Trends in Endocrinology & Metabolism



## Review

# Reassessing the Role of Diacylglycerols in Insulin Resistance

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Skeletal muscle (SM) insulin resistance (IR) plays an important role in the burden of obesity, particularly because it leads to glucose intolerance and type 2 diabetes. Among the mechanisms thought to link IR to obesity is the accumulation, in muscle cells, of different lipid metabolites. Diacylglycerols (DAGs) are subject of particular attention due to reported interactions with the insulin signaling cascade. Given that SM accounts for the majority of insulin-stimulated glucose uptake, this review integrates recent observational and mechanistic works with the sole focus on questioning the role of DAGs in SM IR. Particular attention is given to the subcellular distributions and specific structures of DAGs, highlighting future research directions towards reaching a consensus on the mechanistic role played by DAGs.

# Diacylglycerol and Skeletal Muscle Insulin Resistance: Historical Perspective in the Context of Disease

Obesity and type 2 diabetes (T2D) have reached epidemic proportions [1,2]. **Insulin resistance** (IR, see Glossary) is strongly linked to obesity and is considered to be the cornerstone of T2D [3,4]. IR damages multiple organs including liver, muscle, visceral adipose tissue, and others. At its most severe stage, IR can lead to pancreatic  $\beta$ -cell failure, which results in cessation of insulin production [5,6]. Skeletal muscle (SM) accounts for ~60–80% of glucose uptake in response to insulin [7]. The contribution of SM IR to whole-body IR is supported by a large body of literature [8–11] and by mathematical models [12].

The lipotoxicity theory explains how dysfunctional mitochondria, particularly at the level of the β-oxidation machinery, can be overwhelmed by an excess of **fatty acids** (FAs), leading to the accumulation of FA metabolites in non-adipose tissues (i.e., ectopic fat) [13,14]. Ectopic fat accumulation is seen as a hallmark of lipotoxicity and is thought to be one of the mechanisms linking obesity to IR [15]. The accumulation of intramuscular triglycerides (IMTGs) was considered to be a major player in the development of SM IR in humans [16-19]. However, an exception to this concept is the observation that endurance-trained athletes display paradoxically high levels of insulin sensitivity (IS), despite having a greater content of IMTGs [20,21]. Over the past two decades evidence has shown that IMTGs per se are not incriminated in IR, and that other bioactive lipid intermediates are likely to be involved. Although some evidence currently supports ceramides as contributors [22], the involvement of diacylglycerols (DAGs) in the development of SM IR remains under investigation. We discuss here literature published since our initial review on the same topic in 2012 [23]. Particular attention has been given to human data that incorporate the different stereoisomers and moieties of DAGs, their subcellular localizations, and their relationship to SM IR. Furthermore, animal and cell culture experiments will be discussed to provide further insight into the current status of the field.

### DAG Structure, Pathways, and Mechanisms

DAGs originate either from the hydrolysis of triacylglycerols (TAGs)/phospholipids or appear as a lipid intermediate during the *de novo* synthesis of TAGs from glycerol-3-phosphate, the building

#### Highlights

Insulin resistance (IR), a common feature in obesity, is key to the development of type 2 diabetes (T2D). Lipotoxicity, also known as the lipid metabolite theory, is one of the hypotheses to explain the molecular mechanisms by which obesity leads to IR.

This theory assumes that the accumulation of specific toxic lipid metabolites disrupts the normal functioning of cellular cascades. Among these toxic metabolites, some have been proven to impact specific organs, for example ceramides can lead to liver and skeletal muscle (SM) IR.

Observational and mechanistic studies are so far inconclusive regarding the role of diacylglycerols (DAGs) in inducing IR in SM. Although past research has mostly focused on measuring total DAG levels in whole-muscle lysates, methodological advances now allow the investigation of subcellular localizations and comparisons among different DAG moieties as well as DAG stereoisomers. This level of granularity is necessary to evaluate the DAG-induced SM IR hypothesis while opening new perspectives in the field.

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block of all phospholipids [24]. DAGs can occur in three different stereoisomers: *sn*-1,3 DAG, *sn*-1,2 DAG and *sn*-2,3 DAG (Figure 1). The stereospecific numbering (*sn*) designates the position of the fatty ester on the glycerol backbone. In addition to these structural isomers, 1,2 and 2,3 DAGs have spatial isomerism and are known as **enantiomers** [25]. For this review we use the abbreviated form (without *sn*). Different DAG stereoisomers have distinctive biophysiological characteristics [26]. By 2012, the common notion was that 1,3 and 2,3 DAGs were metabolically inactive [27] and that only 1,2 DAGs were capable of activating **protein kinase C** (PKC) isoforms [28]. Different DAG stereoisomers tend to have specific localizations within the cell. TAG lipolysis-derived 1,3 and 2,3 DAGs are commonly located in **lipid droplets** (LDs), whereas 1,2 DAGs derived from FA esterification and/or *de novo* synthesized tend to accumulate in membranes [29], namely in the sarcolemma and intracellular membranes such as the endoplasmic reticulum (ER) and mitochondria.

Besides their stereospecificity, DAG species differ one from another by the different FA species esterified to the glycerol backbone. In this review we use the terms 'DAG species' or 'DAG moieties' to designate specific DAG molecules that differ from each other by their FA chain length or degree of saturation. By the same logic, the term 'total DAG' is used to designate the complete set of DAG molecules measured in a sample as opposed to specific DAG isomers or species.

Multiple systems for naming FAs are used in the literature. For coherence, in the present review we only use lipid numbers. To ease comparison with other papers, the lipid numbers and common names of the most frequent FAs found in DAG are: C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C20:4 (arachidonic acid), C24:0 (lignoceric acid), and C24:1 (nervonic acid).





#### Glossary

Acipimox: a pharmacological compound that is used to reduce triglyceride levels and increase HDL cholesterol. It is a niacin derivative that acts on the niacin receptor 1 and inhibits the enzyme triglyceride lipase.

#### Adipose triglyceride lipase (ATGL): also known as patatin-like

phospholipase domain-containing protein 2 (PNPLA2). ATGL is one of the major enzymes involved in intracellular degradation of triglycerides. It catalyzes the initial step in triglyceride hydrolysis in adipocyte and

non-adipocyte lipid droplets (LDs) by interaction with CGI-58.

Akt: also known as protein kinase B (PKB). Akt is, a serine/threonine-specific protein kinase belonging to the insulin signaling cascade. The ratio of phosphorylated (p-)Akt to Akt is used to assess Akt activation.

**Diacylglycerol (DAG):** consists of a glycerol backbone linked to two fatty acyl chains. DAGs can exist in three different stereoisomers.

**Enantiomers:** two stereoisomers that are the mirror image of each other and are not superimposable.

Etomoxir: a pharmacological compound used to inhibit fat oxidation. It is an irreversible inhibitor of carnitine palmitoyltransferase 1 on the outer mitochondrial membrane. It prevents the formation of acylcarnitines and thus the transport of FAs from the cytosol to the mitochondria.

