover is assumed to be of relative little quantitative importance during exercise under normal dietary conditions in an overnight fasted state (50, 65, 68, 142). On the basis of measurements of arteriovenous (A-V) plasma TG concentrations and blood flow across the working muscle or limb, lipoprotein-derived TG oxidation has generally been estimated to contribute ∼5–10% of total fat oxidation during moderate-intensity exercise (65, 68, 107, 110, 140, 142, 189). However, their contribution is likely to become substantially greater after ingestion of a mixed meal, after a high-fat diet, and/or after a period of endurance training (65, 68, 107, 108, 110, 140, 142, 189). It should be noted that the reported lipoprotein-derived TG oxidation rates, on the basis of A-V differences, represent maximal estimates because plasma TG uptake does not necessarily equal its rate of oxidation in the muscle.

In most exercise studies, the R_d of a labeled plasma FFA tracer has often been assumed to represent its R_{ox} in the muscle (161). However, at rest and to a lesser extent during exercise, R_d exceeds R_{ox} (193, 196). As such, earlier reports on plasma FFA oxidation rates based on FA tracer R_d have underestimated the use of muscle- and/or lipoprotein-derived TG during exercise by ∼10–15%. More recent studies have applied ^{13}C- or ^{14}C-labeled FA tracers, which are used to directly quantify tracer oxidation rates from the R_a of $^{13}CO_2$ (or $^{14}CO_2$ in case ^{14}C-labeled FA tracers are used) in the expired breath. The main assumption when calculating the R_{ox} of a ^{13}C (or ^{14}C)-labeled FA tracer from the excretion of $^{13}CO_2$ ($^{14}CO_2$) is that the R_a of $^{13}CO_2$ in the expired breath accurately reflects its R_{ox}. This assumption is not always valid because corrections are needed for the proportion of labeled CO_2 that is produced via oxidation but not excreted in the expired breath. In fact, a substantial proportion of labeled CO_2 becomes entrapped within the bicarbonate pool(s) of the body (91, 124, 190). In addition, another substantial amount of carbon label can be (temporarily) fixated via isotopic exchange reactions in the TCA cycle, mainly by way of conversion of α-ketoglutarate to glutamate and glutamine (151, 167). Sidossis et al. (174) were the first to propose that carbon label retention can be quantified by measuring $^{13}CO_2$ ($^{14}CO_2$) production during ^{13}C (or ^{14}C)

acetate infusion. Acetate, converted to acetyl-CoA, immediately enters the TCA cycle. Therefore, the fraction of acetate label retained should equal the fraction of substrate label entrapped between entering the TCA cycle and its appearance in expired CO_2. As such, an acetate correction factor can be applied to correct for both the amount of label entrapped as CO_2 in the bicarbonate pool(s), as well as for label fixated in other metabolites, via isotopic exchange reactions (167, 174, 190, 195). Omission to apply this correction leads to a gross underestimation of plasma FFA oxidation rate at rest and a substantial underestimation during most exercise conditions (193, 195, 196). Because acetate label recovery reaches an apparent plateau value during steady-state exercise conditions, previously published acetate correction values are conveniently used to correct for carbon label retention in ongoing studies performed in different laboratories under different research conditions. The latter could result in serious methodological complications as it has been shown that the plateau value in acetate label recovery during exercise largely depends on the specific tracer used, the implemented exercise protocol, and preexercise infusion time as well as on the individual subject (195, 211).

Several studies applying FA isotope tracers have shown that during moderate-intensity exercise ∼40–60% of whole body fat oxidation is being accounted for by plasma FFA oxidation in endurance-trained subjects after an overnight fast (36, 161, 162, 176, 193, 196). The latter shows that fat sources other than plasma FFA can contribute substantially to total fat oxidation during exercise (Fig. 2). Although the reported contribution of fat sources other than plasma FFA has been shown to vary considerably under various exercise conditions in different populations, stable isotope studies quite consistently show that on a whole body level muscle- and/or lipoprotein-derived TG sources form a significant substrate source during exercise (36,
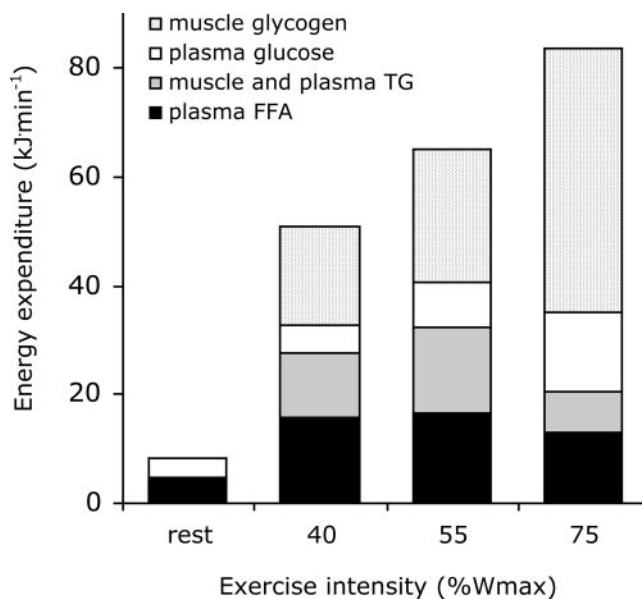

Fig. 2. Energy expenditure as a function of exercise intensity (expressed in percentage of maximal workload capacity). The relative contribution of plasma glucose, muscle glycogen, plasma free fatty acid (FFA), and triacylglycerol (TG) sources (sum of muscle- plus lipoprotein-derived TG) to total energy expenditure are illustrated as described in the legend. [Adapted from van Loon et al. (193).]

54, 65, 68, 130, 131, 149, 161, 162, 168, 169, 174–176, 193, 196). Complementary to the application of stable isotope tracer methodology on a whole body level, others have used a similar methodology over the working muscle or limb (65, 68, 165, 188, 191, 192). By measuring A-V differences in blood metabolites combined with blood flow measurements across the active muscle or limb, these studies have reported considerable plasma FFA uptake and/or oxidation rates, providing important additional information (191). Under resting conditions and during exercise in noncontracting muscle, a substantial portion of plasma FFA uptake has been shown to become reesterified into the IMTG pool (165, 192). However, muscle contraction substantially increases the proportion of FFA uptake that is routed toward oxidation, at the expense of reesterification and storage in muscle TG stores (165). The latter agrees with whole body isotope data showing FA tracer R_d to exceed R_{ox} at rest and to a lesser extent during exercise conditions (193, 196). However, even during moderate-intensity exercise whole body FA tracer R_d generally exceeds R_{ox} by ~10–20%. Recently, we showed that part of this difference can be explained by FFA taken up by noncontracting skeletal muscle (171). Using ^{1}H-MRS to quantitate IMTG content before and after 3 h of cycling exercise, we observed a ~20% net decrease in IMTG content in active vastus lateralis muscle, whereas IMTG content in nonactive biceps brachii had actually increased by ~38% (171). The latter also explains the greater relative difference between R_d and R_{ox} when FA tracer data are compared between studies using whole body methodology and studies measuring A-V differences over the working muscle or limb (165, 192, 196). Although reesterification rates of FFA into the IMTG pool are particularly low during exercise conditions (64), it should be noted that minor reesterification of labeled palmitate into the IMTG pool, followed by subsequent lipolysis and oxidation, would induce a slight overestimation of whole body plasma FFA oxidation rates. The latter would actually lead to an underestimation of whole body muscle- and/or lipoprotein-derived TG use. Therefore, the whole body stable isotope estimates of muscle- plus lipoprotein-derived TG oxidation rates should be considered minimal estimates.

