

REVIEW

The effect of diet and exercise on lipid droplet dynamics in human muscle tissue

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ABSTRACT

The majority of fat in the human body is stored as triacylglycerols in white adipose tissue. In the obese state, adipose tissue mass expands and excess lipids are stored in non-adipose tissues, such as skeletal muscle. Lipids are stored in skeletal muscle in the form of small lipid droplets. Although originally viewed as dull organelles that simply store lipids as a consequence of lipid overflow from adipose tissue, lipid droplets are now recognized as key components in the cell that exert a variety of relevant functions in multiple tissues (including muscle). Here, we review the effect of diet and exercise interventions on myocellular lipid droplets and their putative role in insulin sensitivity from a human perspective. We also provide an overview of lipid droplet biology and identify gaps for future research.

KEY WORDS: Lipotoxicity, PLIN5, PLIN2, Lipolysis, Insulin sensitivity

Introduction

Dietary fatty acids are stored in adipose tissue. However, excess lipid intake leads to hyperlipidemia and elevated plasma levels of triacylglycerol (TAG) and free fatty acids (FFAs). The so-called overflow of lipids promotes the storage of excess fat in non-adipose tissues such as skeletal muscle (van Hees et al., 2010). This phenomenon is referred to as ectopic fat storage. The lipid overflow hypothesis suggests that ectopic fat storage is merely a way of sequestering excess lipids. Thus, excess lipids upon lipid overflow augment the total intramyocellular lipid (IMCL) content. IMCL is dispersed throughout the muscle in small lipid droplets (LDs). In the past, lipid droplets were viewed as inert storage sites; however, they are now considered to be active organelles (Kraher et al., 2009).

Ectopic fat storage has been suggested to interfere with normal cell function, a phenomenon referred to as lipotoxicity (Unger, 2003). Skeletal muscle is an important organ in the storage and release of fatty acids upon changes in supply and demand and, hence, is prone to lipotoxicity. Human skeletal muscle is responsible for approximately 80% of post-prandial glucose uptake (under healthy conditions), which is impeded in muscles with excess IMCL (Blaak, 2004). However, obese individuals or individuals with type 2 diabetes have an elevated IMCL content compared with lean individuals (Goodpaster et al., 2001) and IMCL is also elevated in insulin-resistant offspring of patients with type 2 diabetes (Jacob et al., 1999; Petersen et al., 2004). This supports the notion that excess IMCL can be an early event in type 2 diabetes development. Multiple papers have reported negative correlations of

IMCL content with insulin sensitivity (Anderwald et al., 2002; Goodpaster et al., 2001; Jacob et al., 1999; Krssak et al., 1999; Pan et al., 1997). However, this correlation is absent in trained athletes (Goodpaster et al., 2001; Thamer et al., 2003), a phenomenon referred to as the ‘athlete’s paradox’ (Goodpaster et al., 2001); despite high levels of IMCL, trained athletes remain highly insulin sensitive. However, the mechanistic link between IMCL content and muscle insulin sensitivity remains elusive. It has been suggested that the capacity to readily shuttle excess fatty acids to inert storage in LDs in the form of TAG along with an adjustment of TAG hydrolysis and fatty acid oxidation, ameliorates the insulin desensitizing effects of lipids (Badin et al., 2013). Jointly, these processes are referred to as LD dynamics (Badin et al., 2013).

The dynamic nature of LDs seems to depend on a variety of factors, including nutritional and dietary factors, as well as acute physical exercise and exercise training (Bosma et al., 2012a). Multiple characteristics of LDs have been linked to the dynamic nature of LDs (Fig. 1) and, hence, are suspected to contribute somehow to the intricate relationship between ectopic fat storage and insulin sensitivity. Likewise, the size, number, subcellular distribution, composition and protein coating of LDs, and the interaction of LDs with other subcellular organelles, such as mitochondria and sarcoplasmic reticulum, have all been hypothesized to be determinants of myocellular insulin sensitivity.

Here, we will briefly review basic concepts of LD biology in human skeletal muscle, specifically in relation to insulin sensitivity, with the aim of providing a fundamental understanding of how dietary interventions and physical exercise affect LD dynamics.

Lipid droplet structure, biogenesis and growth

LDs in skeletal muscle are relatively small (0.20–0.50 μm^2) and typically comprise a neutral lipid core composed of lipid esters, including TAGs and, to a lesser extent, cholesterol esters (Walther and Farese, 2012). Usually, myocellular LDs are surrounded by a phospholipid monolayer that provides a base to a variety of proteins involved in storage and release of fatty acids from LDs (Walther and Farese, 2012). Exactly how LDs are formed has not been elucidated. A variety of models for LD formation have been hypothesized (Murphy and Vance, 1999; Ploegh, 2007; Wältermann et al., 2005; Walther and Farese, 2009; Wanner et al., 1981; Zweytick et al., 2000). The classical model describes the ‘budding off’ theory and suggests that globular structures of lipid esters within the endoplasmic reticulum (ER) are budded off, resulting in a LD coated with a phospholipid monolayer (Murphy and Vance, 1999). Another model that has gained momentum over the years involves the accumulation of lipids in membranes of small ER vesicles, resulting in LD formation and release (Walther and Farese, 2009). Although these models are not mutually exclusive, none of the models is underpinned by thorough and complete experimental proof.

Even within a single cell type, such as skeletal muscle, LDs can vary substantially in size under a variety of conditions. How LDs

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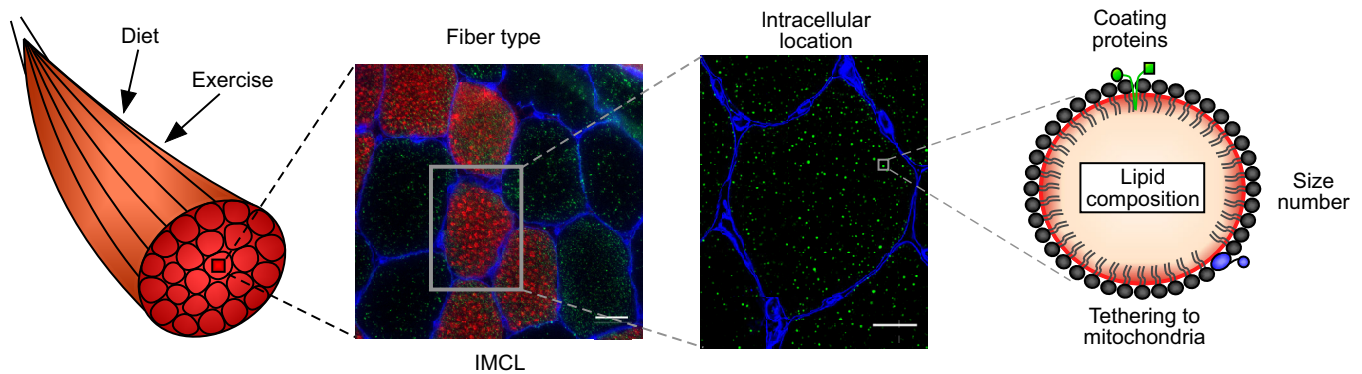