Fatty acid (FA): a carboxylic acid with a long aliphatic chain that can either be saturated or unsaturated.

Glucose tolerance test (GTT): an experimental procedure to assess the response to an oral, intravenous or intraperitoneal load of glucose.

HOMA-IR (homeostatic model assessment of insulin resistance): a mathematical model to assess insulin resistance. Using fasting glucose and insulin, it allows to estimate  $\beta$ -cell insulin secretion and insulin resistance.

#### Hyperinsulinic euglycemic (HE)

**clamp:** a method to assess inulin sensitivity. The technique is based on the principle of 'clamping' plasma glucose concentration while giving a continuous and constant infusion of insulin and maintaining euglycemia through a variable infusion of exogenous glucose. The assumption is that, at steady-state, the rate of glucose infused is equal to the rate of glucose uptaken. This can be



DAGs have been incriminated as being metabolically toxic due to the activation of PKC isoforms which in turn disrupt the insulin signaling cascade (Box 1) [30–32]. For almost four decades the degree of saturation and/or carbon chain length of DAG FAs were thought to be key components of DAG-induced IR [33,34]. Examples encompass studies showing that DAGs containing one FA chain of C18:1 were higher in obesity and T2D [35], that C16:0 was higher in insulin-resistant men [36], or that C16:1 and C22:6 were higher in lean women [37]. Research in the field has evolved with numerous studies since 2012 investigating the different stereoisomers and moieties of DAG in the context of SM IR.

#### Human Studies Showing No Association between SM DAGs and IR

Six cross-sectional studies in the past 6 years did not detect association between DAGs and IR (Table 1). Coen and colleagues reported no difference in total SM DAG content between lean and obese insulin-resistant women [38]. De la Maza and colleagues reported total SM DAGs to be dissociated from obesity, IR and aging [44]. Søgaard and colleagues reported no association between total or several DAG species and IR in different age and training groups [52]. The same group confirmed no differences in IS between sedentary obese younger and older subjects, while pointing to a higher content of total DAGs and particularly of DAGs containing one FA chain of distinct saturated species [53]. Similarly to our previous report on chronically endurance trained athletes [54], Perreault and colleagues confirmed the athlete's paradox with high levels of total DAG in highly insulin sensitive athletes. Further, athlete's total DAG levels were comparable to those of individuals with T2D, who were at the opposite end of the IS scale [51].

Nine intervention studies (Table 1) reported no significant associations between total DAG levels and IR. Liang *et al.* found that pharmacological reduction of circulating free FAs in obese individuals, with or without T2D, caused improvements in insulin signaling without significant change in total DAG content [39], suggesting that DAGs do not play a part in FA-mediated lipotoxicity. Although exercise intervention increased IS, Louche *et al.* reported no change in total DAGs in obese men [41]. Søgaard and colleagues found that total and specific DAG species

#### Box 1. Protein Kinase C (PKC) Isoforms

PKCs are classified into classical, novel and atypical isoforms [88–90]. Classical (or conventional) PKC isoforms include PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II and PKC $\gamma$ . Their activation depends on DAGs and calcium [90,91].

Novel PKC isoforms include PKC $\delta$ , PKC $\epsilon$ , PKC $\eta$  and PKC $\theta$  [83]. The main difference between classical and novel PKCs is that the novel PKCs are calcium-independent. Novel PKCs have a twofold greater affinity for DAGs than do conventional PKCs [92].

Atypical PKC isoforms include PKC $\zeta$ , PKCI and PKC $\lambda$ . These do not require calcium or DAGs and are activated by 3-phosphoinositide-dependent kinase-1-mediated phosphorylation. In this case, the DAGs involved are a byproduct of the process of TAG formation.

Lipid oversupply mediated DAGs accumulation, and subsequent PKC activation, provides one of the main mechanistic explanations for the link between intracellular lipid accumulation and the generation of IR [93]. PKC disrupt the insulin signaling cascade via serine and threonine phosphorylation of the insulin receptor, insulin receptor substrate 1 (IRS-1) and potentially other proteins [82].

1,2-DAGs are potent activators of PKC isoforms [94]. This stereospecificity links PKCs activation to DAGs origin. Lipolytic and lipogenic DAGs are respectively derived from phospholipase C activity at the plasma membrane or are synthesized at the ER membrane as a result of dietary oversupply of lipids [83].

PKC isoforms cross talk in cells, and may be key for the functional integration of signaling networks. These crosstalks between PKC isoforms contribute to their own activation or inhibition, thus bringing another level of complexity to the existing methodological challenges, including state-specific antibodies and assay variations [89].

combined with the use of stable isotope glucose tracer to evaluate endogenous hepatic glucose production.

Insulin receptor substrate 1 (IRS-1): a signaling protein associated with the insulin receptor that acts as a transducer in the insulin-signaling cascade. IRS-1 activation/phosphorylation leads to the interaction with different second messengers (such as

phosphatidylinositol-3-kinase) involved in various pathways mediating the intracellular action of insulin.

**Insulin resistance (IR):** impaired ability of insulin to produce its physiological effects in targeted tissues (SM, fat, liver) usually due to altered insulin signal transduction within the cell. IR can be represented as the opposite of insulin senstivity (IS) or as any reduction of IS.

Insulin tolerance test (ITT): an experimental procedure allowing to assess the response to an intravenous or intraperitoneal load of insulin.

Intramuscular triglycerides (IMTGs): the trigyceride fraction of IMCLs. IMTGs can be measured by

immunohistochemistry using Oil-Red-O that stains neutral lipids. IMTGs are thought to be the most abundant form of lipid within muscle.

Intramyocellular lipids (IMCLs): a general term for fat stored in muscle cells, often used as a synonym for LDs. Lipid droplets (LDs): intracellular organelles consisting of a core of lipids covered by a monolayer of phospholipids and proteins such as perilipins (PLINs) that regulate their storage and use [95]. LDs store INTGs and bioactive lipid intermediates such as

DAGs and ceramides [96]. **Lipotoxicity:** the process by which FAs flow into tissues excessively, overwhelming the β-oxidation machinery and leading to metabolic dvsreoulation.

Matsuda index: an index to evaluate whole-body IS from oral GTT data. Perilipins (PLINs): a family of

lipid-coating proteins that play a vital role in IMTG storage and use.

Protein kinase C (PKC): a family of protein kinase enzymes (Box 1). Very low density lipoproteins

(VLDLs): lipoproteins synthesized in the liver that enable fats and cholesterol to move within the bloodstream.