The measurement of plasma FFA oxidation rates using stable isotope methodology allows an indirect estimation of the use of muscle- and/or lipoprotein-derived TG oxidation during exercise. In general, such studies report a considerable contribution of fat sources other than plasma FFA as a substrate source during exercise, measured both on a whole body level as well as across the active muscle or limb. The contribution of lipoprotein-derived TG oxidation during exercise seems to be of relatively little quantitative importance under normal dietary conditions in an overnight fasted state. Therefore, isotope tracer studies imply that IMTG represents an important substrate source during moderate-intensity exercise both in endurance-trained athletes as well as in lean, healthy men and women not involved in any regular exercise training.

MRS. With the introduction of ^{1}H-MRS, a noninvasive means to quantitate both IMCL and EMCL content has become available (19, 20). The ^{1}H-MRS technique can be used to measure the resonances from methyl and methylene groups of muscle TG. This resonance signal appears as multiple peaks, separated by 0.2 parts $\times 10^{-6}$ (ppm), on the proton spectrum of the skeletal muscle of interest (166). These multiple peaks have been linked to TG in different compartments and were assigned to represent either intramyocellular (peak at 1.3–1.4 ppm) or extramyocellular (peak at 1.5–1.6 ppm) lipid depots (19, 166, 185). In the literature these are generally referred to as IMCL and EMCL, respectively. This differentiation between IMCL and EMCL was validated on the basis of the observation that in humans suffering from generalized lipodystrophy, a disease characterized by absence of interfascial adipose tissue, no EMCL signal was detected, in contrast to the presence of a strong IMCL signal (185).

Although the differentiation between IMCL and EMCL based on a shift in the resonance frequency is not entirely unambiguous, several studies have reported good correlations between IMTG content as determined by ^{1}H-MRS and EM morphometry (86) as well as immunofluorescence microscopy (197). Whereas the IMCL signal is independent of the angle between the leg and the static magnetic field, the amplitude of the EMCL signal has been shown to be highly variable. Consequently, in contrast to the IMCL signal, the EMCL signal should not be regarded as a representative indicator of the actual EMCL content in a muscle (19, 20). It is still questioned what the EMCL signal actually represents. Howald et al. (86) have shown EM photographs of adipocytes located in small clusters between muscle fiber bundles. In accordance, we have occasionally observed the presence of adipocytes near small blood vessels (Ref. 63 as well as personal observations). However, after structural analyses of large numbers of muscle cross sections by using various microscopy techniques, we as well as others (63, 125) have failed to provide evidence for the presence of any substantial EMCL depots and/or large numbers of adipocytes situated among muscle fibers. More research is warranted to investigate the physiological structure(s) responsible for the shift in resonance frequency when ^{1}H-MRS is applied to quantitate IMCL and EMCL in skeletal muscle. For example, although IMCL droplets are generally assumed to represent spherical droplets, more advanced microscopic techniques such as two-photon and/or confocal microscopy should be applied to investigate the structural characteristics of IMCL depots. It could be hypothesized that (part of) the EMCL signal is also associated with the structural characteristics of intramyocellular, nonspherical lipid deposits.

The use of ^{1}H-MRS methodology is noninvasive, offers excellent reproducibility, and seems to represent an accurate quantitative assessment of IMTG content (19, 20, 86, 166, 185, 197). Subsequently, various groups have applied ^{1}H-MRS to quantitate IMTG content in relatively large cohorts in different populations, which has resulted in the proposed relationship between obesity, elevated IMTG contents, and the development of insulin resistance and/or Type 2 diabetes (173). However, an increasing number of groups have also started to apply ^{1}H-MRS as a means to assess IMTG content before and after exercise. In contrast to studies applying biochemical TG extraction analyses on muscle biopsies taken before and after exercise, these ^{1}H-MRS studies are extremely consistent and have all reported substantial (~20–40%) decreases in IMTG content after 1–3 h of moderate-intensity endurance exercise in healthy trained as well as untrained male and female subjects (19, 21, 23, 39, 100, 118, 123, 157, 170, 171, 197, 207). Studies investigating intermittent exercise protocols have either shown a similar decrease in IMTG content after 45 min of interval cycling (208) or reported no significant changes after performing different shuttle-run protocols (156).

In the earlier ^{1}H-MRS studies, IMTG content was generally determined in lower leg muscle (tibialis and/or soleus), which is in contrast to the majority of studies applying either biochemical TG extraction or electron and/or (immuno)fluorescence microscopy techniques on muscle samples collected from the vastus lateralis. It has been speculated that differences in fiber-type composition and/or muscle (group) recruitment could be responsible for the striking difference in the consistency of the data derived by ^{1}H-MRS and by the use of chemical TG extraction analysis. This hypothesis, however, does not hold because more recent ^{1}H-MRS studies also report substantial decreases in IMTG content in the vastus lateralis after moderate-intensity exercise, which are similar to the decreases reported for lower leg muscle (100, 170, 171, 197, 207). Although the relative changes in IMTG content seem to be of similar magnitude in the different leg muscles, these studies report large absolute differences in IMTG content between the various muscle groups within similar subject populations. The absolute IMTG concentrations observed in the vastus lateralis (100, 170, 171, 197, 207) are well above IMTG contents reported for tibialis and/or soleus muscle (39, 90, 156, 157), which can only partly be explained by differences in muscle fiber-type composition (90). These observations imply that local net changes in IMTG content should not be translated to whole body IMTG use.

In accordance with stable isotope data, studies using ^{1}H-MRS to quantify IMTG content all support the contention that IMTG is an important substrate source during moderate-intensity exercise. All studies report substantial (20–40%) net decreases in IMTG content after prolonged endurance exercise in both trained as well as healthy untrained subjects. In addition, these studies report similar net reductions in IMTG content in both male and female subjects, after running as well as cycling exercise, in an overnight fasted as well as in a fed state. As ^{1}H-MRS provides a noninvasive method to quantify IMTG content, it will be the preferred method to determine changes in IMTG content over time during prospective nutritional, exercise, and/or pharmacological interventions in vivo in humans.

Electron microscopy and immunofluorescence microscopy. Both the chemical TG extraction method and the use of ^{1}H-MRS to measure skeletal muscle TG content are restricted to the quantification of net muscle TG content in mixed muscle samples and, as such, do not enable direct localization of muscle lipid depots nor do they enable the differentiation between muscle fiber-type-specific lipid content. The latter, of course, with the exception of chemical TG extraction analyses performed on individually dissected type I and/or II muscle fibers (46, 47). In addition to the aforementioned methods, skeletal muscle lipid metabolism has been investigated by electron microscopy (EM) morphometry or ultrastructural analysis (80–82, 86, 87, 125, 138, 181) and/or the use of histochemical staining of muscle cross sections in combination with conventional light (59, 60, 63, 66, 128, 129) or (immuno)-fluorescence microscopy (115, 196, 197). These techniques have been applied successfully to show IMTG content to be determined by muscle fiber type (66, 87, 128, 129, 196, 197), training status (59, 81, 82), diet (80, 197, 198), obesity, and/or insulin resistance (59, 60, 66, 128, 129). In addition, few studies have also applied this approach to assess the role of the

IMTG pool as a substrate source during exercise (102, 138, 181, 196).