Fig. 1. Schematic overview of the characteristics of muscle lipid droplet (LD) dynamics addressed in this Review. Intramyocellular lipid (IMCL) storage occurs in LDs, which are considered to be highly dynamic organelles with an important role in regulating cellular metabolic health. Multiple dynamic characteristics of LDs, such as cellular localization (muscle fiber type specificity as well as subcellular localization), LD morphology (LD size and number), interactions of LDs with mitochondria and with proteins regulating LD turnover (e.g. the PAT protein family, the lipolytic enzyme adipose triglyceride lipase and its cofactors) and the composition of lipids in the LD have been linked to (patho)physiology, such as insulin sensitivity. Diet and exercise interventions affect the dynamics of the LDs. The widefield microscopy image (left-hand image) and the confocal microscopy image (right-hand image) are cross-sectional views of a cryosection of a biopsy of the vastus lateralis muscle and show human muscle fibers; the basement membrane protein laminin is shown in blue (secondary antibody labeled with Alexa Fluor 405), LDs are stained green (BODIPY 493/503) and type I fibers are shown in red (primary antibody against myosin heavy chain type 1; secondary antibody labeled with Alexa Fluor 555).

change in size is not completely understood. However, three models are currently under consideration (Thiam et al., 2013). (i) The fusion of LDs via pores in the phospholipid monolayer of two LDs results in an exchange of lipids and the fusion of LDs. (ii) An exchange of molecules from one LD to another in a process referred to as ripening, which results in a linear increase in LD size over time. Typically, this process occurs alongside increases in the phospholipids of the LD monolayer. In this model, some LDs grow at the expense of others that shrink, resulting typically in a wider range in LD size. (iii) The expansion of LDs by the cellular uptake of excess fatty acids from the circulation and the incorporation of these fatty acids in the LD as TAG. Given that each of these three processes distinctly affects the lipid interface, the type of process responsible for LD growth likely also affects the physiological properties of large LDs.

Lipid droplet coat proteins of the perilipin family

The TAG molecules making up the LDs in skeletal muscle are subject to enduring cycles of hydrolysis and resynthesis. This dynamic nature is tightly orchestrated by a series of proteins possessing lipolytic activity [e.g. the main TAG lipase, adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004), whose activity is regulated by stimulatory and inhibitory co-factors]. Regulation of LD synthesis and degradation is also under partial control of a set of structurally alike proteins known as PAT proteins that belong to the perilipin family (Kimmel et al., 2010). Among the known PAT proteins, perilipin 2 (PLIN2) (Brasaemle et al., 1997) and PLIN5 (Dalen et al., 2007; Wolins et al., 2006; Yamaguchi et al., 2006) appear most important in skeletal muscle. PLIN2 dissociates lipase from the LD and, hence, prevents lipolysis from occurring (Listenberger et al., 2007). PLIN5 is present at the LD surface, particularly at sites of LDs neighboring mitochondria as well as at LD-mitochondrial contact sites (Bosma et al., 2012b), and is supposed to regulate LD lipolysis in conjunction with ATGL and its co-factors. More specifically, PLIN5 has been suggested to be involved in fine-tuning the release of fatty acids from LD lipolysis to the mitochondrial oxidative demand. In general, the emerging picture is that in the absence of PLINs, muscle becomes devoid of LDs (Bosma et al., 2012a) owing to ongoing lipolysis. Increases in

any of these PLINs results in large LDs and high IMCL content (Bosma et al., 2012b). Thus, proteins of the PAT family are probable candidates to affect the dynamic nature of LDs, resulting in morphological changes such as altered LD number and size and, possibly, lipid composition and interorganelle interaction.

Lipid droplet turnover

Myocellular LDs provide the main lipid reservoir to provide energy by hydrolysis of the TAG they harbor and provide building blocks for membrane synthesis (Farese and Walther, 2009). To fulfill these roles, esterification of fatty acids into TAG and subsequent storage in LDs is essential. It has even been argued that plasma fatty acids are first incorporated in LDs prior to use for oxidation during exercise (Kanaley et al., 2009). Thus, LDs should be highly dynamic and be capable of readily fine-tuning to changes in the supply and demand of fatty acids.

Indeed, the LDs that make up the IMCL pool can exhibit a high turnover during both rest (Kanaley et al., 2009; Sacchetti et al., 2004) and exercise (Guo et al., 2000), whereas the total IMCL content remains unaffected (Guo et al., 2000; Sacchetti et al., 2004). In athletes, the synthesis rate of IMCL is higher than in untrained individuals (Bergman et al., 2010). By contrast, lower rates of IMCL synthesis and oxidation have been reported in obese and pre-diabetic males (Perreault et al., 2010) and in patients with type 2 diabetes (Blaak and Wagenmakers, 2002) than in healthy males. Thus, the IMCL pool in trained athletes represents a highly dynamic pool, whereas the IMCL pool in patients with type 2 diabetes has lost its dynamic character.

A highly dynamic LD pool relies on readily and efficiently sequestering fatty acids in LDs and well-controlled hydrolysis of TAG in the LD to fuel fat oxidation in a timely and adequate fashion. Studies using cell and rodent models have revealed that the absence of PLIN2 (Bosma et al., 2012a) or PLIN5 (Mason et al., 2014) results in a marked reduction in LD number and size. However, under these conditions, the most likely explanation for the loss of LDs is not the compromised incorporation of fatty acids in TAG, but rather uncontrolled and/or continuous lipolysis. However, data from human studies on sequestering and release of fatty acids from LDs are largely lacking.

