ARTICLE

Intramuscular Triacylglycerol in Energy Metabolism during Exercise in Humans

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ROEPSTORFF, C., B. VISTISEN, and B. KIENS. Intramuscular triacylglycerol in energy metabolism during exercise in humans. *Exerc. Sport Sci. Rev.*, Vol. 33, No. 4, pp. 182–188, 2005. *Intramuscular triacylglycerol (IMTG) represents an energy store that can be used during exercise, when it may contribute up to 20% of total energy turnover depending on diet, gender, and exercise type. It is important to consider how measurements of IMTG have been performed. Hormone-sensitive lipase is thought to regulate breakdown of IMTG during exercise.* **Key Words:** gender, exercise training, skeletal muscle, hormone-sensitive lipase, lipid metabolism

INTRODUCTION

Triacylglycerol stored within striated muscle cells (intramuscular triacylglycerol (IMTG)) represents a potentially large energy source (Fig. 1). The fact that muscle cells contain the enzymatic machinery necessary for esterification and hydrolysis of IMTG (Fig. 2) supports the notion that IMTG represents a substrate pool that is readily built up or mobilized depending on the cellular energy status. For example, in conditions of excess substrate supply fatty acids (FA) can be esterified in skeletal muscle to form IMTG via a cascade of reactions in which three FA are coupled to the backbone of glycerol-3-phosphate (Fig. 2, left). Conversely, in a situation of cellular energy need IMTG can be hydrolyzed primarily by hormone-sensitive lipase (HSL) to form FA ready for β-oxidation in the mitochondria (Fig. 2, right). One situation in skeletal muscle that is characterized by increased cellular energy needs is muscle contraction associated with exercise. Because IMTG is localized very close to the mitochondria (Fig. 1), it would be reasonable to think that IMTG contributes to oxidative energy production during exercise. However, many previous studies have not demonstrated IMTG use during exercise. As discussed, this may be caused by the multitude of physiological factors that influence IMTG hydrolysis as well as certain methodological limitations in measuring IMTG content.

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IMTG USE DURING EXERCISE

For many years it has been debated whether IMTG is used during exercise, and several studies have addressed this issue. When having a closer look at the experimental set-up in these studies, it turns out that the exercise trials have varied in intensity, duration, and type of exercise, as well as the training status, dietary status, and gender of the subjects. A strict consideration of these "between-studies" differences may give the opportunity to assess if any of the aforementioned factors could influence IMTG use during exercise (Fig. 3). The drawback is that the several different study designs and the variety of methods used to measure IMTG content sometimes make it very difficult to compare between studies and therefore to answer the overall question: to what extent is IMTG used during exercise?

Effect of Exercise Intensity on IMTG Use during Exercise

The literature on the effect of exercise intensity on IMTG use is scarce. During 120 min of bicycle exercise at 25% V_{O_2}peak, whole-body uptake of plasma FA could account for nearly all fat oxidation, leading the authors to conclude that no significant IMTG use occurred at this low exercise intensity (8). Accordingly, in our laboratory the IMTG content in

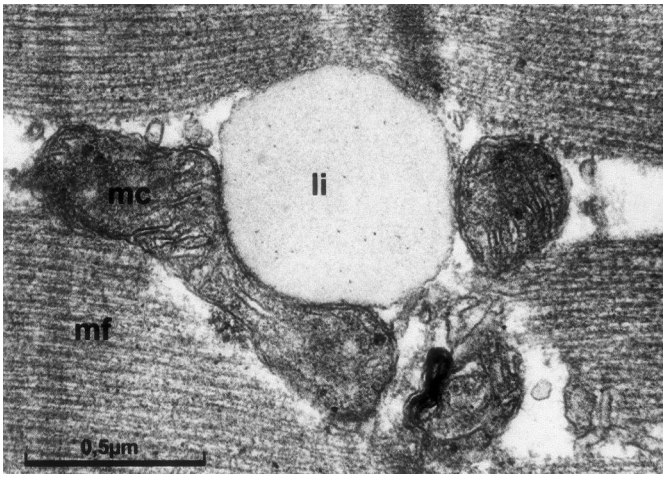

Figure 1. Longitudinal section of human skeletal muscle viewed by electron microscopy. li, lipid droplet; mc, central mitochondria; mf, myofilaments. (Reprinted from H. Hoppeler. Skeletal muscle substrate metabolism. *Int. J. Obes. Relat. Metab. Disord.* 23 Suppl. 3:S7–S10, 1999. Copyright © 1999 Macmillan Publishers Ltd. Used with permission.)