The use of EM allows direct and detailed identification of IMCL droplets (Fig. 1). The volume density of IMCL droplets can be measured and used as a direct measurement of IMTG content, without the possibility of any bias due to the presence of EMCL. Unfortunately, because of the massive magnification ($\times 30,000$), the total sample size analyzed is generally small. In addition, an uneven muscle fiber-type distribution within the small tissue blocks selected for sectioning as well as some observer bias are likely to contribute to the relatively large variability of this method (81, 86). To investigate the validity of the EM approach, Howald et al. (87) determined IMTG content in soleus muscle of 10 healthy subjects by applying EM morphometry, ^{1}H-MRS, and chemical TG extraction analyses. The results from ^{1}H-MRS and EM morphometry were strongly correlated but did not correlate with data obtained from the chemical TG extraction assays (87). This finding seems to agree with the reported methodological difficulties when using TG extraction analysis (200, 206). There are a few studies that used EM to quantitate IMTG content in muscle biopsy samples collected before and after prolonged exhaustive exercise and include measurements after a 100-km run (102, 138) and a full marathon (181). Each of these studies has reported a substantial decrease in the size and/or total volume density of IMCL droplets and confirms the role of the IMTG pool as an important substrate source during prolonged exercise.

Other studies have used oil red O to stain lipid deposits in muscle cross sections and applied conventional light microscopy to determine the total area that was stained as a measure of total IMTG content (59, 60, 63, 66, 128, 129). To enable a more direct and selective quantification of muscle fiber-type-specific IMCL content, we combined the use of oil red O staining of muscle cross sections with (immuno)fluorescence microscopy (196, 197). The latter enables direct visualization of both IMCL deposits and muscle fiber type in the same muscle cross section, allowing direct fiber-type-specific IMTG quantification. The use of lipid staining combined with (conventional light or fluorescence) microscopy provides similar advantages as the use of EM morphometry, i.e., allowing direct localization of IMCL deposits. Because the magnification generally applied ($\times 100$–300) is substantially smaller than with EM, less detailed information is obtained. However, this allows the analyses of larger muscle samples sizes, ranging from 10 to up to >100 individual muscle fibers for each muscle sample analyzed. The use of fluorescence microscopy does not eliminate all problems associated with the heterogeneity of repeat muscle biopsy samples, but an important advantage is that differences in muscle fiber-type composition can directly be visualized and taken into account during the quantification procedures. The use of staining techniques combined with light and/or fluorescence microscopy allows a practical and effective method to compare fiber-type-specific IMTG content between muscle samples. However, it should be noted that all these staining techniques, especially when using fluorescence microscopy, allow only a semiquantitative analysis of IMTG content. The calculated area fraction stained is strongly related to the efficacy of the staining process and in case of fluorescence microscopy directly related to the integration time that is used to record images. Therefore, these techniques should only

be used to compare intervention changes and/or population differences in IMTG content within separate studies under identical image-acquisition settings.

Using fluorescence microscopy on oil red O stained muscle cross sections, we recently observed an approximately three-fold greater lipid content in the type I vs. type II muscle fibers (197). This finding is in agreement with the earlier reports on muscle fiber-type-specific quantification of lipid content (46, 47, 66, 128, 129). In addition, in the same study we also observed a good correlation between the data on IMTG content as determined by ^{1}H-MRS and fluorescence microscopy performed on oil red O-stained muscle cross sections (197). More recently, we applied the same approach within a stable isotope study aimed to elucidate the role of the IMTG pool as a substrate source during exercise (196). The quantification of fiber-type-specific IMTG content is important in the assessment of the proposed role of IMTG as a substrate source during exercise, because muscle fiber-type recruitment during submaximal exercise mainly relies on the use of type I muscle fibers (88). By applying (immuno)fluorescence microscopy on oil red O-stained muscle cross sections prepared from biopsy samples taken from the vastus lateralis before exercise, immediately after 2 h of exercise, and 2 h into postexercise recovery, we observed a >60% net reduction in IMTG content after exercise in the type I muscle fibers only (Fig. 3). The latter seems to be in accordance with the preferred recruitment of type I fibers during prolonged endurance exercise and shows that IMTG depletion has been underestimated in the past because of the inability to differentiate between muscle fiber types (196). Clearly, future research investigating the role of the IMTG pool as a substrate source during exercise will need to consider fiber-type-specific changes. Therefore, fiber-type-specific IMTG quantification using microscopic analyses of stained muscle cross sections will likely become a more frequently applied approach in future studies.

In short, the apparent controversy on the proposed role of intramuscular TG as a potential substrate source during exercise mainly stems from studies that have applied chemical TG extraction analyses on muscle biopsies taken before and after exercise. More recent studies using various other methods to measure or estimate IMTG utilization, including stable isotope
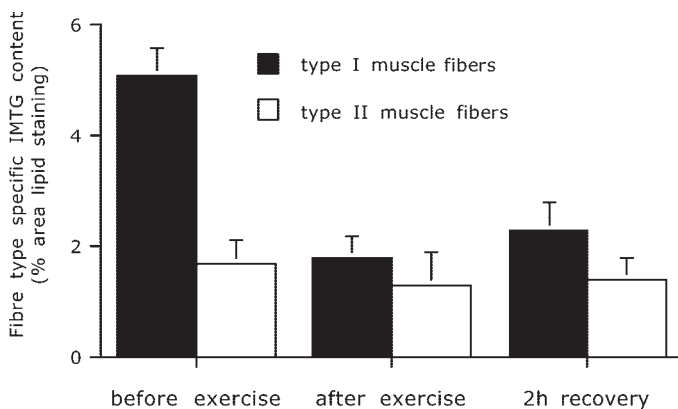

Fig. 3. Mean fiber-type-specific intramyocellular lipid content (%area lipid stained) before and immediately after exercise and 2 h of postexercise recovery as determined by semiquantitative fluorescence microscopy on oil red O-stained muscle cross sections. IMTG, intramyocellular triacylglycerol. Data are means + SE. [Adapted from van Loon et al. (196).]

methodology, ^{1}H-MRS, EM morphometry, and/or fluorescence microscopy on oil red O-stained muscle cross sections, all indicate that the IMTG pool forms an important substrate source during exercise conditions in healthy subjects.

FACTORS THAT DETERMINE IMTG USE DURING EXERCISE

Ample evidence has been provided to support the contention that the IMTG pool can function as an important substrate source during exercise conditions in healthy subjects. However, because of the many different approaches that have been used to estimate IMTG utilization during exercise, it is difficult to compare studies that investigated IMTG use under different exercise conditions. Although there are ample discrepant findings in the literature, factors such as exercise intensity, exercise duration, diet, training status, gender, age, obesity, and/or Type 2 diabetes all seem to affect IMTG use during exercise. It would be likely that muscle HSL, being the rate-limiting enzyme in TG hydrolysis, forms the main site at which IMTG utilization is regulated. However, the provided evidence showing skeletal muscle HSL activity to be stimulated by catecholamines (113, 122, 205) and muscle contraction (113, 119, 121, 202) often does not suffice to elucidate how factors like exercise intensity, exercise duration, diet, training status, gender, and/or age seem to modulate IMTG mobilization.