First author	Year	Design <sup>a</sup>	Subjects <sup>b</sup> ( <i>N</i> ) and gender	Intervention <sup>c</sup> , muscle, and DAG measurement <sup>d</sup>	Results <sup>e,f,g</sup>	Refs
Coen	2013	CS	L (8), OB class I (7), II and III (15), women	Vastus lateralis HPLC-MS/MS	OB class II and III had greater IR (assessed via <b>HOMA-IR</b> ) compared to L and OB class I. Total DAG content and DAG moieties were not different among groups. IR did not correlate with DAGs. FA oxidation was not related to any lipid species but was related to Mito content. DAG species abundances are presented in Table S1.	[38]
Liang	2013	I	L (17), OB (14), OB T2D (12), sedentary women and men	Acipimox 250 mg every 6 h for 8 days. <i>Vastus lateralis</i> Radiolabeling DAG kinase-TLC	OB and T2D had greater IR than L (HOMA-IR and <b>hyperinsulinemic euglycemic clamp</b> - HE clamp). DAG content was similar in all groups at baseline. Acipimox decreased free FAs and improved IS in OB and T2D. Total DAG and ceramide content did not change in response to acipimox.	[39]
Devries	2013	Ι	L (12), OB (11), sedentary women	E 12 week ET <i>Vastus lateralis</i> Radiolabeling DAG kinase-TLC	OB had greater IR than L (HOMA-IR). DAG content was not different between groups at baseline. ET had no effect on total DAGs and IR. ET decreased levels of <b>intramyocellular lipids</b> (IMCLs) in the subsarcolemmal region, increased intermyofibrillar IMCL and increased mito content in both subcellular regions.	[40]
Louche	2013	I	OB men (10)	E 8 week ET <i>Vastus lateralis</i> Gas-LC	ET improved whole-body aerobic capacity but did not influence glucose tolerance (assessed by oral <b>glucose</b> <b>tolerance test</b> - GTT), or plasma lipids. ET reduced IMTG, improved FA oxidation and lipases content, but did not change total DAG content.	[41]
Chow	2014	Ι	L sedentary (13), A (15), women and men	LI Vastus lateralis LC-MS	A had higher IS than L. Baseline total TAG, total DAG, and saturated DAG were not different between groups. Ll produced similar elevations of free FAs and declines of IS (HE clamp) in both groups. Ll increased total DAGs and DAGs containing C18:1, C18:2, and C18:3 in L but not in A.	[42]
Hussey	2014	I	Sedentary L NGT (12), women and men	Ll Vastus lateralis LC-MS/MS	LI reduced IS (HE clamp and HOMA IR) and p-IRS-1 <sup>Tyr612</sup> without affecting total DAGs. LI modified two DAG species in opposite directions: C14:0-C18:1 and C18:0-C20:4 (text and graphs show different directions, Table S1).	[43]
De la Maza	2015	CS	Healthy sedentary males (56)	Anterior abdominal oblique LC-MS/MS	Total DAGs did not relate to obesity, IR, or age. DAG species were not different when subjects were stratified by abdominal adiposity. DAG species abundance is presented in Table S1.	[44]
Søgaard	2016	1	Sedentary L (16), T2D offspring (19), women and men	E 10 week ET <i>Vastus lateralis</i> UPLC-MS	IS (HE clamp) was higher in L compared to offspring despite similar total DAGs or DAG species. IS and total DAGs were not related at baseline. IS was improved in both groups after ET without any change in the levels of total DAGs or DAG species. Changes in IS with intervention were not related to changes in total DAG content. PKC0 or p-PKC0 <sup>Ser676</sup> protein expression were similar in both groups before or after ET. DAG species abundance is presented in Table S1.	[45]
Goossens	2016	CS	IFG (12), IGT (14), women and men	Vastus lateralis TLC	IGT had lower IS than IFG (HE clamp). IGT had higher TAG and lower DAG contents than IFG. IGT increased SM <b>very low density lipoprotein</b> (VLDL)-TAG extraction and reduced lipid turnover of saturated FAs in response to a high-fat meal compared to IFG.	[46]

#### Table 1. Studies Showing No Associations between Muscle DAG Content and IR



#### Table 1. (continued)

First author	Year	Design <sup>a</sup>	Subjects <sup>b</sup> (N) and gender	Intervention <sup>c</sup> , muscle, and DAG measurement <sup>d</sup>	Results <sup>e,f,g</sup>	Refs
Shepherd	2017	1	OB sedentary men (16)	E Randomized 4 weeks of SIT or MICT <i>Vastus lateralis</i> Electrospray ionization-MS	IS ( <b>Matsuda index</b> ) was increased by both SIT (11%) and MICT (24%), with no significant difference between groups. IMTGs and total DAGs did not change with either training protocols. Mito content increased similarly in both groups as well as IMGTs in contact with Mito. DAG containing C18:1-C18:2 and C18:0-C18:2 increased similarly in both groups.	[47]
Lundsgaard	2017	I	Healthy men (9)	D Randomized crossover 3 Days hypercaloric high unsaturated FA (UNSAT), hypercaloric carbohydrate (CHO), eucaloric diet (CON) <i>Vastus lateralis</i> TLC	UNSAT (compared to CON) decreased whole-body IS and insulin-stimulated leg glucose uptake (HE clamp). UNSAT elevated IMTG content (52%) and 1,3 DAG levels (37%) without changes in insulin signaling cascade (p-Akt <sup>Thr308</sup> ). Decreases in p-HSL <sup>Ser680</sup> suggested reduced DAG hydrolysis.	[48]
Bak	2018	1	L (9) and OB (9) men	D Randomized crossover 72 h versus 12 h overnight fast <i>Vastus lateralis</i> Non-targeted gas chromatography-MS and LC-MS	OB had ~50% lower whole-body IS compared to L after 12 h fasting. Prolonged fasting decreased further IS by ~50% in L and ~25% in OB, reaching similar absolute rates of glucose infusion after 72 h of fasting. Similar increments in IMTGs were observed in both groups in response to 72 h of fasting. 1,2 DAG and 1,3 DAG levels were not elevated in OB compared to L and were not modified after 72 h of fasting in any of the groups. Insulin-stimulated p-Akt <sup>Ser473</sup> and <sup>Thr308</sup> were lower in OB than L after 12 h, but were similar between groups after 72 h of fasting.	[49]
Bergman	2018	CS	OB (14),T2D (15), A (15), women and men	Vastus lateralis HPLC-MS	A were more IS than OB and T2D (ivGTT). Palmitate oxidation was similar at rest and increased in all groups during an acute bout of exercise, with a greater increment in A. IMTGs were similar in all groups at rest. During exercise IMTGs did not change in OB and T2D, but decreased in A. IMTG synthesis at rest was greater in A. During exercise IMTG synthesis increased in all groups and decreased during recovery. DAG contents were similar in all groups at all times. Resting IMTG synthesis was associated with IS and with cytosolic DAG content, but not with total DAG or membrane DAG content.	[50]
Perreault	2018	CS	Sedentary L (15), OB (15), T2D (12), A (16), women and men	Vastus lateralis HPLC-MS	T2D were more IR than OB, which were more IR than L, which were more IR than A (HE clamp). A and T2D had the highest total DAG content. Only 1,2 DAGs were different among groups, with higher contents in A and T2D. Sarcolemma DAGs were higher in A and T2D than in L. Membrane 1,2 DAGs were higher in A, OB and T2D compared to L. Sarcolemma disaturated 1,2-DAGs were negatively related with IS if A were removed (not significant across the whole cohort). Mito and ER DAGs, particularly 1,2 DAGs, were more abundant in L and A compared to OB. Mito and ER 1,2 DAGs inversely associated with IS. Desaturated 1,2 DAGs inversely associated with IS. Nuclear DAGs were greater in A compared to L and OB, with a positive relationship with IS.	[51]