the vastus lateralis muscle was unaffected by 45 min of two-legged knee-extensor exercise at 25% of maximal power (9). During whole-body exercise at intensities between 50 and 70% $\dot{V}O_2$peak, some studies have demonstrated significant IMTG use after 60–120 min of exercise when using Folch extraction of triacylglycerol (TG) in muscle biopsy samples (6,12), oil red O (ORO) staining (11), ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) (1), or indirect estimation of IMTG use (8). In contrast, other studies have not detected any significant IMTG hydrolysis under such exercise conditions when using TG extraction in muscle biopsy specimens (2,10,13,15). When bicycling was performed at 85% $\dot{V}O_2$peak for 30 min, whole-body uptake of plasma FA was significantly lower than fat oxidation (8). Therefore, it was concluded that IMTG was used during exercise at this high intensity (8). However, 90 min of intermittent bicycle exercise alternating between 2-min bouts at 50 and 90% $\dot{V}O_2$peak did not reduce IMTG content in the vastus lateralis muscle (5). Also, when exercise was allocated to the knee extensors for 35 min at 85% of maximal power, any significant IMTG hydrolysis did not occur (9). It appears from these studies that if any IMTG is used during exercise, this occurs primarily during moderate-intensity exercise with little or no IMTG use at low or high exercise intensities. This would not be surprising in light of the fact that the absolute fat oxidation rate is maximal during exercise between 50 and 70% $\dot{V}O_2$peak (8).

Effect of Exercise Duration on IMTG Use during Exercise

Only very few studies have directly investigated the time course of IMTG use during prolonged moderate-intensity exercise. During 120 min of continuous bicycling at 65% $\dot{V}O_2$peak, the whole-body IMTG oxidation rate, estimated as fat oxidation rate minus uptake rate of plasma FA, peaked during the first hour and then declined in the second hour of exercise (8). Accordingly, the IMTG content in the vastus lateralis muscle was reduced during the first 120 min of bicycle exercise at 60% $\dot{V}O_2$peak and did not decrease further from 120 to 240 min of exercise (12). This suggests that IMTG is primarily used early in exercise when oxidation of the alternative fat source, plasma FA, may be limited by the supply of FA from the circulation. In the later stages of exercise, the arterial plasma FA concentration increases and as a consequence oxidation of plasma FA may be able to cover the majority of oxidative fat metabolism. Therefore, IMTG use is reduced. Such a scenario is supported by studies showing that IMTG hydrolysis occurs from 90 to 180 min of bicycle exercise at 60% $\dot{V}O_2$peak only when the arterial plasma FA concentration is suppressed by nicotinic acid but not in a control trial in which the arterial plasma FA concentration increases gradually as usual (13). In other words, IMTG appears to act as a lipid substrate buffer that provides FA for oxidation primarily when these are not delivered in sufficient amounts from the circulation.

Effect of the Preexercise IMTG Level on IMTG Use during Exercise

The level of IMTG at initiation of exercise may determine the extent to which IMTG is used during exercise. Supporting this, in 42 women and men the initial IMTG content was shown to correlate with IMTG use during 90 min of bicycle exercise at 60% $\dot{V}O_2$peak (10) (Fig. 4). One factor that influences IMTG content is the dietary macronutrient composition. For example, it has been demonstrated that ingestion of a fat-rich diet induces an increase in IMTG content in contrast to ingestion of a carbohydrate-rich diet, after which the IMTG stores are either unchanged or even reduced (2). Another factor that determines the IMTG content is the muscle fiber-type composition. This is so because the IMTG content is twofold to threefold higher in type I than in type II muscle fibers. Accordingly, we found a positive correlation between the percentage of type I fibers expressed relative to fiber area and the IMTG content in the vastus lateralis muscle of 42 women and men (10). It seems that in healthy individuals, preexercise dietary status as well as muscle fiber-type composition can indirectly determine IMTG use during exercise by affecting the preexercise IMTG content.