Exercise intensity. Most information on the effects of exercise intensity on skeletal muscle substrate use has been derived from Romijn et al. (161). In their study, [^{2}H$_2$]palmitate and [6,6-^{2}H$_2$]glucose tracers were applied to estimate endogenous substrate source utilization rates during exercise at three different workloads. Because they assumed tracer R_d to equal R_{ox}, their reported values for plasma FFA and glucose oxidation rates should be considered maximal estimates, whereas the reported values for muscle- plus lipoprotein-derived TG and glycogen use need to be considered minimal estimates (161). Using more contemporary stable isotope methodology, we applied a [U-^{13}C]palmitate tracer to determine plasma FFA oxidation rates directly from the rate of appearance of ^{13}CO$_2$ in the expired air, with corrections made for carbon label retention using an [1,2-^{13}C]acetate correction factor (193). The latter showed that plasma FFA R_{ox} represents between 80 and 90% of R_d during exercise of various intensities, which confirms that the oxidation rate of muscle- plus lipoprotein-derived TG has been underestimated in the past. In contrast, plasma glucose R_d has been shown to equal its R_{ox} during moderate-intensity exercise (99) and therefore represents an accurate estimate of plasma glucose oxidation rate. Substrate source utilization as measured at rest and during exercise at a workload representing 40, 55, and 75% of maximal workload capacity (Wmax) is illustrated in Fig. 2.

Generally, plasma FFA oxidation provides the majority of the energy needs during low-intensity exercise [<30% of maximal oxygen uptake capacity ($\dot{V}_{O_2 max}$)], with little or no net utilization of intramuscular plus lipoprotein-derived TG (77, 114, 161, 162). During moderate-intensity exercise (40–65% $\dot{V}_{O_2 max}$), fat oxidation, from an absolute point of view, reaches maximal rates and generally contributes between 40 and 60% of total energy expenditure (37, 77, 84, 130, 161, 162, 193, 196). About 50–70% of total fat oxidation is generally reported to be derived from plasma FFA, leaving muscle- and/or lipoprotein-derived TG to provide the other part of total

fat oxidation. When exercise intensity is further increased (up to 70–90% $\dot{V}_{O_2 \, max}$), total fat oxidation rates decline substantially, which is accounted for by a decrease in the use of both plasma FFA as well as muscle- and/or lipoprotein-derived TG (161–163, 175, 193). Several studies have indicated that this limitation in fat oxidative capacity mainly exists at the level of long-chain FA transport over the mitochondrial membrane (139, 163, 175, 209, 210) and as such would be unrelated to either FFA uptake and/or IMTG hydrolysis. The latter has been supported by Kiens et al. (112), who observed an increase in intramuscular long-chain FA concentrations after incremental exercise (112). There is still intense debate on the precise mechanism(s) responsible for the reduced capacity to transport long-chain FA over the mitochondrial membrane during moderate- to high-intensity exercise. First, it was hypothesized that the high glycolytic flux rates during high-intensity exercise could lead to the accumulation of acetyl-CoA, which via the subsequent increase in the cytosolic production of malonyl-CoA could lead to an increased inhibition of carnitine (long-chain) acyltransferase I activity. However, muscle malonyl-CoA concentrations have been found not to increase in rat or in human skeletal muscle during high-intensity exercise (139, 153). Alternative mechanisms that could be responsible for the downregulation of carnitine acyltransferase I activity during high-intensity exercise include a decrease in intramyocellular free carnitine availability (193) and/or a decrease in intramyocellular pH (182). Our data tend to suggest that IMTG plus lipoprotein-derived TG use declines more than plasma FFA oxidation rate during high-intensity exercise (Fig. 2). The latter would be in line with the contention that increased intramyocellular FA and/or FA metabolite concentrations downregulate HSL by negative feedback inhibition (98, 201).

Exercise duration. Although various studies have estimated skeletal muscle fuel selection during moderate-intensity exercise, only little information is available on the changes in substrate use with the duration of exercise. The latter stems from the fact that most studies have implemented exercise trials lasting <1–2 h and/or applied a method that does not enable repeated measurements over time during exercise conditions (as in the case of ^{1}H-MRS and muscle biopsy sampling performed before and after exercise). Total fat oxidation has been shown to increase with the duration of exercise (2, 149, 161, 188, 197). The latter has been attributed to a progressive increase in peripheral lipolytic rate with concomitant increases in plasma FFA R_a, R_d, and/or R_{ox} (2, 149, 161, 188, 197).

By applying continuous infusions with a [U-^{13}C]palmitate tracer with corrections made for carbon label retention using a [1,2-^{13}C]acetate correction factor determined in a separate trial, we have reported a progressive increase in plasma FFA R_a, R_d, and R_{ox} during moderate-intensity exercise in trained athletes (197). The increase in plasma FFA oxidation strongly correlated with the increase in plasma FFA concentrations, which agrees with the contention that plasma FFA availability forms one of the factors that regulate FFA oxidation during exercise (42, 43, 84, 152, 163). Concomitant with the progressive increase in plasma FFA R_a, R_d, and R_{ox}, the rate of muscle- and/or lipoprotein-derived TG oxidation was shown to decline during the second hour of exercise. These findings confirmed earlier estimations by Romijn and colleagues (161, 163) and more recent suggestions by Watt et al. (200). The latter studied IMTG use during 4 h of moderate-intensity

exercise by measuring muscle TG content in muscle biopsies collected before and after 2 and 4 h of exercise. Using the chemical TG extraction analyses, they reported a substantial decrease in IMTG content during the first 2 h of exercise, with no further decrease in IMTG content during the latter 2 h (200). Together these findings seem to support the contention that a progressive increase in peripheral lipolytic rate and subsequent increase in plasma FFA concentration suppress IMTG hydrolysis and its subsequent rate of oxidation during the latter stages of prolonged exercise. To confirm this hypothesis, we recently investigated substrate utilization during 2 h of moderate-intensity exercise in trained athletes with a protocol identical to the study mentioned above (197) but with the inclusion of a trial in which we reduced plasma FFA availability by administration of acipimox (a nicotinic acid analog that can be used to inhibit adipose tissue lipolysis). Acipimox administration effectively lowered plasma FFA R_a, R_d, and R_{ox} and prevented the decline in muscle- and/or lipoprotein-derived TG use during the latter stages of the exercise trial (recent unpublished observations). As such, a reduction in plasma FFA availability was shown to stimulate IMTG use, which supports the contention that there is an inverse relationship between the uptake and/or oxidation of plasma-derived FFA and the use of the intramuscular TG stores. Although more research is warranted, it seems likely to assume that the IMTG pool acts as a buffer to maintain intracellular FA (metabolite) concentrations and, as such, can prevent an excessive accumulation of FA (metabolites) during conditions of increased plasma FFA provision. With HSL (120) as a likely site at which IMTG hydrolysis and/or oxidation is regulated, it could be speculated that HSL is not only stimulated by muscle contraction and increased plasma epinephrine concentrations (113, 121, 122) but may also be inhibited by an increase in intramyocellular long-chain fatty acyl-CoA concentrations and/or other FA metabolites, secondary to an increase in plasma FFA uptake. Indirect evidence for such a mechanism has been provided by the in vitro observation that HSL is inhibited in a noncompetitive manner by oleoyl-CoA in bovine adipose tissue (98) and more recent observations of reduced HSL activity and elevated long-chain fatty acyl-CoA concentrations during the second hour of a prolonged moderate-intensity exercise trial (201). More research is warranted to investigate the apparent relationship between intramyocellular FA (metabolite) availability and IMTG metabolism.