Rare, genetic disorders resulting in compromised ATGL activity (Fischer et al., 2007), either directly by affecting ATGL protein content or function, or by reducing the function of its co-factor comparative gene identification 58 (CGI-58; also known as α - β hydrolase domain-containing protein 5, ABHD5) (Lass et al., 2006; Schweiger et al., 2009, 2008), greatly compromise LD dynamics and result in severe metabolic aberrations. Following the reasoning that inert storage of fatty acids in LDs does not necessarily impede cell function, the severity of the metabolic aberrations observed in patients with ATGL or CGI-58 mutations is somewhat surprising. Studies in rodents have revealed that mice lacking ATGL had severely compromised mitochondrial function and consequently possessed low fat oxidative capacity (Haemmerle et al., 2006). Given that fatty acids are ligands for peroxisome proliferator-activated receptor (PPAR)-mediated gene expression, it has been hypothesized that in the absence of LD hydrolysis, the limited availability of fatty acids for PPAR activation may be the underlying factor for the compromised oxidative gene expression observed when ATGL function is limited (Haemmerle et al., 2011). This notion was substantiated by the observation that the administration of synthetic PPAR ligands to mice (Haemmerle et al., 2011) and ATGL mutant humans (van de Weijer et al., 2013) promoted the oxidative phenotype, without major effects on LD number or size or TAG hydrolysis (Haemmerle et al., 2011; van de Weijer et al., 2013). These observations indicate that the maintenance of TAG hydrolysis capacity is an essential aspect of LD dynamics, and links proper LD dynamics to proper cell function, including the maintenance of an oxidative phenotype (see also the 'Role of oxidative capacity and mitochondria' section).

Role of oxidative capacity and mitochondria

IMCL is an important substrate during exercise in endurance-trained athletes (Stellingwerff et al., 2007). In athletes, a high IMCL level coexists with a high oxidative capacity and the large contribution of lipids to total energy provision. This is in contrast with sedentary subjects, where lipid supply often exceeds the oxidative demand or capacity. In athletes, the increased demand may result in high lipid (droplet) turnover, which may affect important parameters such as levels of specific lipid species, the cellular localization of lipids, LD protein coating and LD–organelle interactions. Moreover, mitochondrial dysfunction has been implicated in insulin

resistance in type 2 diabetes, further compromising the ability to oxidize fat. Thus, mitochondrial deficits may lead to incomplete β -oxidation, resulting in an accumulation of lipid intermediates such as diacylglycerol (DAGs), ceramides or other toxic lipid species (see also the 'Lipid composition and intermediates' section).

Maintaining a tight match between lipid supply and lipid usage appears to be important to prevent lipotoxicity. Therefore, a tight tethering of LDs and mitochondria could be key for effectively shuttling fatty acids into mitochondria. Indeed, LDs and mitochondria show a close spatial approximation (Hoppeler, 1999; Shaw et al., 2008). PLIN5 is specifically located at the LD–mitochondrial interface (Bosma et al., 2012b), which suggests that this protein has a role in shuttling fatty acids from LD to mitochondria. The exact nature of LD–mitochondrial interactions remains elusive.

Although mitochondria are no longer viewed as single organelles, they can form an organized network, which can be observed both in cultured muscle cells and muscle tissue. Just like LDs, the mitochondrial network is also thought to be highly dynamic and can adapt to cellular energy status via the fission and fusion of mitochondria. Interestingly, LDs appear to be located within the mitochondrial reticular network (Fig. 2). A properly fused mitochondrial network, and the embedding of LDs within this network, is thought to be relevant for the efficient and homogenous distribution of fatty acids coming from these LDs across the mitochondrial population. Indeed, it has been shown in the case of cellular starvation, when cells rely heavily on oxidation of cellular stored lipid for energy production, that when mitochondrial fusion is inhibited, fatty acids were not homogeneously distributed across mitochondria and fatty acid oxidation was reduced (Rambold et al., 2015).

Lipid composition and intermediates

The negative relationship between IMCL and insulin sensitivity has been suggested to originate from an accumulation of toxic lipid intermediates. LDs may protect against lipid-induced insulin resistance via the sequestration of insulin-desensitizing lipid intermediates. In recent years, much attention has been given to the putative role of specific lipid classes, mainly DAG and ceramides, which impede insulin signaling *in vitro* and in animal studies (Montell et al., 2001; Schmitz-Peiffer et al., 1999). The

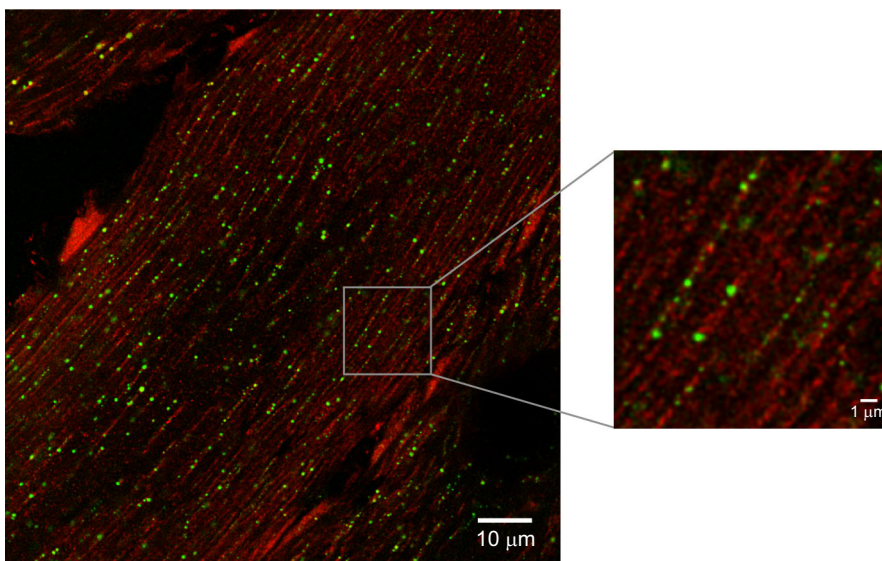


Fig. 2. Lipid droplets (LDs) appear to be located within the mitochondrial network. The embedding of LDs within strings of mitochondria may facilitate the efficient shuttling of fatty acids from the LDs toward the mitochondrial network in times of elevated energy demand. Immunofluorescence image of a longitudinal cryosection of a muscle cell in a human muscle biopsy of the vastus lateralis muscle. Mitochondria were stained using an antibody against a protein located in the outer mitochondrial membrane (translocase of the outer mitochondrial membrane-20, TOMM20). A secondary antibody labeled with Alexa Fluor 555 was used to visualize TOMM20. LDs have been stained green using BODIPY 493/503.

relationship between these lipid classes and insulin resistance is not straightforward. Recent advances in lipidomics triggered an examination of specific lipid intermediates with respect to fatty acid chain length, saturation and position on the glycerol backbone.

Diacylglycerols

Our knowledge of the potential mechanisms by which DAGs may affect insulin resistance is primarily derived from *in vitro* and animal data. The prevailing idea is that DAG activates protein kinase C (PKC) isoforms, promoting serine phosphorylation of the insulin-receptor substrate 1 (IRS-1), thereby blunting insulin-stimulated tyrosine phosphorylation and activation by phosphatidylinositol-3 kinase, reducing the localization of the glucose transporter GLUT4 to the cell membrane, with compromised glucose uptake (Erion and Shulman, 2010).