(continued on next page)



#### Table 1. (continued)

First author	Year	Design <sup>a</sup>	Subjects <sup>b</sup> (N) and gender	Intervention <sup>c</sup> , muscle, and DAG measurement <sup>d</sup>	Results <sup>e,f,g</sup>	Refs
					Cytosolic DAGs were not different among the groups and there was no relationship with IS. <i>De novo</i> DAG synthesis did not explain DAG repartition and accumulation. PKCε activation was higher in OB and T2D compared to L and A, without significant differences for PKCθ, PKCδ, or PKCβII. Positive relationship between PKCε and sarcolemma 1,2 DAG C16:0-C18:2. There were no significant relationships between 1,2-DAGs in any other compartment and PKCε. No significant relationships were found between sarcolemmal 1,2-DAGs and PKCθ, PKCδ, or PKCβII. 1,2 DAGs were more abundant (60%) than 1,3 and 2,3 DAGs. The more abundant 1,2 DAGs in Mito and ER were 16:0-18:1, then di-C18:1 and 16:0-18:2 (all higher in A). Similar abundances were found in membrane, nuclear and cytosolic DAGs but without significant differences between groups. DAG species abundance presented in supplemental table S1.	
Søgaard	2019	CS	Young untrained (11) and trained (16), aged untrained (18) and trained (15) men	Vastus lateralis TLC	Aged had higher IR (HOMA-IR) than young. DAGs were not different across trained and untrained states. Only DAGs containing C24:0 were higher in young, whereas C16:1n7 were higher in the aged. None of the DAG FAs or total DAGs correlated with HOMA-IR.	[52]

Footnotes:

<sup>a</sup>Design: CS, cross-sectional; I intervention.

<sup>b</sup>Subjects: A, chronically endurance trained/athletes; IFG, impaired fasting glucose; IGT, impaired glucose-tolerant; L, lean; NGT, normal glucose-tolerant; OB, obese; T2D, individuals with type 2 diabetes.

<sup>c</sup>Intervention: E, exercise; D, dietary; ET, endurance training; LI, lipid infusion; MICT, moderate intensity continuous training; SIT, sprint interval training.

<sup>d</sup>DAG measurement/technique: HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; TLC, thin layer chromatography; UPLC, ultra-performance liquid chromatography.

<sup>e</sup>Outcome: DAG, diacylglycerol; FA, fatty acid; IMCL, intramyocellular lipid; IMTG, intramuscular triglyceride; IR, insulin resistance/resistant; IS, insulin sensitivity/sensitive; LD, lipid droplet; TAG, triacylglycerol; PKC, protein kinase C; VLDL, very low density lipoprotein.

<sup>f</sup>Localization: Mito, mitochondria.; ER, endoplasmic reticulum.

<sup>9</sup>Outcome measurement: GTT, glucose tolerance test; HE clamp, hyperinsulinemic euglycemic clamp; HOMA-IR, homeostatic model assessment; iv, intravenous.

were not different in SM of offspring of patients with diabetes compared to age and gendermatched controls and did not explain IS improvements in response to endurance training [45]. Chow and colleagues demonstrated similar reductions in IS upon lipid infusion in sedentary or trained subjects, despite increases in total and specific DAGs in the sedentary [42]. Using a dual stable-isotope approach to differentiate between metabolic fates of dietary versus endogenous FAs, Goossens and colleagues reported that individuals with impaired glucose tolerance have lower accumulation of DAG despite having lower levels of IS compared to individuals with impaired fasting glucose. The authors concluded that it may not be DAG accumulation *per se* but rather disturbances in SM FA handling that contribute to IR [46]. Taken together, these studies disconnect improvement or deterioration of IS from muscle DAG content.

To end this section, it is important to note recent studies that looked at DAG stereoisomers (Table 1). Lundsgaard and colleagues reported that a hypercaloric diet high in unsaturated fat increased 1,3 DAG levels but reduced IS by downregulating muscle glucose uptake rather than by interfering with insulin signaling [48]. Bak *et al.* reported no differences in 1,2 and 1,3 DAGs in SM of obese and lean individuals, neither at baseline, nor in response to 72 h of fasting, even though this intervention decreased whole-body IS in both groups [49]. In a cross-sectional



comparison between lean, endurance-trained athletes, obese with T2D and obese without T2D, Perreault *et al.* found no differences in 1,3 DAGs. Athletes had the highest level of IS and higher total DAG and 1,2 DAG content compared to both lean and obese, with levels similar to those observed in T2D [51]. These studies suggest that IR can occur without elevation of muscle DAGs and that IS can occur despite elevation of muscle DAGs.

# Human Studies Showing an Association between SM DAGs and IR: Focus on Subcellular Localization

Since our previous review [23], many studies have examined the role of DAG subcellular distribution (Table 2). These works will be discussed with three distinct focuses: subcellular localization, DAG moieties, and DAG stereoisomers.

A recurring theme in the current literature is that membrane DAGs are elevated in individuals with T2D [55,56,58]. Bergman *et al.* reported that both total and membrane DAGs, particularly saturated membrane DAGs, are associated with IR in a cross-sectional comparison among sedentary obese controls, individuals with T2D and lean endurance-trained athletes [55]. Jocken *et al.* and Nowotny *et al.* both observed a significant positive relationship between membrane DAGs and IR [56,57]. Szendroedi and colleagues reported that acute induction of IR through lipid infusion in lean insulin sensitive individuals increased total and membrane DAG content [58], further leading to preferential increments in distinct membrane DAGs [57].

The consensus is less evident for cytosolic DAGs. Lipid infusion studies show increments in cytosolic DAGs [58], whereas cross-sectional studies show higher [58], lower [55], or similar [56] cytosolic DAG contents in individuals with T2D compared to normal glucose-tolerant subjects. Correlations between cytosolic DAGs and IS were reported to be negative [58] or non-significant [51,55].