Diet. IMTG content has been shown to be strongly related to fat intake and can be substantially increased (50–100%) after the use of high-fat diets, with fat intake varying from 40 to 65% of total energy intake (68, 69, 80, 100, 108, 198, 213). In contrast, the use of high-carbohydrate diets, with fat intake varying from 2 to 25% of total energy intake, can substantially decrease (10–30%) IMTG storage (36, 100, 180). Numerous studies have shown that both short- and long-term adaptation to a high-fat diet substantially increases fat oxidation during exercise conditions (25, 67). However, only little information is available on the changes in the relative contribution of the different endogenous fat sources to total energy expenditure after adaptation to either a low- or high-fat diet.

Schrauwen et al. (169), using whole body stable isotope methodology, observed a ~40% increase in total fat oxidation rate during moderate-intensity exercise after a high-fat diet (60% fat) for 7 days. As plasma FFA R_a, R_d, as well as R_{ox}

remained unchanged after the high-fat diet, the increase in total fat oxidation was entirely attributed to an increase in muscle- and/or lipoprotein-derived TG use. Combining both exercise training with either a low-fat (20%) or high-fat (62%) diet over a more prolonged period of 7 wk, Helge et al. (68) observed a ~80% greater total fat oxidation rate during exercise after using a high-fat diet. The latter was accounted for by a substantial increase in both plasma FFA (~50%) as well as muscle- and/or lipoprotein-derived TG (~100%) use. The increase in plasma FFA oxidation after the high-fat diet was associated with elevated plasma FFA R_a and higher circulating plasma FFA levels compared with the low-fat diet. In their study, A-V measurements were also performed over the active leg, showing a severalfold greater VLDL-TG uptake by the active limb after the high-fat diet. Assuming VLDL-TG uptake to match their rate of oxidation, the authors concluded that VLDL-TG represents an important substrate source during exercise after adaptation to a high-fat diet. After estimating the contribution of VLDL-TG oxidation to total fat oxidation, they suggested that IMTG use is of only minor quantitative importance. In agreement, the authors failed to report a significant decline in muscle TG content after 1 h of cycling exercise after adaptation to either the low-fat (from 40 ± 6 to 40 ± 5 mmol/kg dry wt) or high-fat diet (from 69 ± 8 to 62 ± 4 mmol/kg dry wt). Although the latter did not reach statistical significance, such a decrease in IMTG content of ~7 mmol/kg dry wt would account for a substantial portion of total fat oxidation during a 1-h exercise trial.

More recent studies have provided convincing evidence showing an increase in IMTG use during exercise after short-term adaptation to a high-fat diet (100, 213). Johnson et al. (100), applying ^{1}H-MRS in the vastus lateralis muscle, measured a ~50% increase in preexercise IMTG content after 2 days of a high-fat vs. a low-fat diet. After a 3-h time trial on a cycle ergometer, IMTG content had decreased by ~60% in both trials, representing a much greater absolute net decrease in IMTG content on the high-fat vs. the low-fat diet (6.63 ± 1.08 vs. 3.63 ± 1.80 μmol/g wet wt, respectively). Also Zderic et al. (213) provided convincing evidence showing the increase in total fat oxidation after adaptation to a high-fat diet to be largely attributed to an increase in the use of IMTG as a substrate source. After 2 days of adaptation to a high-fat diet (60%), they observed a 36% greater preexercise IMTG content compared with the control situation in which an isoenergetic control diet (22% fat) was used. During a subsequent 1-h exercise trial, total fat oxidation (~72%) as well as whole body lipolysis rate (79%; estimated by R_a glycerol) were substantially elevated on the high-fat diet compared with the control diet. Interestingly, similar findings were obtained when the same trials were repeated after the administration of Acipimox. So, even under conditions where plasma FFA availability was substantially reduced in both trials, fat oxidation was shown to be substantially greater after the high-fat diet. Combined with the fact that nicotinic acid administration has been reported to effectively reduce VLDL synthesis (199), it was concluded that elevated IMTG content and a concomitant increase in IMTG utilization represent the major contributing factors to the observed increase in fat oxidation after (short-term) adaptation to a high-fat diet. These conclusions seem to be in accordance with several other studies, all reporting IMTG utilization during exercise to be correlated with preexercise IMTG content

(48, 179, 183, 196). Clearly, the mass effect of IMTG content seems to represent a strong regulatory factor, stimulating IMTG oxidation in healthy subjects and capable of overriding potential inhibitory effects of elevated plasma FFA availability on IMTG mobilization and/or oxidation during exercise. In accordance to the observed responses after adaptation to a high-fat diet, the use of low-fat diets has been shown to result in reduced IMTG content and a substantial reduction in fat oxidation rate (36, 100, 180, 197). The reduction in total fat oxidation has been associated with a reduction in the use of muscle- and/or lipoprotein-derived TG use, reduced lipolysis and lower plasma FFA R_a and R_d (36).

Studies investigating the acute effects of carbohydrate and/or fat feeding on fat source utilization during exercise are generally lacking. Only few studies have investigated the acute effects of carbohydrate ingestion on plasma FFA and/or IMTG utilization (37, 204). Coyle et al. (37) reported an equal reduction in plasma FFA oxidation rate and muscle- plus lipoprotein-derived TG use during moderate-intensity exercise after preexercise glucose ingestion. In contrast, Watt et al. (204) reported a substantial decrease in plasma FFA oxidation rates without any change in estimated IMTG use during prolonged exercise when ingesting glucose. Clearly, more studies are warranted to investigate how acute as well as more prolonged nutritional interventions can modulate IMTG metabolism during exercise. Such nutritional interventions could be of clinical relevance as means to improve the apparent beneficial effects of regular physical exercise on IMTG turnover and/or skeletal muscle insulin resistance.

Since the recognition of preexercise muscle glycogen content as an important factor determining endurance performance capacity (9, 11), numerous studies have investigated the efficacy of dietary interventions to optimize muscle glycogen storage (93). These have led to the recommendation for endurance athletes to use high-carbohydrate, low-fat diets (3). However, with the observation that IMTG content can be reduced by >60–70% after only 2–3 h of submaximal exercise in endurance-trained athletes (100, 196), much interest is now being directed toward the role of nutrition in postexercise IMTG repletion. Recent ^{1}H-MRS studies have shown that the use of low-fat diets can substantially impair postexercise IMTG repletion (38, 39, 123). However, in these studies, fat intake has been set as low as 10–15% of total energy intake in the low-fat trials, which is considerably less than the reported fat intake in elite endurance athletes (23 En%, where En% is percentage of total energy intake). The latter, of course, is still well below fat intake in a normal Western diet (~35–40 En%). Recently, we studied postexercise IMTG repletion after 3 h of cycling exercise, and observed no repletion for at least 48 h when using a high-carbohydrate diet containing only 24% fat (197). In contrast, when a moderate fat containing diet (39%) was used, IMTG stores returned to preexercise levels between 24 and 48 h of postexercise recovery. More research is warranted to determine the importance of postexercise IMTG repletion in maintaining endurance exercise performance during periods in which optimal performance is required during consecutive days of competition.