Human intervention and cross-sectional studies investigating the possible association between DAG and insulin resistance have revealed conflicting results. Several studies have examined the role of DAG in acute lipid-induced insulin resistance. A pioneering study in humans, in which lipids were infused, revealed elevated DAG levels and a concomitant drop in insulin sensitivity (Itani et al., 2002). However, other studies failed to show elevated levels of DAG after lipid infusion while insulin sensitivity was clearly compromised (Vistisen et al., 2008). These observations dissociate DAG and acute lipid-induced insulin resistance.

Although several cross-sectional studies have shown higher total muscle DAG levels in obese and/or diabetic subjects and a negative correlation between total DAG levels and insulin sensitivity (Bergman et al., 2012; Moro et al., 2009; Straczkowski et al., 2007), other studies have failed to do so (Anastasiou et al., 2009; Perreault et al., 2010). The role of exercise training in modulating lipid composition has also shown conflicting results. Acute exercise blunted lipid infusion-mediated increases in DAG (Schenk and Horowitz, 2007; Vistisen et al., 2008). However, in cross-sectional studies in endurance-trained subjects, DAG content was found to be similar (Bergman et al., 2010) or elevated (Amati et al., 2011) compared with that of sedentary subjects. Controversially, a moderate exercise training program in obese sedentary volunteers reduced total DAG levels (Dube et al., 2008).

Part of these inconsistencies may originate from differences in subspecies of DAG. Early biochemical studies highlighted the importance of both saturation and the chain length of fatty acid moieties in the bioactivity of DAG (Molloy and Rando, 1988; Mori et al., 1982). In insulin-resistant men, DAGs have a higher level of saturation compared with DAGs of insulin-sensitive controls. This was predominantly reflected by higher C16:0 and lower C18:1 DAG (van Hees et al., 2011). However, a significant increase in 18:1 DAG was found in obese versus non-obese subjects (Moro et al., 2009). Acute lipid-induced insulin resistance via lipid infusion led to an increase in DAG containing C16:0, C18:0, C18:1, C18:2 and C20:4; these changes correlated with the activation of PKC θ and insulin resistance. In another study, these DAG species were also associated with insulin resistance in obese and type 2 diabetes subjects (Szendroedi et al., 2014). In this study, membrane bound as cytosolic DAG was elevated (Szendroedi et al., 2014) and correlations with insulin resistance were most prominent for membrane fractions of DAG containing C18:1, C18:2 and C20:4. This observation matches another study reporting a negative correlation between membrane-bound DAG and insulin sensitivity (Bergman et al., 2012). An analysis of specific DAG subspecies in both compartments revealed elevated membrane DAG species C18:0/C20:4, di-C16:0 and di-C18:0 in type 2 diabetes subjects,

with di-C18:0 showing a significant positive correlation with both insulin sensitivity and PKC ϵ (Bergman et al., 2012). These studies highlight the importance of the cellular localization of lipid species (see also the 'Location of lipids' section).

DAG species can be distinguished on the basis of their fatty acid moieties and the position of these moieties on the glycerol backbone, namely 1,2-DAG, 1,3-DAG and 2,3-DAG. The location and production of these isomers is different. In general, three locations can be distinguished. DAG located at the plasma membrane consists of 1,2-DAG and is mainly generated from phospholipids. In addition, 1,2-DAG can be found at the ER and Golgi network and comes primarily from *de novo* lipogenesis. 2,3-DAG and 1,3-DAG are produced from TAG hydrolysis and, therefore, are present in cytosolic LDs (Eichmann and Lass, 2015). Interestingly, early biochemical studies showed that only 1,2-DAG (not 1,3-DAG or 2,3-DAG) has the ability to activate PKC (Boni and Rando, 1985; Hannun et al., 1986) and, hence, impede insulin signaling and contribute to insulin resistance. However, no such data exist specifically for intact cells systems, let alone for skeletal muscle. To date, no human studies have reported on the specific positions of the fatty acid moieties on the glycerol backbone. Some indication of the importance of fatty acid position comes from a study using hormone-sensitive lipase (HSL) knock-out mice. After a treadmill exercise, knock-out mice showed increased insulin-stimulated glucose uptake along with higher (rather than lower) total DAG levels compared with wild-type mice. DAG subtype analysis revealed that 1,3-DAG increased after exercise whereas 1,2-DAG decreased after exercise (Serup et al., 2016).

Ceramides

Ceramides have a central role in sphingolipid metabolism given that they are a precursor for complex sphingolipids. They can be produced *de novo* from serine and saturated fatty acids, mainly palmitate (C16), and from sphingomyelin hydrolysis, or they are reformed via the salvage pathway from complex sphingolipids (Bellini et al., 2015). As *de novo* synthesis of ceramides requires fatty acids such as palmitate, the rate of ceramide synthesis is dependent on the availability of these fatty acids. In this respect, a diet high in saturated fat can influence intramyocellular ceramide accumulation (Blachnio-Zabielska et al., 2010). As with DAGs, the mechanism by which ceramides are thought to induce insulin resistance is mainly derived from *in vitro* and animal studies. Ceramides may inhibit insulin-stimulated glucose uptake via the inhibition of Akt/protein kinase B (PKB) activation. Two routes have been proposed, namely the activation of phosphatase 2A by ceramides, leading to dephosphorylation of Akt/PKB (Stratford et al., 2004), and by activation of the atypical PKC isoform PKC ζ , which binds, phosphorylates and sequesters Akt/PKB in caveolin-enriched microdomains (Fox et al., 2007; Powell et al., 2003).

Several studies in humans support a relationship between ceramide accumulation and insulin resistance. An inverse relationship between total muscle ceramide content and insulin sensitivity was found in lean, insulin-sensitive subjects (Straczkowski et al., 2004). In addition, ceramide levels increased in the muscle of obese subjects (Adams et al., 2004; Coen et al., 2010; Straczkowski et al., 2007) and the offspring of type 2 diabetes patients (Straczkowski et al., 2007) compared with lean subjects. Two studies have shown that participation in a moderate exercise training program can reduce the total ceramide levels of obese sedentary subjects and improve insulin sensitivity (Bruce et al., 2006; Dube et al., 2008).

However, other studies have not reported a link between ceramides and insulin resistance. Upon examining muscle ceramide content in patients with a wide range of insulin sensitivity, only a weak borderline significant correlation between ceramide levels and insulin sensitivity was observed (Skovbro et al., 2008). Acute induction of insulin resistance by lipid infusion was observed without changes in ceramide levels (Hoeks et al., 2012). In addition, several intervention studies, including weight loss and/or exercise training, failed to show any changes in muscle ceramides (Dube et al., 2011; Helge et al., 2011; Johnson et al., 2016; Sogaard et al., 2016).