It is important to acknowledge here that fractionation techniques have evolved rapidly. Indeed, while studies published in 2010–2014 separated membranes versus cytosolic DAGs, recent protocols allow separation of different membranes, where specific DAG content can be measured for example in the cell membrane (sarcolemma), mitochondria or endoplasmic reticulum (ER). This fine-tuned ability to measure DAG in different subcellular localizations may explain discrepancies between earlier studies. A good example is the work done by Bergman and Perreault in their successive publications. Following their 2012 observation on DAG localization [55], Bergman et al. reported that total DAG content did not differ between obese subjects, individuals with T2D and athletes at rest or in response to an acute exercise bout. As they observed a higher rate of IMTG synthesis in the athletes at rest, which correlated with higher cytosolic accumulation of DAGs and with IS, the authors concluded that chronic endurance exercise promotes high rates of IMTG synthesis, which alters intramuscular lipid localization and may explain the athlete's paradox [50]. Recently, the same authors reported that subjects with T2D and endurance-trained athletes have similar and higher amounts of total DAGs and sarcolemma DAGs compared to obese non-diabetic or lean sedentary volunteers, but without differences in cytosolic DAGs [51]. Looking at other subcellular compartments, they also observed that total DAGs and 1,2 DAGs in mitochondria and ER, as well as in the nucleus, were higher in athletes and were positively associated with IS. Overall, these findings highlight the importance of investigating the subcellular localization of the different DAG species. This compartmentalization may, in part, explain the athlete's paradox which was initially considered bound to LDs and IMTGs [21], later extended to specific lipid metabolites such as DAGs [51,54], which, in light of these recent studies using organelle fractionation, appear to be bound to membranes and include specific DAG moieties [51].



First author	Year	Design <sup>a</sup>	Subjects <sup>b</sup> (N) and gender	Intervention <sup>c</sup> , muscle, and DAG measurement <sup>d</sup>	Results <sup>e,f,g</sup>	Refs
Bergman	2012	CS	OB (6), T2D (6), A (10), males and females (only males for T2D)	Vastus lateralis HPLC-MS	OB and T2D had higher IR than A (ivGTT). Total DAGs and membrane DAGs were higher in OB and T2D than A. Cytosolic DAGs were lower in T2D compared to OB and A. Specific membrane DAG species higher in T2D: C18:0-C20:4, di-C16:0, and di-C18:0. There were no group differences in cytosolic species. DAG species abundances are presented in Table S1. Total and cytosolic DAG species did not correlate with IS. Only membrane DAGs and di-C18:0 correlated negatively with IS. Cytosolic DAG content was negatively, and membrane DAG positively, associated with PKCe. Specific species leading these associations in the cytosol: C18:0-C18:1, di-C14:0, di-C16:0, and di-C18:0, and in the membrane: C16:0-C18:1 and C16:1-C18:1. No associations with PKC0 activation. Only saturated membrane DAGs were related to IR. DAG species abundance presented in supplemental table S1.	[55]
Jocken	2013	CS	NGT (11) and T2D (9), men	Vastus lateralis TLC	T2D had higher IR than NGT (HOMA-IR and HE clamp). Total and membrane DAGs were higher in T2D. Specific membrane DAG species higher in T2D contained: C16:0, C17:0, C18.0, C22:0, and <i>trans</i> C18:1. Cytosolic DAGs were not different between groups. Saturated membrane DAGs were inversely associated with IS and positively associated with PKCδ activation.	[56]
Nowotny	2013	I	L IS (16), men and women	Acute IR induction, randomized crossover, four conditions: LI, oral fat, LPS, and control <i>Vastus lateralis</i> LC-MS/MS	LI, oral fat and LPS reduced IS (HE clamp). LI and oral fat increased PKC0 activation. Membrane di-C18:2 DAG was increased after LI but not after oral fat or LPS. Overall membrane DAGs and membrane di-C18:2 DAG correlated positively with PKC0 activation after oral fat but not LI or LPS. LPS raised IR by stimulating inflammatory pathways. DAG species abundance presented in supplemental table S1.	[57]
Szendroedi	2014	CS	Sedentary L NGT (36), OB IR (10), OB T2D (10), women and men	LI in L Vastus lateralis LC-MS/MS	OB and T2D were similarly more IR than L (HE clamp) and had higher contents of total and cytosolic DAGs than L. Membrane DAGs were higher in T2D than in OB and L. Specific cytosolic DAGs higher in OB and T2D: C16:0-C18:2, di-C18:2, C18:1-C18:2, C18:0-C16:0, di-C16:0, C18:1-C18:0, C18:2-C18:0, C18:0-C20:4, C16:0-C20:4, C18:1-C16:0. Specific membrane DAGs higher in OB and T2D were di-C18:0 and the same as above but not di-C16:0 and C18:1-C16:0. Total cytosolic DAGs correlated negatively with IS, as did cytosolic species containing C18:0-C18:2 and C16:0-C18:2 and membrane species containing C18:0-C20:4, C18:2-C18:0, C18:1-C18:2, di-C18:2, C16:0-C18:2. Other-membrane DAGs containing C20:4 and C20:5 correlated positively with IS. Activation of PKC6 was higher in OB and T2D than in L, without differences in PKC6 and PKCε activity. PKCθ activation correlated negatively with IS and positively with total cytosolic and membrane DAGs, and with the following species at both locations: C16:0-C20:4, C16:0-C18:2. In L, acute induction of IR through 4 h of LI increased total DAGs, cytosolic DAGs and membrane DAGs. Specific cytosolic species increased: C16:0-C18:2, di-C18:2, C16:0-C20:4. Membrane species that increased are the same as the cytosolic species and C18:0-C18:2, C18:1-C18:2. LI activated PKC6, but not PKC6 or PKCε, and increased p-IRS1 <sup>Ser1101</sup> . DAG species abundance presented in supplemental table S1.	[58]

#### Table 2. Studies Showing an Association between Skeletal Muscle DAG and IR



#### Table 2. (continued)

First author	Year	Design <sup>a</sup>	Subjects <sup>b</sup> (N) and gender	Intervention <sup>c</sup> , muscle, and DAG measurement <sup>d</sup>	Results <sup>e,f,g</sup>	Refs
Tonks	2016	CS	Sedentary L (23), overweight/OB (14), or OB IR (14), women and men	Vastus lateralis LC-electrospray ionization MS	Di-C18:2 DAG was the only DAG that differed between groups, with a lower content in OB IR compared to L. DAG species abundances are presented in Table S1.	[59]

#### Footnotes:

<sup>a</sup>Design: CS, cross-sectional; I intervention.

<sup>b</sup>Subjects: A, chronically endurance trained/athletes; L, lean; NGT, normal glucose tolerant; OB, obese; T2D, individuals with type 2 diabetes. <sup>c</sup>Intervention: LI, lipid infusion; LPS, intravenous endotoxin.