Effect of Training Status on IMTG Use during Exercise

It is controversial whether IMTG use during exercise depends on the training status of the subjects. When men performed 90 min of bicycle exercise at 60% of pretraining $\dot{V}O_2$peak before and after 31 d of exercise training and IMTG content was measured by Folch extraction of TG in muscle biopsy specimens from the vastus lateralis muscle, the IMTG use during exercise was enhanced by training (6). Also, during running at 50% $\dot{V}O_2$peak, the degradation of intramyocellular lipids (IMCL) in the tibialis anterior muscle, measured by ^{1}H-MRS, was larger in endurance trained than in untrained men (1). However, studies in our laboratory

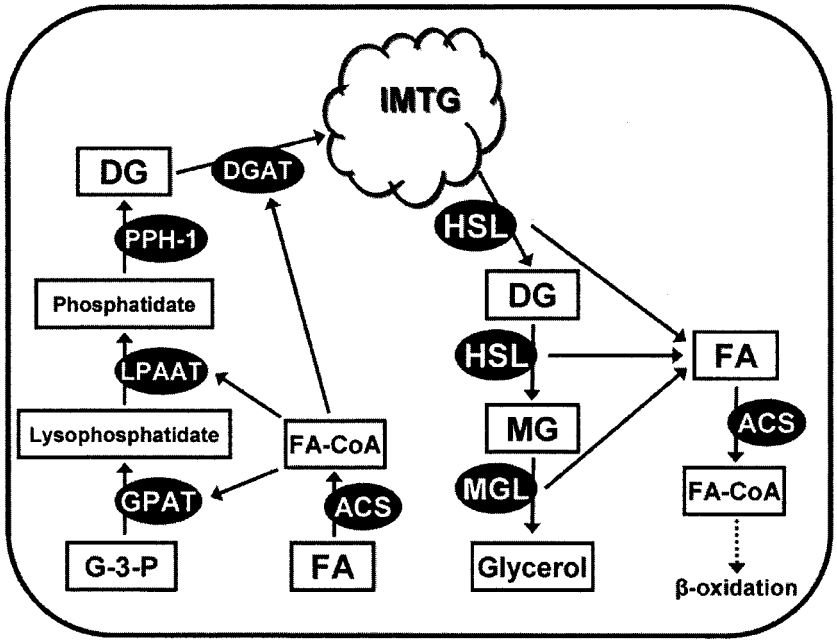

Figure 2. Simplified schematic overview of intramuscular triacylglycerol (IMTG) turnover in human skeletal muscle. G-3-P, glycerol-3-phosphate; FA-CoA, fatty acyl-CoA; DG, diacylglycerol; MG, monoacylglycerol; ACS, acyl-CoA synthetase; MGL, monoacylglycerol lipase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; PPH-1, phosphatidate phosphohydrolase; DGAT, diacylglycerol acyltransferase.

using TG extraction in muscle biopsy samples to measure IMTG content have not found any effect of training status on IMTG use during exercise at the same absolute or relative workload (4,10). Apparently, it is difficult based on the current literature to conclude whether IMTG use during exercise is affected by training status. Additional well-controlled studies are needed in this area. Such studies should focus on a longitudinal exercise training design with strict dietary control and determination of IMTG content by several of the available methods.

Effect of Gender on IMTG Use during Exercise

During the past few decades, several studies have shown that different aspects of muscle metabolism are influenced by gender. Recently, studies in our laboratory have suggested that also IMTG use during exercise is gender-dependent. Thus, in 42 well-matched women and men representing a broad range of aerobic capacities, IMTG use during 90 min of bicycle exercise at 60% $\dot{V}O_2$peak was higher in women than in men (10) (Fig. 5). In women the IMTG concentration was significantly reduced by approximately 24% during the exercise bout, whereas no significant change occurred in men (Fig. 5). Our findings suggest that, irrespective of training status, IMTG is an important energy source during prolonged moderate-intensity exercise in women, whereas in men IMTG is used to a quantitatively lesser extent. This prompted us to look for possible mechanisms that could explain this marked gender difference in IMTG use during exercise. As discussed, preexercise IMTG content appears to partly determine IMTG use during exercise (Fig. 4). Interestingly, we observed that women had higher basal IMTG levels than did men (10). It follows that the higher IMTG use during exercise in women compared with men can at least partly be explained by the higher preexercise IMTG levels in women. Because IMTG hydrolysis is thought to be catalyzed by HSL, gender differences in skeletal muscle HSL regulation could also contribute to the higher IMTG use seen during exercise in women compared with men. Studies to compare exercise regulation of HSL activity in skeletal muscle of women and men are currently in progress in our laboratory.