Rather than total ceramide content, specific ceramide species may be responsible for the effect on insulin sensitivity. Thus, although the total muscle ceramide levels of type 2 diabetes patients, obese individuals and endurance-trained athletes are the same, C18:0 ceramide has been reported to be higher in the muscle of type 2 diabetes patients relative to that of obese individuals and endurance-trained athletes. Moreover, other C18:0 sphingolipids (dihydroceramide and glucosylceramide) have been associated with insulin resistance (Bergman et al., 2016). The association between C18:0 ceramides and insulin resistance was extended by a study showing associations between insulin resistance and ceramide di18:1/18:0 and C18:0 sphingolipids dihexosylceramide and trihexosylceramide (Tonks et al., 2016). Furthermore, ceramides C14:0, C16:0 and C18:0 were increased in obese insulin-resistant women compared with those of obese insulin-sensitive women (Coen et al., 2010). In addition to these long-chain ceramides, very long-chain ceramides [20:0, 20:4 (de la Maza et al., 2015), c20:1 and C22:1 (Thrush et al., 2009)] have also been associated with obesity and glucose intolerance. As well as the ceramides subtype, the subcellular location of ceramides might also be of importance. For example, ceramides have been implicated in mitochondrial membranes and contribute to mitochondrial dysfunction (Di Paola et al., 2000; Yu et al., 2007), which indirectly links ceramides to insulin resistance. However, there are no data on the subcellular location of ceramides in human skeletal muscle.

In general, the evidence for a direct role of diacylglycerols and ceramides in human muscle insulin resistance is inconsistent at best. With recent advances in lipidomics, it has become evident that specific lipid intermediates may be important, which may explain inconsistencies between earlier studies looking at total DAG or ceramides. In addition, most data, particularly in human studies, are correlative and causality is difficult to prove. Moreover, the metabolism of lipid intermediates is highly intertwined, which complicates the identification of 'malign' insulin-desensitizing lipid moieties. Besides this, the role of specific lipid intermediates might be gender-, situation-, intervention- or even (epi)genetic-specific (Kitessa and Abeywardena, 2016).

Location of lipids

Another complicating factor for the interpretation of changes in insulin-desensitizing bioactive lipids is the observation that the subcellular location of these lipid moieties appears to be of relevance. Skeletal muscle contains glycolytic non-oxidative muscle fibers (type II fibers) and oxidative muscle fibers that are well equipped for lipid oxidation (type I fibers). The fiber type specificity of LD distribution (with more LDs being present in type I fibers) can be readily observed in histology (Fig. 3A–C). Indeed, skeletal muscle fiber typology is related to lipid content, oxidative capacity (He et al., 2001) and insulin sensitivity (Albers et al., 2015). Although several studies in rodents have indicated

differences in the lipid storage pattern between oxidative and glycolytic muscle (Holloway et al., 2014; Lally et al., 2012), human studies looking at fiber type-specific IMCL storage and its relation to insulin sensitivity are limited. Two studies showed larger and fewer LDs in type I fibers of type 2 diabetes patients than in lean sedentary individuals and endurance-trained athletes (He et al., 2004; van Loon et al., 2004). In addition, IMCL content was higher in type I fibers in insulin-resistant subjects than in insulin-sensitive obese subjects. Moreover, IMCL content correlated negatively with insulin sensitivity only in type I fibers (Coen et al., 2010). Furthermore, the subcellular location of LDs [in the intermyofibrillar (IMF) space and in the vicinity of mitochondria or in the subsarcolemmal (SS) space, Fig. 3D] may be a determinant of insulin sensitivity.

The importance of subcellular location has already been illustrated above by the possible differences in the subcellular location of DAGs. Other studies also hint at the importance of subcellular LD location with respect to insulin sensitivity. Type 2 diabetes patients have been shown to have increased IMCL content, compared with obese and endurance-trained subjects, in the SS region. The presence of SS LDs negatively correlated with insulin resistance in type 2 diabetes patients (Nielsen et al., 2010). Furthermore, a training intervention decreased LD content in the SS area in lean, obese (Li et al., 2014; Samjoo et al., 2013) and type 2 diabetes subjects (Nielsen et al., 2010). However, exactly how LD location affects insulin sensitivity remains elusive.

The location of LDs is also a determinant of the interaction of LDs with other cellular organelles (such as the ER). Thus, LD location also affects subcellular lipid handling and metabolism. For organelle interactions to occur, organelle motility is essential to bring organelles into close proximity and direct membrane connections are warranted. ER–LD contacts may facilitate the transfer of bioactive lipids and of proteins with metabolic or signaling functions (Barbosa et al., 2015). As an example, several TAG synthetic enzymes such as diacylglycerol-*O*-acyltransferase 2 are bound to the ER membrane and move to the LD to promote TAG synthesis (Barbosa et al., 2015; Kuerschner et al., 2008). Although data on the motility of LDs in human skeletal muscle are scarce, LD motility is anticipated to facilitate fluctuations in energy supply and demand, e.g. during exercise (Badin et al., 2013).

Classically, lipid composition analysis entails tissue lipid extraction, followed by conventional mass spectrometry. Unavoidably, this procedure results in the loss of all spatial information. However, several techniques are emerging that enable both chemical and spatial information to be analyzed. Advances in both vibrational microscopy, e.g. coherent anti-stokes Raman scattering, and imaging mass spectrometry, e.g. secondary ion mass spectrometry, could enable the role of specific lipid species in their subcellular localization to be studied in the near future (Daemen et al., 2016).

Dietary effects on lipid droplets and intramyocellular lipid content

A sustained positive energy balance (e.g. via excess caloric intake) results in an individual becoming overweight and/or obese. Obesity development and the concomitant expansion of white adipose tissue mass promotes the storage of fat in a variety of tissues, including skeletal muscle. Thus, elevated IMCL content is commonly observed in the obese state. Somewhat counterintuitively, prolonged fasting or alternate day fasting also seems to augment IMCL content. The following section aims to outline how dietary interventions can affect IMCL content.