<sup>d</sup>DAG measurement/technique: HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; TLC, thin layer chromatography; UPLC, ultra-performance liquid chromatography.

e Outcome: DAG, diacylglycerol; FA, fatty acid; IR, insulin resistance/resistant; IS, insulin sensitivity/sensitive; PKC, protein kinase C.

<sup>f</sup>Localization: ER, endoplasmic reticulum; Mito, mitochondria.

<sup>9</sup>Outcome measurement: GTT, glucose tolerance test; HE clamp, hyperinsulinemic euglycemic clamp; HOMA-IR, homeostatic model assessment; iv, intravenous.

## Human Studies Showing an Association between SM DAGs and IR: Focus on DAG Moieties and DAG Stereoisomers

In the past 6 years research has also focused on DAG moieties and their specific contribution and association with IR (Table 2). In Table S1, we combined all available observations on human muscle, ranking DAG moieties by abundance. When quantitative data were not available, the ranking was estimated from figures. Before comparing specific DAGs across different studies, it is important to acknowledge methodological issues (Tables 1 and 2). While most recent studies used lipidomic techniques such as liquid chromatography tandem mass spectrometry with or without fragmentation, other studies used thin layer chromatography with or without radiolabeling through kinase assays [60,61]. Importantly, none of these methods have been validated to differentiate DAG enantiomers.

As previously observed [54,62], current literature confirms that the most frequent (top three) DAG moieties are di-C18:0, C16:0-C18:0 and C16:0-C18:1, with some discrepancies among studies. In three independent studies the most abundant DAG was C16:0-C18:0 [38,44,55], for two reports it was DAG C16:0-C18:1 [43,45], and all other studies have their own champion. Only two studies looked at the different moieties within different compartments, and here too abundances varied [55,58].

Many species of DAGs have been thought to modulate IS but there is no consensus on those that specifically alter IS (Table S1). We only found three moieties for which data were confirmed in at least two independent studies: di-C18:2, di-C14:0, and C18:0-C20:4. Di-C18:2 was found to be higher in obese insulin-resistant individuals [59], in the cytosolic and membrane fractions of T2D and obese subjects [58], and following lipid infusion and high-fat diet (HFD) [57,58]. Di-C14:0 was found higher in obese individuals in one study [54] and correlated with PKC $\epsilon$  activation in another study [55]. C18:0-C20:4 was observed to be higher in the membrane fraction of obese and individuals with T2D [55,58] and in the cytosolic fraction [58]. This observation questions the role of FA composition of the diet given the fact that 20:4 is one of the long-chain  $\omega$ 3 polyunsaturated essential FA that cannot be synthesized *de novo* by humans and that are thought to play an important role in human health by reducing the risk of chronic diseases [63].

Nine DAG moieties have diverging, or even opposing, results across studies. A good example is C16:0-C18:1 which was observed to be higher in the cytosol of obese and subjects with T2D [58]



but was also reported as being higher in athletes [54] and particularly in the membrane fraction of athletes [51]. Yet in another study, membrane C16:0-C18:1 correlated with PKCɛ activation without being significantly different in individuals with T2D compared to obese subjects and athletes [55]. Other examples are DAG C18:1-C18:2 and C18:0-C18:2 that were found to increase in response to exercise intervention without improvements in IS [47], or were increased after lipid infusion [58]. Finally, Perreault *et al.* reported a positive relationship between PKCɛ activation and sarcolemma 1,2 DAG C16:0-C18:2 [51].

Taken together, these observations point to the importance of doing systematic comparisons among studies, measurement methods and human variability. Table S1 will be useful in comparing and discussing relevant results with an integrative approach as proposed in the concluding remarks.

#### DAGs and IR: Findings from In Vitro Studies

*In vitro* evidence regarding the association between DAGs and IR in recent years is also equivocal (Table 3). We summarize here all studies from 2012 to date that report measures of DAGs and insulin signaling or IS in SM cell lines or primary myotubes. Importantly, we consciously decided not to incorporate studies using cultures of cardiomyocytes or cardiomyoblasts because these have histological, physiological and metabolic properties that are distinct from SM cells. We prefer not to risk inferences from these different models.

Two studies in which rat L6 myoblasts were incubated with palmitate (C16:0) observed impaired insulin signaling, but one found no associated changes in total DAGs [69] while the other observed increases in total and specific DAGs [67]. Pillon and colleagues found a positive

First author	Year	<i>In vitro</i> model <sup>a</sup>	Experiments <sup>b</sup>	Results <sup>c</sup>	Refs
Bosma	2012	C2C12	PLIN2 OE and KD, incubation with/without C16:0 or C18:1	PLIN2 KD incubated with C16:0 lowered IMTGs and increased C16:0 DAGs, without compromising in insulin signaling. PLIN 2 OE incubated with C16:0 increased IMTGs, increased C16:0 DAGs, and improved IS, thus protecting against C16:0-induced impairments in insulin-stimulated glucose uptake compared to control cells.	[64]
Newsom	2015	C2C12, human primary cells	Incubation with different concentrations and proportions of FA mixtures	C2C12: incubation with 100% C16:0 increased DAG content in a dose-dependent manner and impaired insulin signaling (decreased p-Akt <sup>Thr308</sup> /Akt). The FA composition of DAG resembled that of the FAs provided in the incubation media. Myotubes: incubation with 100% C16:0 increased DAG concentration without significant impairments of insulin signaling.	[65]
Capel	2016	C2C12	Incubation with C16:0 with/without different doses of C18:1	C16:0 incorporation into DAG was reduced by concomitant C18:1. C18:1 coincubation decreased the impact of C16:0 on the insulin signaling cascade and PKC0 phosphorylation in a dose-dependent manner.	[66]
Miklosz	2017	L6	Incubation with/without C16:0	Incubation with C16:0 increased total DAGs, the incorporation into DAG of C16:0, C16:1, C18:0, C18:2, C18:3, C20:4, C24:0 and C20:5, and decreased insulin signaling (p-Akt/Akt ratio).	[67]
Jefferson	2017	C2C12	Incubation with/without calcitriol	Calcitriol increased insulin-stimulated p-Akt, increased total DAGs, and increased specifically di-C18:0, C18:1-C20:0, C18:0-C20.4, di-C18:1, C16:0-C18:1, di-C14:0, di-C16:0, and C18:0-C18:2.	[68]
Pillon	2018	L6	Incubation with C16:0 or C16:1	Incubation with C16:0, but not C16:1, impaired insulin signaling and IS without differences in total DAGs. DAGs containing 16:1 were positively associated with IS.	[69]

#### Table 3. Mechanistic Studies (In Vitro) Showing Associations and Dissociations between Muscle DAG Content and IR

Footnotes:

<sup>a</sup> In vitro models: C2C12, cell line from mouse myoblasts [research resource identifier (RRID): CVCL\_0188]; L6, cell line from rat myoblasts (RRID: CVCL\_0385). <sup>b</sup>Experiments: KD, knockdown; OE, overexpression; PLIN, perilipin.