METHODS TO MEASURE IMTG CONCENTRATION

An important aspect to consider when interpreting previous findings on IMTG use during exercise is that the methods used to measure IMTG content and/or use have varied be-

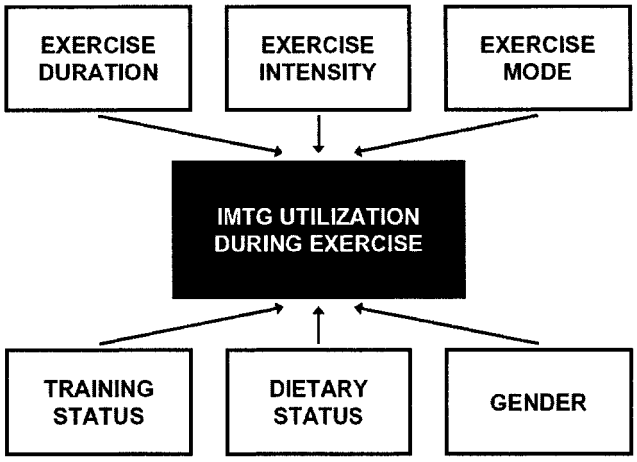

Figure 3. Schematic overview of factors that may determine IMTG use during exercise.

tween studies. This may have contributed to the disparity in the literature when it comes to the extent of IMTG use during exercise. Of the five methods that have been used to quantify IMTG content and/or IMTG use, one is based on biochemical TG extraction in muscle biopsies. Another two use microscopical determination of IMTG content in muscle biopsy specimens. A fourth method is based on ^{1}H-MRS. Finally, whole-body IMTG oxidation can be indirectly calculated from the difference between total lipid oxidation and plasma FA oxidation. As discussed, each of these techniques has its pros and cons and the appropriate choice of technique may depend on specific study conditions.

Biochemical Extraction of IMTG in Muscle Biopsies

The most often used method to determine IMTG content has been to extract TG, by Folch extraction, from freeze-dried muscle that has been dissected free of visible adipose tissue, connective tissue, and blood under a microscope. After hydrolysis of TG in the muscle sample, measurement of glycerol content is assumed to reflect IMTG content. This technique has been criticized for having a large variability as described by Wendling *et al.* (15). The laborious and repeated steps performed in the Folch extraction of tissue lipids may contribute to this variability. Instead, we have used a single-step incubation of tissue samples with tetraethylammoniumhydroxide, which selectively cleaves TG to produce glycerol, which then reflects IMTG content. With the latter method, recovery of TG is improved compared with the Folch extraction and assay variability is therefore also reduced. Still, variability in the IMTG assay has also been thought to stem from invisible contamination of the muscle fibers with adipocytes. Such contamination would highly influence the sample TG concentration because TG content in adipocytes is much higher than in myocytes. To test whether carefully dissected muscle samples are contaminated with adipocytes, we used Western blotting against perilipin,

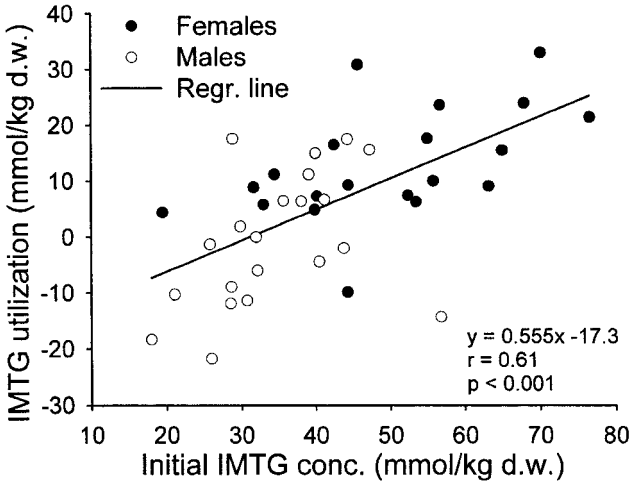

Figure 4. Relationship between initial IMTG concentration and IMTG use during 90 min of bicycle exercise at 60% $\dot{V}o_2$peak in females and males. d.w., dry weight; Regr. line, regression line. (Reprinted from Steffensen, C.H., C. Roepstorff, M. Madsen, and B. Kiens. Myocellular triacylglycerol breakdown in females but not in males during exercise. *Am. J. Physiol.* 282:E634–E642, 2002. Copyright © 2002 the American Physiological Society. Used with permission.)