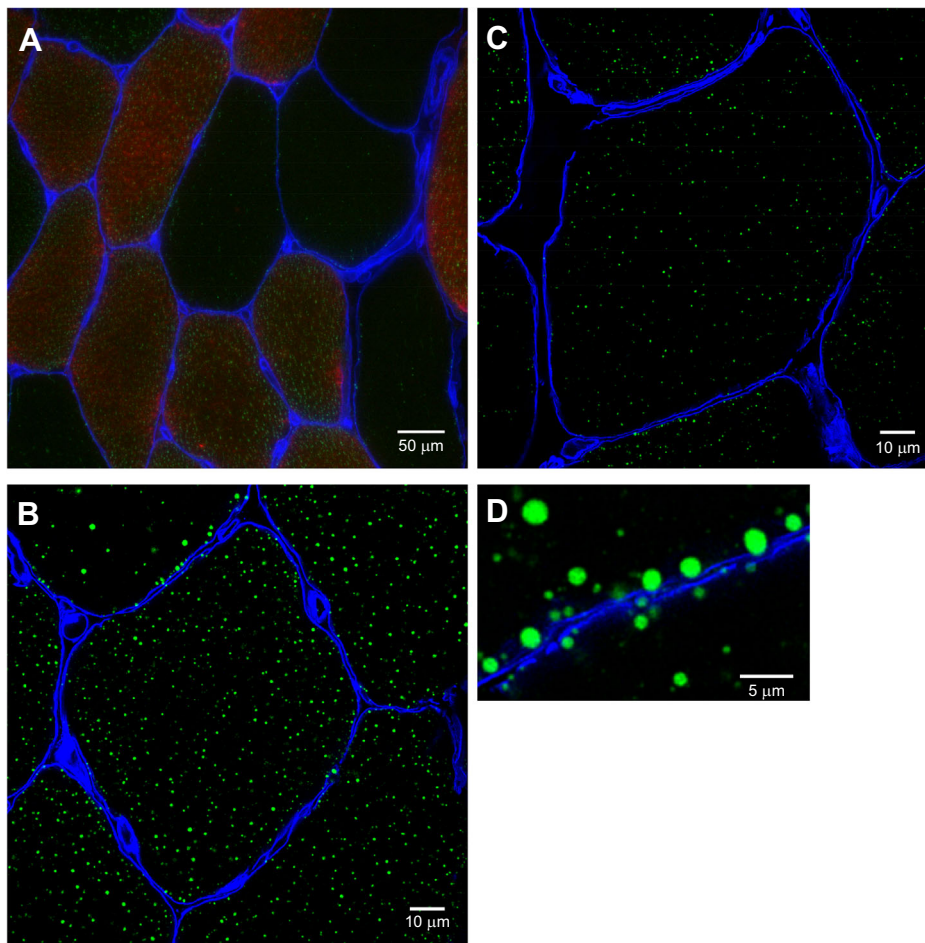


Fig. 3. Distribution of lipid droplets (LDs) in skeletal muscle. (A) Muscle lipid storage differs between muscle fiber types. Widefield microscopy image of human skeletal muscle fibers (vastus lateralis muscle) in cross-sectional view. The basement membrane protein laminin is shown in blue (secondary antibody labeled with Alexa Fluor 405), LDs are stained green (BODIPY 493/503), and type I fibers are shown in red (primary antibody against myosin heavy chain type 1; secondary antibody labeled with Alexa Fluor 555). (B and C) Confocal images of human muscle fibers in cross-sectional view. In human skeletal muscle, LDs are more abundant in type I muscle fibers (B) than in type II fibers (C). The staining procedures were similar to the staining described for 3A. (D) Subcellularly, LDs are dispersed alongside the contractile elements but are also in the vicinity of the muscle cell membrane.

High-fat diet and lipid infusion

A hypercaloric high-fat diet (HFD) can promote IMCL content in resting muscle within days (Bachmann et al., 2001; Larson-Meyer et al., 2008; Sakurai et al., 2011; Schrauwen-Hinderling et al., 2005; Van Proeyen et al., 2011; Zderic et al., 2004; Zehnder et al., 2006). If provided after an exercise session, a HFD rapidly restores the exercise-mediated drop in IMCL (Decombaz et al., 2001; Larson-Meyer et al., 2002; van Loon et al., 2003b). Thus, the consumption of a HFD promotes myocellular fat deposition, even during periods that are too short to significantly affect body mass.

Nevertheless, it is interesting to examine models in which IMCL content was acutely (within hours) elevated by the infusion of lipid emulsions. These models compared the effects of intravenous lipid emulsion infusions on IMCL content with those of saline or glycerol infusions. Lipid emulsions were co-infused with or without heparin to stimulate endothelial lipoprotein lipase (LPL) activity. Typically, this results in elevated plasma FFA levels up to the millimolar range (compared with 200–300 μmol under control conditions). These studies have robustly and consistently shown that infusion of lipid emulsions results in a significant increase in IMCL content (Bachmann et al., 2001; Brechtel et al., 2001a; Brehm et al., 2010; Hoeks et al., 2012; Lee et al., 2013) in the type I and type II muscle fibers of healthy male individuals (Hoeks et al., 2012) and highly trained athletes (Phielix et al., 2012). In these lipid emulsion infusion studies, the increase in IMCL content was paralleled by a decrease in insulin-stimulated glucose uptake in muscle. Interestingly, in trained athletes, lipid-induced insulin resistance was ameliorated (Phielix et al., 2012) even though IMCL content

was significantly increased. Studies involving a high-protein/low-carbohydrate diet did not alter plasma FFA levels and failed to affect IMCL content or whole-body insulin sensitivity (Green et al., 2010).

Thus, elevating circulatory FFA levels in situations of a positive energy balance seems to promote TAG storage in skeletal muscle and compromise insulin sensitivity in men with normal to low oxidative capacity as well as in athletes. Cycling exercise for 3 h in the fasted state reduces the IMCL content in the active muscles and is paralleled by increased plasma FFA levels and by increased IMCL content in the inactive muscle (Schrauwen-Hinderling et al., 2003b). This indicates that IMCL not only represents a very mobile lipid pool but also supports the notion that elevated plasma FFA levels may drive IMCL content.

Calorie restriction and diet-induced weight loss

Calorie restriction is an effective non-pharmaceutical intervention to improve life span and age-related diseases in various organisms. In rodents, a 30–50% reduction in calorie intake can lead to improved health (Carmona and Michan, 2016; Guarente, 2006). The influence of calorie reduction or weight loss interventions on IMCL has been studied in relation to insulin sensitivity in several populations.

Overweight and obese people subjected to a diet-induced weight loss (DIWL) intervention (16 weeks) showed a drop in IMCL and a parallel improvement in insulin sensitivity (Toledo et al., 2008). In another study with a similar design, the overweight individuals not only showed a reduction in total IMCL but also a parallel drop in

DAG content and in specific ceramides (although the total ceramide level remained unchanged) (Dube et al., 2011). These data, along with a study showing similar effects upon DIWL (Lara-Castro et al., 2008), indicate that DIWL potentially reduces IMCL content and affects its composition. These changes appear to occur in a muscle fiber type-independent fashion (Gray et al., 2003) and occur in parallel to a substantial improvement in insulin sensitivity (Larson-Meyer et al., 2006).