<sup>c</sup>Outcomes: Akt, protein kinase B; DAG, diacylglycerol; FA, fatty acid; IMTG, intramyocellular triglyceride; IS, insulin sensitivity; p-, phosphorylated; PKC, protein kinase C.



association between C16:1 DAG levels and IS after incubating these cells with palmitoleate (C16:1) [69].

Two studies using mice C2C12 myoblasts demonstrated that the FA composition of DAGs resembles FAs provided in the incubation media. When incubating these cells with palmitate (C16:0), cellular DAG content increased in conjunction with impairments of the insulin signaling cascade [65,66]. Coincubating palmitate (C16:0) with oleate (C18:1) decreased DAG content, impacted PKC0 phosphorylation, and improved insulin signaling in a dose-dependent manner [66]. A recent work using the same model presented arguments disconnecting DAGs, and specific DAG species, from the insulin signaling cascade [67]. While studying the positive effect of vitamin D on IS, Jefferson and colleagues found that calcitriol, the active metabolite of vitamin D, increased insulin-stimulation of phospho (p-)**Akt** while increasing total DAGs and many DAG species [68]. Finally, Bosma and colleagues showed a disconnect between DAG and IS in two separate experiments [64]. When incubating C2C12 cells with C16:0 and modulating **perilipin** (PLIN)2 content by knocking down or overexpression, they observed increases in C16:0-containing DAGs without compromising insulin signaling (when they knocked down PLIN2) or improving IS (when overexpressing PLIN2).

#### DAGs and IR: Evidence from Animal Studies

Similarly to human studies and *in vitro* investigations, *in vivo* animal studies have failed to confirm a relationship between SM DAGs and IR (Table 4).

Timmers and colleagues found that treating mice with **etomoxir** improved insulin signaling and IS despite the accumulation of DAGs [70]. Thus, blocking FAs from entering the mitochondria and the subsequent DAGs accumulation does not necessarily lead to IR. Selathurai *et al.* observed that very high DAG content, particularly membrane DAGs, do not cause IR. Indeed, they created muscle-specific knockout (KO) mice lacking one of the phospholipids biosynthesis pathways (CDP–ethanolamine pathway) which resulted in a 200% increase in DAG content [78]. Exploring the influence of DAG stereoisomers, Serup and colleagues observed that in hormone-sensitive lipase (HSL) KO mice, the accumulation of 1,3 DAGs, which originate from **adipose triglyceride lipase** (ATGL)-mediated lipolysis after an acute bout of exercise, did not inhibit insulin-stimulated glucose disposal [80]. Taken together, these works point to situations were DAGs and IS have independent behaviors from one another.

In parallel to their *in vitro* observations mentioned above, Bosma and colleagues modulated PLIN2 expression *in vivo* in rats and observed that improvements in IS may be accompanied with increases in IMTGs without modifications in DAG content [64]. Matravadia *et al.* demonstrated that linoleic acid and  $\alpha$ -linoleic acid supplementation mediate glucose homeostasis preservation in obese Zucker rats while at the same time increasing total DAGs and many DAG species [79].

In different mouse strains, Badin *et al.* [73] and Turner *et al.* [74] found that HFD supplementation increased total DAGs, impaired insulin signaling and contributed to the initial development of IR. Bruce *et al.* reported that treatment with sphingosine-1-phosphate analog FTY720, in conjunction with HFD, prevented the accumulation of total and specific DAGs, and resulted in improved glucose homeostasis [75].

Zabielski and colleagues identified specific DAG species that responded to HFD alone and/or to the combination of HFD with metformin in conjunction with the deterioration or amelioration of IS in Zucker rats [81]. Intriguingly, some DAG species were elevated in both conditions, while



First author	Year	Animal model <sup>a</sup>	Diet and/or intervention <sup>b</sup>	Results <sup>c</sup>	Refs
Timmers	2012	14 weeks old male C57BL/6 mice	HFD for 14 days. Etomoxir injections and GTT.	Etomoxir increased IMCL and DAG content without increasing PKC0. Glucose tolerance (ipGTT), insulin signaling and insulin-stimulated GLUT4 translocation improved despite DAGs accumulation in response to etomoxir.	[70]
Franko	2012	5–7 months old male mice either: (i) <i>ob/ob</i> normoglycemic (ii) HFD C57BL/6 (iii) STZ C57BL/6 (iv) MIRKO	For (ii) HFD during 6 months starting at 3 months of age. For (iii) STZ injected at 3 months of age.	HFD mice were glucose intolerant (ipGTT), IR estimated by <b>insulin tolerance test</b> (ITT) and had higher levels of 1,2 and 1,3 DAGs. STZ mice were glucose intolerant (ipGTT), IR (ipITT) but did not accumulate either 1,2 or 1,3 DAGs.	[71]
Bosma	2012	7 weeks old male Wistar rats	Low-fat (10% energy from fat) or HFD for 3 weeks. <i>In vivo</i> muscle-specific PLIN2 OE (electroporation).	PLIN2 OE increased IMTGs and improved IS (HE clamp) in HFD conditions, without affecting DAG content.	[64]
Bosma	2013	8 weeks old male Wistar rats	HFD. In vivo muscle-specific PLIN5 OE.	PLIN5 OE increased IMTGs without affecting IS (HE clamp) or DAG content.	[72]
Badin	2013	5 weeks old male C3H mice and female HSL null mice	HFD or chow for 4 weeks. HSL KO mice received normal chow for 7 weeks.	C3H mice: HFD increased total DAG content, PKC membrane translocation, impaired insulin signaling and induced IR (ipITT). HFD mice had higher membrane/cytosol ratio of PKC0 and PKC2. HSL KO mice: higher DAG content, higher C16:0 incorporation into DAG, and impaired insulin signaling.	[73]
Turner	2013	8–12 weeks old male C57BL/6 mice	HFD versus control diet, from 3 days to 16 weeks.	Total DAG content was increased at the time that IR (HE clamp) developed (3 weeks of HFD). At 3 weeks of HFD, the following DAG species were increased compared to control diet: C16:0-C18:1, C16:0-C18:2, C16:1-C18:1, di-C18:1, C181-C18:2. At 16 weeks of HFD, IR was similar to 3 weeks but the following DAG species were differentially elevated compared to control diet: C16:0-C18:1, C18:0-C18:1, C18:1-C18:2, C16:0-C20.4, C18:0-C20.4, C16:0-C22.6.	[74]
Bruce	2013	8 weeks old male C57BL/6 mice	HFD versus control diet for 12 weeks with/without FTY720 (5 mg/kg) daily for the last 6 weeks.	In addition to preventing SM ceramide increases with HFD, FTY720, a sphingosine-1-phosphate analog, prevented increases in total DAGs and the following species: C16:0-C18:1, C16:0-C18:2, C16:0-C20:4, C18:0-C20:4 and C18:1-C20.4. FTY720 improved glucose homeostasis, reduced plasma insulin, improved glucose tolerance (ipGTT), increased insulin-stimulated glucose uptake, and Akt phosphorylation.	[75]
Holloway	2014	L and OB female Zucker rats (age unavailable)	Chronic contraction: <i>in vivo</i> unilateral electrical stimulation 6 h/day for 6 days, versus sham.	At baseline, OB had lower insulin-stimulated glucose uptake, higher IMTGs and higher total DAGs in both red and white muscle. The content of DAG moieties depended on the type of muscle. In red muscle OB had higher DAGs containing C14:0, C16:0, C16:1, C18:0 and C22:0, but lower C18:n3 and C20:4n6. In white muscle, OB had higher levels of almost all DAGs measured (12 of 14). Only DAGs containing C20:4n6 and C20:5n3 were similar in L and OB.	[76]