Training status. Christensen and Hansen (28), in their classic study, observed that endurance training increases the capacity to utilize fat as a substrate source during exercise and reduces the reliance on endogenous carbohydrate stores. This training-

induced increase in fat oxidative capacity has since been confirmed by numerous exercise studies (5, 7, 30, 32, 33, 41, 57, 58, 72, 73, 75, 76, 89, 96, 101, 107, 130, 132, 149). However, data on the effects of endurance training on the contribution of the different endogenous fat sources during exercise are less consistent. Several training studies applying either labeled FA tracers in combination with indirect calorimetry (130, 149, 168) or chemical TG extraction analyses on muscle biopsies taken before and after exercise (89) have reported a substantial increase in muscle- plus lipoprotein-derived TG use during moderate-intensity exercise after 1–3 mo of endurance training. In accordance, cross-sectional studies comparing substrate use between trained and untrained subjects have reported similar results (31, 114, 176).

In a study by Schrauwen et al. (168), a group of sedentary, lean subjects participated in a 12-wk supervised training program. Before and after the training period, endogenous fat source utilization was measured during a 1-h exercise trial (at 50% pretraining Wmax) using a [U-^{13}C]palmitate tracer, with an appropriate [1,2-^{13}C]acetate recovery factor being determined in a separate trial. The observed increase in total fat oxidation rate after training was mainly accounted for by an increase in the use of muscle- plus lipoprotein-derived TG use. Interestingly, comparing these findings with substrate oxidation rates measured in highly trained endurance athletes through identical stable isotope methodology (196) strongly suggests that the capacity to utilize muscle- and/or lipoprotein-derived TG during exercise is strongly related to training status (Fig. 4). Although the applied stable isotope methodology does not enable the differentiation between muscle- and lipoprotein-derived TG use, it is likely that part of the training effect is accounted for by an increase in FA uptake from circulating lipoprotein particles. The latter would agree with the reported increase in skeletal muscle lipoprotein lipase activity after

endurance training (110, 134) and could be representative of an increased capacity to utilize lipoprotein-derived TG as a substrate source (141, 142). Because the contribution of lipoprotein-derived TG use is generally assumed to be of little quantitative importance during exercise in an overnight fasted state (50, 65, 68, 142), it would be likely that the greater part of the elevated TG use in the trained state is accounted for by an increased capacity to mobilize and/or oxidize the IMTG. More direct evidence is provided by Hurley et al. (89), who observed a substantially greater net decrease in IMTG content after 2 h of exercise at 64% of pretraining $\dot{V}_{O_2 max}$ after 12 wk of strenuous endurance training.

There are also studies that report greater plasma FFA uptake and/or oxidation rate instead of an increase in the use of TG sources during exercise in the endurance-trained state (7, 52, 54, 73, 107, 188). Some of these studies have applied the one-legged knee-extensor exercise model and measured substrate use over the working muscle (73, 107, 188). In these studies, in which relatively high plasma FFA concentrations were reached during prolonged one-legged exercise, plasma FFA uptake was shown to be greater in the endurance-trained leg. More specifically, Turcotte et al. (188) reported a leveling off in net plasma FFA uptake in the untrained leg but demonstrated a further increase in plasma FFA uptake in the trained leg after >2 h of exercise. The latter suggests that the capacity for (carrier-mediated) FFA transport from the vascular space into the intramyocellular compartment can, at some stage, become limiting. Endurance training is therefore also believed to lead to an increased capacity to take up plasma FFA, which is supported by the observation that plasma membrane bound FA binding protein content is increased in trained vs. untrained muscle (109). The fact that these studies do not report a substantial contribution from TG utilization is, aside from methodological considerations, likely because of the fact that IMTG use is reduced during the latter stages of prolonged exercise when plasma FFA levels are increased (see *Exercise duration*). In addition, it has been speculated that a less pronounced hormonal or sympathetic activation in the one-legged exercise model, because of the relatively small amount of muscle that is recruited, could also be responsible for a lack of IMTG hydrolysis and oxidation observed in these studies. In line with these findings, Coggan et al. (31) reported both higher plasma FFA oxidation rates as well as increased use of muscle-plus lipoprotein-derived TG during 30 min of high-intensity exercise (75–80% $\dot{V}_{O_2 max}$) in endurance-trained athletes vs. healthy but untrained controls.

Endurance training generally results in a less pronounced increase in plasma FFA concentrations during moderate-intensity exercise of the same relative as well as absolute workload. The latter has been attributed to a reduction in circulating (nor)epinephrine due to the altered neuroendocrine response to exercise, thereby inhibiting adipose tissue lipolysis in the trained state (149). Because it has been speculated that a subsequent reduction in plasma FFA availability stimulates IMTG hydrolysis, this could (partly) explain an increase in IMTG use during the initial stages of moderate-intensity exercise in an endurance-trained state. Interestingly, studies that failed to report lower plasma FFA concentrations during exercise after endurance training also failed to observe an increase in muscle- (plus lipoprotein-) derived TG use in the trained state (7, 52, 54).
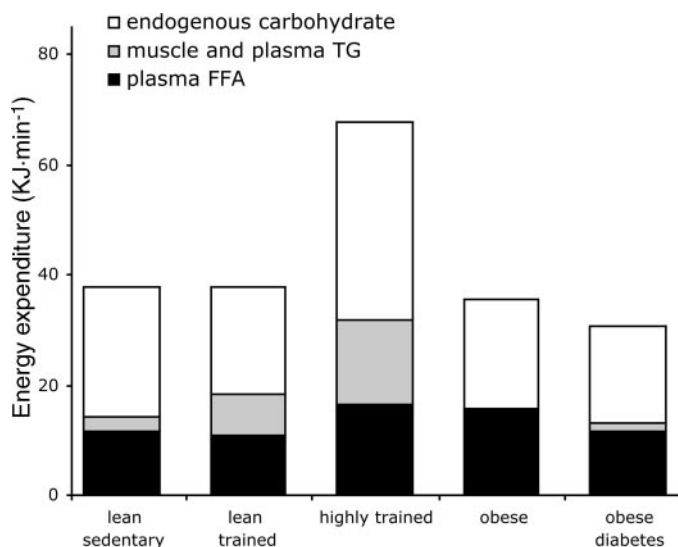

Fig. 4. Substrate utilization in sedentary, lean subjects before and after 3 mo of low-intensity endurance training (168), in highly trained endurance athletes (193), and in obese, Type 2 diabetes patients and their obese controls (15) during moderate-intensity exercise (50–60% maximal oxygen uptake capacity). Total fat and carbohydrate oxidation rates were determined by indirect calorimetry, and plasma FFA oxidation rates were measured by the intravenous [U-^{13}C]palmitate infusion with corrections for ^{13}C-label retention by using an [1,2-^{13}C]acetate correction factor, determined in independent additional trials.

Besides the proposed increased capacity to utilize IMTG and plasma-derived FFA as substrate sources during exercise, endurance training has also been associated with greater IMTG storage (19, 39, 59, 81, 133, 170, 187, 194). This increased IMTG content in the endurance-trained state likely represents an adaptive response to compensate for the increased oxidative capacity (79, 81) and the concomitant regular depletion and repletion of the IMCL stores (196, 197). Such an increased IMTG storage capacity would be in line with similar observations of increased glycogen storage in the endurance-trained state (75, 81). In accordance, we recently observed that preexercise IMTG content in both the type I and II fibers correlated with maximal workload capacity, net IMCL depletion (histochemistry), as well as average muscle- (plus lipoprotein-) derived TG oxidation rates (stable isotope methodology) during exercise in trained athletes (196). These findings are in agreement with earlier findings (48, 179) and seem to imply that the capacity to utilize IMTG is indeed linked with an increased IMTG storage. In accordance, Steffensen et al. (183) also reported a significant correlation between preexercise IMTG content and net IMTG depletion during exercise. However, this correlation was only observed in female subjects and not in men.