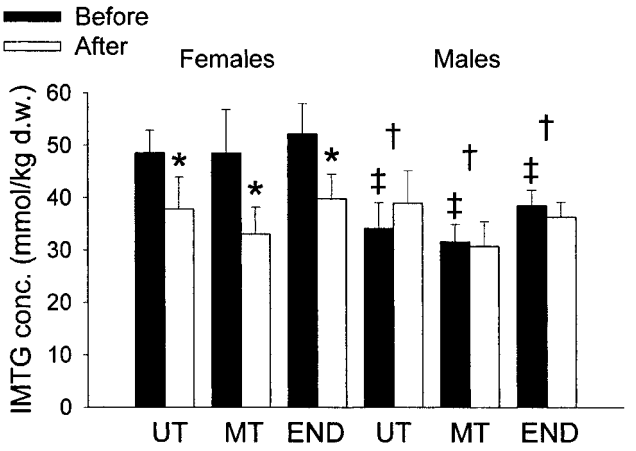

Figure 5. IMTG concentration in the vastus lateralis muscle before (black bars) and after (white bars) 90 min of bicycle exercise at 60% $\dot{V}o_2$peak in females and males. UT, untrained; MT, moderately trained; END, endurance trained; d.w., dry weight; *Different from before exercise, $P < 0.001$; †Different from females in net hydrolysis during exercise, $P < 0.01$; ‡Different from females, $P < 0.001$. (Reprinted from Steffensen, C. H., C. Roepstorff, M. Madsen, and B. Kiens. Myocellular triacylglycerol breakdown in females but not in males during exercise. *Am. J. Physiol.* 282:E634–E642, 2002. Copyright © 2002 the American Physiological Society. Used with permission.)

a specific adipocyte marker protein. As expected, perilipin was detected in human paraumbilical adipose tissue but, importantly, not in any of the dissected muscle samples (Fig. 6), suggesting that adipocyte contamination of human muscle samples does not occur after careful dissection of the freeze-dried muscle under a microscope. An alternative cause of variability in IMTG measurements on muscle homogenates could stem from the fact that IMTG content is two- to threefold higher in type I than in type II muscle fibers (Fig. 7). Therefore, differences in fiber-type composition between pieces of muscle tissue obtained from the same subject will indirectly introduce some variability in the IMTG measurement. One way to partly overcome this source of variation is to mix a large sample (8–10 mg dry weight) of muscle tissue and use a small subpool (1–2 mg dry weight) for IMTG measurement (10). Still, even with our optimization of the IMTG assay procedure, the variability remains somewhat high, with the coefficient of variation being 18.8% between IMTG concentrations in three biopsy samples obtained at the same time in the same leg in moderately trained men.

Morphological Techniques

Two different morphological techniques have been used to visualize and measure the appearance of lipids in skeletal muscle. First, electron microscopy offers very detailed information on the structure of the muscle including the size and subcellular localization of IMTG droplets (Fig. 1). However, the large magnification makes it laborious to examine an adequately large number of muscle cells. Second, ORO staining of neutral lipids in muscle sections offers another technique to measure IMTG. Originally, ORO staining has been detected by light microscopy but based on the excitability of ORO at wavelengths between 540 and 580 nm the ORO staining technique has been optimized for detection by fluorescence microscopy. With this modification, myosin heavy