Caloric restriction not only reduces IMCL content in healthy people. Insulin-resistant offspring of patients with type 2 diabetes on a hypocaloric diet for 9 weeks also showed a reduced total IMCL content (owing to reductions in LD size, not number) and improved insulin sensitivity (Petersen et al., 2012). In patients with type 2 diabetes, a 6-day very low-calorie diet reduced IMCL content by 53%, whereas insulin-stimulated glucose uptake increased by 9% (Lara-Castro et al., 2008). In obese patients with type 2 diabetes, a very low-calorie diet with targeted weight loss reduced IMCL and improved insulin sensitivity (Jazet et al., 2008). In morbidly obese patients eligible for gastric bypass surgery, the intervention reduced their body mass index by 43% with a concomitant drop in IMCL of 30% and improvements in glucose homeostasis (Gray et al., 2003). In most of these studies, body mass also decreased significantly. Thus, it is premature to conclude that the improvement in glucose homeostasis observed after caloric restriction entirely originates from a reduction in IMCL.

Fasting

In models of caloric restriction (e.g. alternate day fasting, very low-calorie diet or gastric bypass), food is still consumed and, hence, there are transient cycles of carbohydrate and insulin peaks. During prolonged fasting, these oscillations are absent and the body relies on endogenous energy stores. Thus, hydrolysis of TAG in adipose tissue results in elevated plasma FFA levels. This elevation of plasma FFA levels is probably responsible for the observation that (in contrast to caloric restriction) prolonged fasting (>48 h) augments IMCL levels in healthy individuals (Browning et al., 2012; Gemmink et al., 2016; Green et al., 2010; Hoeks et al., 2010; Stannard et al., 2002; Wietek et al., 2004). Shorter episodes of fasting (<12 h), requiring only a modest activation of adipose tissue lipolysis, did not result in increased IMCL levels (Machann et al., 2011). This apparent discrepancy is explained by lower plasma FFA levels in the early phase of fasting (Browning et al., 2012; Green et al., 2010; Hoeks et al., 2010; Stannard et al., 2002; Wietek et al., 2004). Recently, we showed that following prolonged (62 h) fasting, when plasma FFA levels were substantially elevated, LDs coated with PLIN5 significantly increased in size compared with LDs that were not coated with PLIN5 (Gemmink et al., 2016). We also observed that participants that showed the greatest increase in LD size were least insulin resistant upon fasting (Gemmink et al., 2016). Thus, prolonged fasting results in elevated plasma FFA levels and augments IMCL content; however, if IMCL is stored in PLIN5-coated LDs, the increase in IMCL does not compromise insulin sensitivity.

Other dietary interventions

Most commonly, the macronutrient composition of the diet is changed to modulate IMCL levels (changes in total caloric intake, or changes in the fat component of the diet). Alternatively, potentially bioactive food compounds can be selectively administered. We previously observed that obese normoglycemic patients administered with polyphenol resveratrol for 30 days showed a significant increase in IMCL content (Timmers et al.,

2011). The increase in IMCL was most pronounced in type I muscle fibers, and a parallel increase in the protein content of the LD coat protein PLIN5 and a decrease in hepatic fat content were also observed (Timmers et al., 2011). Similar observations were made in a follow-up study in patients with type 2 diabetes (Timmers et al., 2016). How resveratrol exerts these effects is unknown. Intriguingly, the low hepatic fat content and elevated IMCL levels in type I muscle fibers, along with the induction of PLIN5, mimic an exercise signature for LD remodeling (Koves et al., 2013), suggesting that resveratrol affects IMCL levels in a manner that is commonly associated with health benefits.

Thus, there is a growing body of literature that recognizes the importance of nutrition in LD synthesis and the modulation of the total IMCL pool in muscle. However, most of the research on the effects of diet and IMCL have focused on static measures of IMCL and have not taken into account factors involved in the dynamic nature of LDs. In a study involving a combined exercise training and dietary intervention, LD size was reduced (He et al., 2004). This drop in LD size correlated with an improvement in insulin sensitivity (He et al., 2004), suggesting that the size of LDs somehow affects insulin sensitivity. How dietary interventions affect LD dynamics and their coating proteins in relation to insulin sensitivity remains elusive.

Effects of acute exercise on IMCL

After acute exercise at moderate intensity, most studies report not only a decrease in IMCL in healthy and sedentary subjects (Bucher et al., 2014; Decombaz et al., 2001; Egger et al., 2013; Schrauwen-Hinderling et al., 2003a; White et al., 2003) but also in endurance-trained athletes (Brechtel et al., 2001b; De Bock et al., 2007; Decombaz et al., 2001; Johnson et al., 2003; Krssak et al., 2000; Rico-Sanz et al., 2000; van Loon et al., 2003a; van Loon et al., 2003b; Vermathen et al., 2012; Zehnder et al., 2005). Importantly, the utilization of IMCL during exercise depends upon the duration and intensity of exercise and the training status of the participant. All these factors affect the degree of stimulation of lipolysis of IMCL and fat oxidative capacity. During prolonged, moderate-intensity endurance exercise, fat oxidation is maximal (Kiens et al., 2011). However, even at maximal rates of fat oxidation, there is still a substantial contribution of plasma-derived fatty acids to total fat oxidation (van Loon et al., 2003a). The relative contribution of IMCL and plasma sources to lipid oxidation during exercise remains a subject of investigation and may vary with nutritional and training status and exercise intensity.

With respect to dietary status, subjects showed a reduction in IMCL after a 2 h cycling exercise at 75% $\dot{V}_{O_{2,max}}$ only under fasting conditions and not when fed carbohydrates. Moreover, this decrease was only seen in type I fibers (De Bock et al., 2005). Another study examined the effect on subjects of 48 h of high- or low-dietary carbohydrate before exercise and found higher net IMCL usage in the low-carbohydrate condition, mainly owing to higher IMCL levels before exercise started (Johnson et al., 2003). Furthermore, it was shown that a HFD increased net IMCL degradation during exercise, an effect that was not altered by an exercise training program (Van Proeyen et al., 2011), again possibly owing to increased basal IMCL values. Indeed, several studies indicate that it may be basal IMCL values that are the determinant of IMCL usage during exercise (Steffensen et al., 2002; Vermathen et al., 2012; Zehnder et al., 2006), so higher IMCL levels at the onset of exercise may result in higher IMCL utilization. For example, athletes given 1.5 days of lipid supplementation before an acute cycling exercise showed increased pre-exercise IMCL levels; the IMCL

depletion during exercise was strongly correlated with these pre-exercise IMCL levels (Zehnder et al., 2006). Furthermore, training status can influence the amount of IMCL used during exercise: for example, trained subjects showed higher levels of fat oxidation during 30 min of exercise at 75–80% of $\dot{V}_{O_{2,max}}$ than untrained subjects, which could not be completely explained by higher usage of plasma FFA (Coggan et al., 2000). This additional lipid substrate must therefore come from intramuscular fat stores, presumably IMCL.