Gender. There has been much controversy on the role of gender on substrate metabolism during exercise. Whereas most studies have reported a greater reliance on lipid as a fuel source in female vs. male subjects (27, 55, 85, 186), others have failed to observe such differences (24, 34, 53, 131, 160). In women, a greater reliance on fat as a fuel source during exercise has generally been associated with an elevated adipose tissue lipolytic response to exercise (14, 27, 53, 70, 131). In subjects matched for adiposity and aerobic fitness, Mittendorfer et al. (131) reported greater plasma FFA and glycerol release during moderate-intensity exercise (50% peak oxygen uptake) in women compared with men. In accordance, whole body plasma FFA R_a, R_d, and R_{ox} were substantially greater in women compared with men. Because total fat oxidation rates were observed to be similar between men and women, muscle- (plus lipoprotein-) derived TG utilization rates were higher in men. The latter would be in line with the proposed reciprocal relationship between plasma FFA availability and IMTG hydrolysis and could suggest that women generally oxidize less IMTG than men during moderate-intensity exercise at the same absolute and/or relative workload.

In contrast, others have suggested that IMTG actually plays a more significant role as a substrate source during moderate-intensity exercise in females as opposed to males (160, 183). After applying biochemical TG extraction analyses on muscle biopsies collected from 21 male and 21 female subjects before and immediately after exercise (90 min; 60% peak oxygen uptake), Steffensen and colleagues (160, 183) reported a 25% net decrease in IMTG content in female subjects, regardless of training status, whereas in the male subjects IMTG content remained unaffected. In their study, resting IMTG contents were shown to be substantially (~40%) greater in women compared with men. Interestingly, the observed net decreases in IMTG content during exercise correlated with preexercise IMTG levels ($r = 0.61$; $P < 0.001$). The latter corresponds with other reports, showing similar positive correlations between preexercise IMTG content and net decline in IMTG content and/or calculated muscle- (plus lipoprotein-) derived

TG oxidation rates (48, 179, 196). Therefore, it is possible that the reported gender differences in the use of IMTG as a substrate source can (partly) be attributed to differences in preexercise IMTG content. The greater IMTG content in women vs. men could be related to their greater fat mass-to-lean body mass ratio and in combination with lower basal fat oxidation rates (14) could likely explain greater IMTG storage in women.

Age. A functional relationship between age, IMTG accumulation, and the development of skeletal muscle insulin resistance has not yet been established. Studies using ^{1}H-MRS have failed to show a significant correlation between age and IMTG content (117, 187). The latter is not surprising because high IMTG contents have also been reported in (obese) children at an age of only 11–16 yr (178). Clearly, factors like diet, training status, and body composition are likely to play a major role in modulating the possible relationship between age and IMTG storage. Unfortunately, data on the role of age and the capacity to use IMTG as a substrate source during exercise are currently unavailable. The latter is likely to become an important topic because of the fact that an age-related decline in mitochondrial function has been associated with elevated IMTG storage and the development of skeletal muscle insulin resistance (104, 147, 173).

Obesity and Type 2 diabetes. The proposed relationship between IMTG accumulation and skeletal muscle insulin resistance is not new and has been reported for many years in both animal (116, 136, 137, 184) and human studies (143, 149, 177). In most of these earlier studies, IMTG content was determined by chemical TG extraction analyses, which made it impossible to differentiate between IMCL and EMCL deposits. Since the introduction of ^{1}H-MRS to quantify IMTG content, numerous studies have confirmed that IMCL accretion is associated with obesity (49, 145) and the development of skeletal muscle insulin resistance and/or Type 2 diabetes (94, 146). Furthermore, direct visualization of IMTG aggregates by the application of oil red O staining of muscle cross sections has provided evidence showing greater IMCL content in obese (128) and/or Type 2 diabetes patients (59, 60, 66) compared with healthy, lean controls. These findings are often generalized, leading to the assumption that elevated IMTG stores should be considered a risk factor for the development of skeletal muscle insulin resistance and/or Type 2 diabetes. However, as already mentioned under INTRAMUSCULAR LIPID CONTENT, correlations between IMTG content and insulin resistance (117, 143, 148) disappear with the inclusion of endurance-trained athletes in such studies (59, 187) because these athletes are highly insulin sensitive (44, 127, 159), despite an elevated IMCL storage (59, 81, 187). To explain the apparent metabolic paradox, it has been speculated that the association between IMCL content and insulin resistance is mediated by muscle oxidative capacity (59, 66, 187).

Muscle lipid content as well as oxidative capacity are both strongly determined by muscle fiber-type composition (47, 66, 90, 128, 194, 196, 197). Type I muscle fibers are considered to be more insulin sensitive (71, 95, 106) and contain about threefold more lipid than type II muscle fibers (47, 66, 90, 128, 194, 196, 197). A reduced proportion of type I muscle fibers has been reported in muscle from obese and/or Type 2 diabetes patients (74, 135), whereas a greater proportion of type I muscle fibers is generally observed in highly trained endurance

athletes (4, 6). Therefore, differences in fiber-type composition and/or fiber-type lipid content between trained athletes and obese and/or diabetes patients could likely explain the metabolic paradox. Therefore, we recently compared fiber-type-specific IMTG content between overweight Type 2 diabetes patients, weight-matched healthy men, and endurance-trained athletes (194). The latter showed ~75% greater IMTG content in the endurance-trained athletes compared with diabetes patients and weight-matched sedentary men. A greater proportion of type I muscle fibers in the trained athletes compared with the Type 2 diabetes patients accounted for over 40% of the difference in mixed muscle lipid content, with the remaining difference attributed to a significantly greater IMTG content in the type I muscle fibers in the endurance-trained athletes. In contrast to earlier suggestions (128), no further structural differences in IMCL aggregate size and/or relative distribution pattern of the IMTG deposits were observed between groups (194). As such, our data as well as the findings of others (39, 164, 187, 212) imply that merely elevated IMTG contents are unlikely to be directly responsible for inducing skeletal muscle insulin resistance.

The increase in IMTG storage capacity in the trained athlete likely represents one of the many metabolic responses to endurance training to compensate for increased oxidative capacity (79, 81) and the concomitant regular depletion and repletion of the IMCL stores (196, 197). In contrast, in the obese and/or Type 2 diabetes patient, excess IMTG accretion is more likely a consequence of elevated plasma FFA availability (144, 154, 173) and/or a reduced capacity to oxidize plasma FFA (15, 16, 105). Elevated FFA availability and/or impaired FA oxidation are likely to result in intramyocellular accumulation of TG and FA metabolites (such as fatty acyl-CoA, ceramides, and diacylglycerol), of which the latter have been shown to be able to induce defects in the insulin signaling cascade (1, 18, 40, 45, 92, 158, 173, 212). In addition, Russell et al. (164) recently reported a higher degree of lipid peroxidation in the IMTG pool of obese subjects compared with IMTG in muscle samples obtained from trained endurance athletes. As such, it has also been speculated that lipid peroxidation could likely represent an important factor in the development of skeletal muscle insulin resistance (164). The development of insulin resistance can subsequently lead to the hyperglycemic and hyperinsulinemic state that is associated with Type 2 diabetes and accompanied by major disturbances in skeletal muscle substrate metabolism (97, 153, 209). These disturbances include increased, rather than decreased, muscle glucose oxidation under basal resting and/or exercise conditions and decreased glucose oxidation under insulin-stimulated circumstances, producing a state of metabolic inflexibility (103). The concomitant impaired capacity to augment FA oxidation during fasting conditions likely forms one of the mechanisms that further stimulate IMTG storage in insulin-resistant muscle.