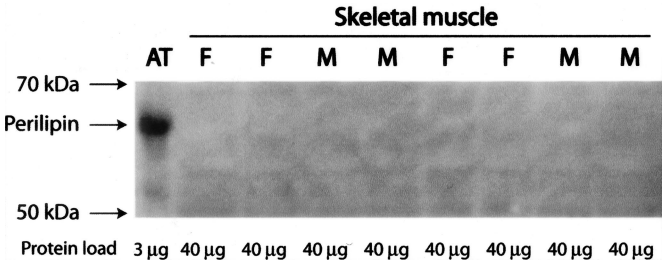

Figure 6. Western blot against perilipin in freeze-dried human vastus lateralis muscle dissected free of visible adipose tissue, connective tissue, and blood. Muscle homogenate corresponding to 40 μg protein were loaded in each lane. Left lane is human paraumbilical adipose tissue used as a positive control (3 μg protein loaded). AT, adipose tissue; F, female; M, male.

chain isoforms can be immunolabeled at the same time to detect muscle fiber type specific IMTG content (Fig. 7). In contrast to electron microscopy, ORO staining provides the opportunity to analyze a large number of muscle fibers from the same biopsy sample. However, it is important to realize that detection of IMTG droplets by either electron microscopy or ORO staining only gives semiquantitative measures of IMTG concentration because of the lack of reference IMTG concentration and the two-dimensional nature of the quantification procedure.

^{1}H-Magnetic Resonance Spectroscopy

^{1}H-MRS is based on measurement of resonances from methyl and methylene protons of TG in skeletal muscle tissue. The resulting peaks in the proton spectrum are 0.2 parts per million apart and represent IMCL (1.4 parts per million) and extramyocellular lipids (1.6 parts per million), respectively. ^{1}H-MRS provides a noninvasive determination of muscle TG content and, like the morphological techniques, it can distinguish true IMTG from TG localized in intermyocellular adipocytes. However, ^{1}H-MRS has a number of drawbacks. First, limitations arise with this technique because of the difficulty in positioning the muscle volume of interest to minimize contribution of vasculature and subcutaneous adipose tissue and to ensure orientation of the muscle fibers along the magnetic field. Second, the presence of extramyocellular lipids can cross-contaminate the IMCL signal and can thereby reduce the accuracy of the IMCL estimation. Third, a scan takes at least 15 min and therefore causes a long interruption in exercise experiments. Also, if the scan is extended into recovery from exercise, the IMCL measurement, thought to reflect exercise IMCL use, may be influenced by postexercise IMTG use (5). Fourth, most ^{1}H-MRS studies to measure IMCL content have been restricted to the soleus and/or the tibialis anterior muscles, whereas only a few studies have included the vastus lateralis muscle. This is probably so because several technical requirements, related to the positioning of the muscle in the magnet bore, are easier fulfilled when measuring on lower leg muscles compared with thigh muscles. It appears that ^{1}H-MRS is a suitable technique to noninvasively measure IMTG content under certain conditions. However, technical considerations may limit its use in dynamic exercise experiments in which thigh muscles are of interest and in which measurements of immediate postexercise IMTG concentrations have high priority.

Combined Indirect Calorimetry and Isotope Tracer Methodology

A common method to estimate whole-body IMTG oxidation during exercise is based on the combination of indirect calorimetry to determine total lipid oxidation and isotope tracer methodology to measure plasma FA uptake or oxidation. With this methodology, the difference between total lipid oxidation and plasma FA uptake/oxidation is taken to reflect IMTG oxidation. A possible advantage of this method is that it provides an estimate of IMTG oxidation rather than IMTG net hydrolysis. In other words, the contribution of reesterification to IMTG turnover is not an issue. However, the method is based on several assumptions that may not all be fulfilled. First, some studies have used plasma FA uptake to reflect plasma FA oxidation. This assumption does not hold true during low- to moderate-intensity exercise. Of course, this concern does not pertain to those studies that have directly measured plasma FA oxidation. Second, the contribution of very low-density lipoprotein triacylglycerol to energy turnover is completely neglected, which may lead to overestimation of IMTG oxidation. Depending on training status and dietary status, very low-density lipoprotein triac-