Although the effects of an acute exercise bout on total IMCL content have been extensively studied, the effect of exercise on LD dynamics and their coating proteins during acute exercise in humans has not yet been studied in great detail. One study showed that 60 min of cycling at 60% of $\dot{V}_{O_{2,max}}$ did not alter the interaction of PLIN5, ATGL and its co-activator with LDs (Mason et al., 2014). Another study showed that LDs coated with PLIN5 were depleted during exercise, whereas LDs without PLIN5 were not (Shepherd et al., 2012). This selective use was maintained by a 6-week sprint interval training or endurance training intervention (Shepherd et al., 2012). Similarly, PLIN2-coated LDs were used during acute exercise in contrast to LDs devoid of PLIN2 (Shepherd et al., 2012, 2013). In contrast to PLIN5, the preference for utilizing PLIN2-positive LDs over LDs devoid of PLIN2 faded upon 6-week sprint interval training, as well as after the endurance training program (Shepherd et al., 2013).

Thus, during acute physical exercise, IMCL is used to fuel the increased energy demand of exercise and, hence, IMCL levels drop. The level of IMCL utilization during acute exercise depends on nutritional status, training status, absolute and relative exercise intensity and the IMCL levels at the onset of exercise. More detailed analyses of LDs during acute exercise indicate that coating LDs with PLIN2 or PLIN5 also affects the utilization of IMCL during exercise.

Long-term exercise training effects

In most populations, chronic exercise training augments IMCL storage (Dube et al., 2008; Phillips et al., 1996; Pruchnic et al., 2004; Schrauwen-Hinderling et al., 2003a; Shepherd et al., 2013; Tarnopolsky et al., 2007). This appears to be in line with the ‘athlete’s paradox’ and the notion that IMCL fuels exercise of moderate intensity. However, in type 2 diabetes patients and impaired glucose-tolerant subjects, the effects of exercise training on IMCL content are less consistent. Although some studies report augmented IMCL storage upon exercise training (Meex et al., 2010; Peters et al., 2012), others have reported no change (Jonker et al., 2013) or a reduction (Bruce et al., 2004; Solomon et al., 2008). Although these apparent differences may originate from different training protocols, they might also be a reflection of the limited exercise capacity of patients with type 2 diabetes (Stephens and Sparks, 2015). The lower exercise capacity of type 2 diabetes patients blunts the reliance on IMCL as a substrate (Ghanassia et al., 2006) and, hence, may not suffice as a trigger to augment IMCL content. However, even in the absence of reductions in IMCL, training improves insulin sensitivity (Bruce et al., 2004; Dube et al., 2008; Nielsen et al., 2010; Solomon et al., 2008).

The insulin-sensitizing effect of exercise training does not require changes in total IMCL content. The emerging view is that alterations in LD morphology, coating LDs with proteins of the perilipin family, the subcellular localization of the LD and the LD–mitochondrial interaction are more directly related to the insulin-sensitizing effect of exercise than to total IMCL content. In lean men and women, 7 weeks of endurance exercise training increased

IMCL content owing to an increase in the number of LDs rather than an increase in LD size (Tarnopolsky et al., 2007). This increase in LD number rather than LD size was also observed in another study (Shepherd et al., 2013). Furthermore, several studies have observed an improvement in the contact between LDs and mitochondria (Devries et al., 2013; Tarnopolsky et al., 2007). With regard to the coating of LDs, the expression of PLIN2 and PLIN5 increases upon exercise training (Gjelstad et al., 2012; Peters et al., 2012; Shaw et al., 2012; Shepherd et al., 2013); one study also reported an increase in the expression of PLIN3 (Gjelstad et al., 2012). However, the fraction of PLIN2 and PLIN5 co-localizing with IMCL remained unchanged (Shepherd et al., 2013).

Exercise training also appears to lead to a remodeling of cellular lipid distribution. In both obese non-diabetic and lean women, the redistribution of lipid from SS to IMF was observed after training, alongside an increased LD–mitochondrial interaction, together most likely favoring lipid oxidation during exercise (Devries et al., 2013). In overweight dysglycemic and normal weight control subjects, SS LDs, as opposed to IMF LDs, decreased both in size and number (Bucher et al., 2014). Moreover, type 2 diabetes patients showed higher baseline SS lipid levels compared with obese controls and endurance-trained athletes. The training intervention reduced the SS lipid levels by half in these diabetic patients, reaching the levels of non-diabetic controls (Nielsen et al., 2010).

Although the effect of acute exercise and exercise training on total IMCL levels has been extensively studied, further studies are needed to elucidate the role of proteins involved in LD dynamics on IMCL turnover during exercise, and to explore whether these processes are affected in the insulin-resistant state.

Conclusions and future perspectives

In contrast to what has been the dogma for years, IMCL represents a highly dynamic pool of lipids that can readily adjust to alterations in energy supply and demand. A sustained positive energy balance promotes IMCL, whereas intermittent periods of caloric restriction or short-term fasting reduces the IMCL pool size. In general, high IMCL levels are associated with impeded insulin sensitivity; however, this is not always the case. If, for example, IMCL is elevated because of exercise training, the insulin desensitizing effect is not apparent. In the trained state, the IMCL pool undergoes continuous cycles of depletion and repletion and seems to have more intimate interaction with organelles such as mitochondria and the sarcoplasmic reticulum. These adaptive responses enable rapid esterification of fatty acids entering the myocyte and rapid oxidation of fatty acids released from the LD upon lipase activity. To maintain the dynamic nature of LDs, an orchestrated interaction of lipases such as HSL and ATGL, their co-activators, and the myocellular members of the PLIN family (PLIN2, PLIN3 and PLIN5) is essential. We are just beginning to understand how these proteins respond to dietary and exercise interventions and how they affect LD dynamics. However, to date, the results seem to indicate that by modulating these proteins either by diet, nutritional compounds or exercise, the LD phenotype can be affected such that expansion of IMCL can occur without negative effects on cellular function and/or insulin sensitivity. Clearly, more work needs to be done to determine how and why alterations in proteins involved in LD dynamics affect LD and cellular function and under which conditions these changes can be beneficial to human health.

Competing interests

The authors declare no competing or financial interests.

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