As such, in contrast to the endurance-trained athlete, elevated IMTG contents in sedentary, obese, and/or Type 2 diabetes patients are likely indicative of a structural imbalance between FA uptake, TG storage, and FA oxidation, resulting in excess IMTG accretion. The latter implies that not the actual size of the IMTG pool but rather the imbalance between IMTG storage and oxidation could be responsible for the development of skeletal muscle insulin resistance. It could be speculated that

the flexibility of the IMTG pool to respond to changes in intracellular FA availability represents a key factor in determining the association between IMTG content and insulin resistance. A greater metabolic flexibility combined with an elevated IMTG turnover rate may prevent an excessive accumulation of intramyocellular FA (metabolites) and could likely reduce the degree of lipid peroxidation due to a decreased resident time of the IMCLs. It seems obvious that physical activity plays a main role in increasing IMTG turnover and/or metabolic flexibility of the IMTG pool. Unfortunately, most interest is currently directed toward the relationship between IMTG accretion and the development of insulin resistance, whereas studies investigating the potential to increase IMTG utilization at rest and during different exercise conditions in both healthy and clinical populations are generally lacking (22). Interestingly, such studies would provide important information relevant to the prevention and treatment of skeletal muscle insulin resistance and/or Type 2 diabetes. Figure 4 shows a comparison between stable isotope tracer data on whole body substrate use during exercise as obtained in our laboratory using identical methodology in Type 2 diabetic patients and obese controls (15) with data in a group of sedentary, lean subjects before and after a 3-mo training program (168) and data in highly trained endurance athletes (193). When combined, these data seem to suggest that the capacity to oxidize IMTG (plus lipoprotein-derived TG) during exercise is greatly reduced in the obese and/or Type 2 diabetes patients and can be augmented in healthy, sedentary lean subjects by participation in an exercise training program. In contrast to these findings, others have also reported increased total fat oxidation rates during moderate-intensity exercise in both obese men (61) and women (83) compared with sedentary lean controls, which were shown to be entirely attributed to greater muscle- (plus lipoprotein-) derived TG utilization rates. Studies are warranted to determine fat source utilization at rest and during exercise conditions in both obese and/or Type 2 diabetes patients.

The apparent progressive accumulation of IMTG in sedentary, obese, and/or Type 2 diabetes patients should form a major therapeutic target, and efforts should be made to develop effective exercise, nutritional, and/or pharmacological interventions to prevent excess IMTG accretion and/or stimulate IMTG oxidation. Physical exercise stimulates IMTG turnover and could represent an effective means to improve the balance between muscle FFA uptake and FA oxidation. Therefore, measurements of IMTG oxidative capacity should be performed in obese and/or Type 2 diabetes patients to confirm the speculations on their reduced IMTG oxidative capacity. The mechanisms responsible for this proposed reduced capacity to mobilize and/or oxidize IMTG need to be elucidated. From the data presented before, it could be speculated that elevated plasma FFA concentrations, associated with obesity and/or Type 2 diabetes, could be responsible for inhibiting HSL activity, thereby reducing IMTG use. As such, combining exercise training with plasma FFA-lowering interventions could form an effective approach to stimulate IMTG use in this population. Clearly, we first need to study the efficacy of exercise and/or exercise training in combination with nutritional and/or pharmacological interventions as tools to improve the balance be-

tween FA uptake and oxidation and, as such, improve skeletal muscle insulin sensitivity.

FUTURE RESEARCH

There has been much speculation on the potential role of the IMTG pool as a substrate source during exercise. However, the use of novel methods in metabolic research has resulted in an increasing number of studies showing IMTG stores to be readily oxidized during exercise conditions. Because total fat oxidation rates can increase >10-fold during the transition from rest to moderate-intensity exercise, it seems likely that the IMTG pool represents a buffer of readily available FA for oxidative purposes. In reviewing the literature, IMTG utilization during exercise seems to be determined by factors such as exercise intensity, exercise duration, training status, diet, gender, age, obesity, and/or Type 2 diabetes. In many of these cases, reduced plasma FFA availability and/or increased IMTG content seem to be associated with elevated IMTG oxidation rates. Although the mechanisms responsible for the actual rate of IMTG mobilization and/or oxidation remain to be elucidated, it seems reasonable to assume that muscle HSL plays a key role in this process. However, HSL activity does not always predict IMTG mobilization and/or oxidation, which has led to the belief that additional downstream sites of regulation exist in the regulation of IMTG metabolism. These likely include allosteric regulation of HSL by changes in intramyocellular long-chain fatty acyl-CoA concentrations, translocation of the HSL (complex) toward the lipid droplet membrane, the presence of regulatory sites for HSL docking or fusing at the lipid droplet membrane, as well as many other possibilities. Future research will need to unravel the regulatory mechanisms responsible for IMTG metabolism at the myocellular level. Furthermore, it remains possible that structural characteristics of IMCL aggregates play an important role in the (patho)physiological regulation of IMTG metabolism. Although we have not detected any structural differences in lipid aggregate size or spatial distribution between muscle cross sections collected from endurance-trained athletes, Type 2 diabetes patients, and weight-matched sedentary controls, more advanced visualization techniques like confocal or two-photon microscopy should be applied to investigate three-dimensional shape and structure of IMCL deposits in human skeletal muscle tissue in more detail.

On a whole body or limb level, future research is warranted to provide insight in the contribution of IMTG oxidation to energy expenditure at rest and during various exercise conditions in different populations. Because of the proposed association between IMTG accretion and the development of insulin resistance and/or Type 2 diabetes, it will be important to determine the role of nutrition, obesity, and especially physical inactivity in stimulating IMTG accretion and reducing IMTG oxidative capacity. The latter could provide information that could be of use in the development of effective intervention strategies to prevent excess IMTG accretion. Studies should also be performed to evaluate whether IMTG oxidative capacity is indeed reduced in the obese and/or insulin-resistant state. If so, exercise training combined with nutritional and/or pharmacological interventions should be developed with the intention to improve the balance between IMTG accretion and oxidation. Restoring this balance likely represents an effective means to improve skeletal muscle insulin sensitivity. Clearly, such studies should focus on getting maximal benefits from combined nutritional, pharmacological, and/or exercise intervention programs to prevent and/or treat insulin resistance.

Because of the proposed role of the IMTG pool during prolonged exercise in trained athletes more research is also warranted to elucidate the importance of preexercise IMTG storage in maximizing endurance performance capacity. With the use of ^{1}H-MRS to noninvasively quantify IMTG content, prospective studies on the effects of exercise and nutrition on the depletion and repletion of the IMTG pool are now applicable. Studies should be performed to determine whether preexercise IMTG contents are associated with performance capacity. The latter should be followed by exploring the role of (sports) nutrition in optimizing IMTG repletion without reducing muscle glycogen stores.

In summary, now that we have become aware that the IMTG pool can function as a dynamic fuel store during exercise, more research is starting to focus on the mechanisms that regulate IMTG storage and oxidation. An increasing amount of techniques has become available to study IMTG metabolism. Combined with the reported association between IMTG accretion and the development of insulin resistance, this provides an exciting area of metabolic research.

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