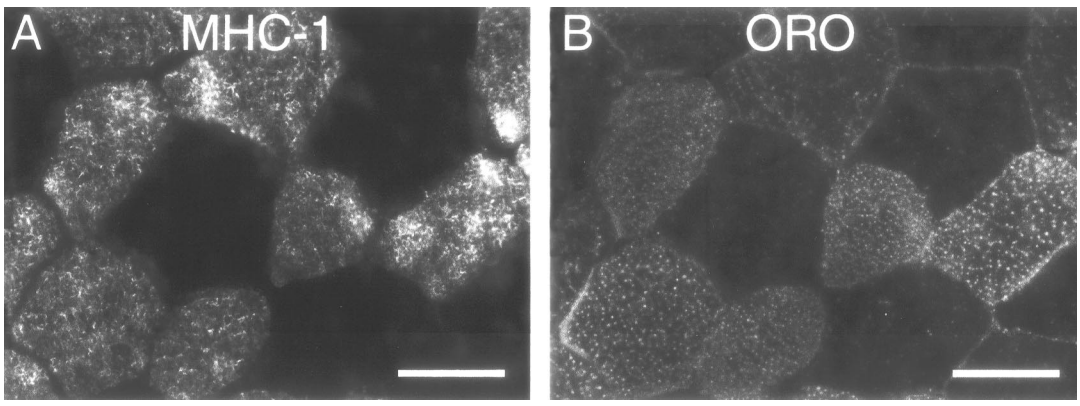

Figure 7. Immunostaining against myosin heavy chain-I (MHC-I) to mark type I muscle fibers (A) and oil red O staining to visualize IMTG (B) on a cross-section of human vastus lateralis muscle. It is evident that IMTG content is much higher in type I than in type II muscle fibers. Scale bar, 50 μm.

ylglycerol contributes 5–25% to oxidative energy turnover (2,4), so neglecting the contribution of very low-density lipoprotein triacylglycerol may in some cases cause a considerable error in the estimation of IMTG oxidation, whereas in other circumstances it should not lead to any concern. Third, the method is applied on the whole-body level and may therefore not very accurately reflect IMTG oxidation in the active muscles, although it should be recognized that during exercise a considerable fraction of whole-body energy turnover occurs in the contracting muscles. In conclusion, this method appears to be inadequate to estimate IMTG use in several exercise conditions.

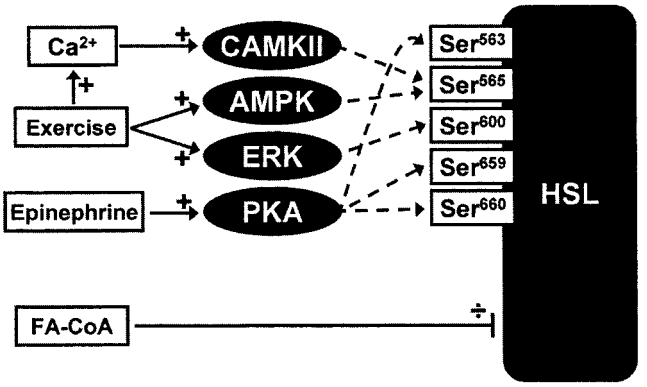

Figure 8. Schematic overview of factors thought to regulate hormone-sensitive lipase activity in skeletal muscle. CAMK II, Ca^{2+}-calmodulin-dependent kinase II; ERK, extracellular signal-regulated kinase; +, positive effect; −, negative effect.

ROLE OF HSL IN REGULATING IMTG USE DURING EXERCISE

It is apparent from the first section that controversy exists on the IMTG use measured during exercise. An alternative approach to elucidate the regulation of IMTG use during exercise is to address the regulatory mechanisms governing IMTG hydrolysis. It has been thought that HSL is the primary enzyme responsible for TG hydrolysis in skeletal muscle as is the case in adipose tissue. Very recently, it has been shown that HSL is indeed expressed in human skeletal muscle (7). Moreover, total neutral lipase activity in human skeletal muscle is increased by exercise (7,14) and this increase in neutral lipase activity can be completely accounted for by an elevated HSL activity (7). Interestingly, the HSL activation by exercise appears to be transient (7), which is in accordance with studies indicating that IMTG hydrolysis occurs primarily early in exercise (8,12).

To help elucidate the regulation of HSL activity, and consequently TG hydrolysis, in skeletal muscle during exercise, investigation of the molecular mechanisms governing HSL activity could prove to be helpful. HSL activity in skeletal muscle is thought to be regulated covalently (by phosphorylation) and allosterically. Covalent regulation of HSL activity may be elucidated through the use of antibodies against site-specific phosphorylation of HSL. Studies on adipocyte HSL have identified five phosphorylation sites on HSL (Fig. 8) (3). These phosphorylation sites are thought to be targets for cAMP-dependent protein kinase A (PKA) (HSL Ser (563), Ser (659), and Ser (660)), extracellular signal-regulated kinase (HSL Ser (600)), and AMP-activated protein kinase (AMPK) and Ca^{2+}–calmodulin-dependent kinase II (HSL Ser (565)) (Fig. 8) (3).

So far, we have investigated HSL Ser (563) and Ser (565) phosphorylation in human vastus lateralis muscle during exercise (7). Because HSL Ser (563) was expected to be a protein kinase A target site also in skeletal muscle, we hypothesized that HSL Ser (563) phosphorylation would increase by exercise, which is known to enhance circulating epinephrine levels. But surprisingly, despite an approximate fivefold increase in the arterial epinephrine concentration from rest to exercise, HSL Ser (563) phosphorylation was not enhanced compared with rest (7). These findings suggest that epinephrine is not an important regulator of HSL Ser (563) phosphorylation in skeletal muscle during exercise.

In adipocyte HSL, phosphorylation of Ser (565) by AMPK has an antilipolytic effect by inhibiting HSL Ser (563) phosphorylation (3). However, in skeletal muscle an anti-lipolytic effect of AMPK is at odds with the presumed role of AMPK as a cellular fuel gauge that acts to speed energy-producing processes in conditions of energy crisis. Accordingly, we recently demonstrated that changes in α_2AMPK activity during exercise was associated with similar changes in HSL Ser (565) phosphorylation but that HSL activity and IMTG hydrolysis did not reflect AMPK activity and HSL Ser (565) phosphorylation (7). These findings suggest that AMPK can phosphorylate HSL on Ser (565) but that AMPK is of minor importance as a regulator of HSL activity and IMTG breakdown in human skeletal muscle during exercise (7).

TG hydrolysis in skeletal muscle also appears to be regulated allosterically by long-chain fatty acyl-CoA (LCFA-CoA) (Fig. 8). Thus, an approximate 20% reduction in total neutral lipase activity was found in homogenates from resting rat soleus muscle when 10 μmol · L^{-1} palmitoyl-CoA was added (13). Intramuscular concentrations of LCFA-CoA and FA are high during intense exercise and during the late stages of low- to moderate-intensity exercise. This may explain why IMTG appears not to be used to any major extent during high-intensity exercise (9) and why the rate of IMTG use declines during prolonged low- to moderate-intensity exercise beyond approximately 60–90 min (8,12,13). An allosteric inhibitory effect of LCFA-CoA on HSL activity and IMTG hydrolysis is also supported by studies showing that a reduction in the arterial plasma FA concentration during exercise, by nicotinic acid, elevates IMTG hydrolysis compared with a control trial with normal plasma FA concentrations (13).

To summarize, covalent regulation of HSL by HSL Ser (563) or Ser (565) phosphorylation appear not to be major determinants of HSL activity in human skeletal muscle during exercise. However, allosteric inhibition of HSL activity by LCFA-CoA may be a potent mechanism to regulate HSL activity in skeletal muscle *in vivo*. Still, the role of HSL Ser (600), Ser (659), and Ser (660) phosphorylation in regulation of HSL activity in human skeletal muscle needs to be investigated.

CONCLUSION

Available studies do not give a clear picture of the extent of IMTG use during exercise. This may be because of certain technical limitations in measuring IMTG content. Nevertheless, when taking an overview of the available literature, it appears that if IMTG is used at all during exercise in men, this occurs primarily during moderate-intensity exercise rather than during low- and high-intensity exercise. Also, IMTG use seems to occur early in exercise and to decline during prolonged submaximal exercise. Interestingly, it has been suggested that women use more IMTG during submaximal exercise than do men. In overnight-fasted men the contribution of IMTG to oxidative metabolism during exercise may account for up to 10% of energy provision, whereas in the same situation in women IMTG appears to contribute approximately 20% to energy turnover. In the future, a new approach to elucidate the regulation of IMTG use is to address the regulatory mechanisms governing IMTG hydrolysis. Along these lines, investigation of the molecular regulation of HSL activity is now in progress.

Acknowledgments

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