#### Table 4. Mechanistic Studies (Animal Models) Showing Associations and Dissociations between Muscle DAG Content and IR

## Trends in Endocrinology & Metabolism



#### Table 4. (continued)

First author	Year	Animal model <sup>a</sup>	Diet and/or intervention <sup>b</sup>	Results <sup>c</sup>	Refs
				Contraction increased insulin-stimulated glucose uptake in all muscles in both OB and L. IMTGs increased in all muscles with +127% in L white, +57% in OB white, +74% in L red and +32% in O red. Neither total nor specific DAGs were modified in L muscles or OB white muscle compared to non-stimulated controls. In OB red muscle, contraction decreased total DAGs by –17%, increased DAGs containing C16:1 (+54%), and decreased DAGs containing C20:0 (–43%).	
Mason	2014	16 weeks old PLIN5 KO male mice versus control	Chow.	PLIN5 KO mice were more glucose tolerant (ipGTT) but more IR (HE clamp) and had similar total DAG content to controls.	[77]
Selathurai	2015	18 weeks old male muscle-specific ECT KO mice versus control	Chow.	ECT KO mice: 200% accumulation of total DAGs, membrane DAGs, and specific species including C14:0-C16:0, C14:0-C18:1, di-C16:0, C16:0-C18:2, C16:1-C18:1, C16:0-C18:0, di-C18:2, C18:1-C18:2, C18:0-C18:2, di-C18:1, C18:0-C18:2, C18:0-C18:2, C18:0-C18:2, C18:0-C18:1, IS or oxidative capacity.	[78]
Matravadia	2015	6 weeks old male OB Zucker rats	12 weeks of diet containing supplements of C18:2 (LA), C18:3 (ALA) versus controls.	LA and ALA did not change total DAGs but decreased DAGs containing C14:0 and C18:0. LA increased DAGs containing C18:2 and decreased C22:6. ALA decreased DAGs containing C20:4 and increased DAGs containing C18:3. ALA and LA prevented the elevation of fasting blood glucose. ALA prevented glucose and insulin intolerance (ipGTT and ipITT). Insulin signaling (IRS1) was decreased similarly in all conditions.	[79]
Serup	2016	16-25 weeks old female HSL KO mice versus control	Acute endurance exercise (running).	After exercise: HSL KO mice had higher insulin-stimulated glucose uptake and higher 1,3 DAGs compared to controls.	[80]
Zabielski	2017	6 weeks old male Wistar rats	8 weeks of control diet, HFD or HFD with metformin (Met).	Met prevented HFD-induced glucose and insulin intolerance (ipGTT and ipITT), IR (HOMA-IR) and deteriorations in insulin signaling. HFD increased total DAGs, increased DAG C16:0-C18:2, di-C18:0, C18:0-C18:2, di-C18:2, and C18:1-C18:2, and decreased DAG C16:0-C18:0, C16:0-C18:1, and di-C18:1. Met decreased total DAGs, decreased DAG di-C16:0, and increased DAG di-C18-2 compared to controls. Met decreased DAG di-C16:0, C16:0-C18:0, C16:0-C18:2, C18:0-C18:1, C18:0-C18:2, C18:1-C18:2, and di-C18:2 compared to HFD alone.	[81]

Footnotes:

<sup>a</sup>Model: ECT, phosphoethanolamine cytidylyltransferase; HSL, hormone-sensitive lipase; KO, knockout; L, lean; MIRKO, muscle-specific insulin receptor knockout mice; OB, obese; *ob/ob*: obese mice; PLIN, perilipin; STZ, streptozotocin.

<sup>b</sup>Intervention: ALA,  $\alpha$ -linolenic acid; HFD, high-fat diet; LA, linoleic acid; OE, overexpression.

<sup>c</sup>Outcomes: Akt, protein kinase B; DAG, diacylglycerol; GLUT4, glucose transporter type 4; GTT, glucose tolerance test; HE clamp, hyperinsulinemic euglycemic clamp; IMCL, intramyocellular lipids; ip, intraperitoneal; IR, insulin resistance; IS, insulin sensitivity; ITT, insulin tolerance test; PKC, protein kinase C; SM, skeletal muscle; TAG, triacylglycerol.



others were decreased. In the same puzzling impression, Turner and colleagues pointed to the relationship between SM-specific DAG content and HFD-induced IR in mice, but perplexingly specific DAGs varied at different timepoints for the same degree of IR [74]. Similarly unclear are the results from Holloway *et al.* who reported the effects of *in vivo* muscle contraction secondary to unilateral electrical stimulation in obese and lean Zucker rats [76]. When looking at the absolute values reported in the supplemental data, specific DAG moieties were higher or lower depending on the type of muscle measured. With chronic contraction, insulin-stimulated glucose uptake was increased in both lean and obese, white and red stimulated muscles compared to their contralateral sham controls. While IMTGs increased in all muscles, DAG content did not change significantly in lean white and red muscles nor did it change in white obese muscle. Chronic contraction changed total DAGs. Among all the DAG moieties measured, the only significant modifications with chronic contractions were an increase in DAGs containing C16:1 and a reduction in DAGs containing C20:0. Taken together, these works point to the difficulty of identifying specific DAG properties linked to IR even in animal models.

#### PKC Isoforms Involved in DAG-Induced SM IR

Classification and differences among PKC isoforms are described in Box 1. Despite considerable research, which PKC isoform is the most pertinent in DAG-mediated IR remains to be elucidated. This section emphasizes the lack of consensus raised by the current state of investigation. Before expanding on the differences between studies, it is important to acknowledge a potential reporting bias. Indeed, studies not supporting the DAG-induced IR theory do not report PKC data. Out of the five human studies that reported an association between SM DAGs and IR, three reported links with PKC $\theta$ , one with PKC $\delta$  and one with PKC $\epsilon$ . Among the six animal studies, only one reported a link with PKC (PKC $\theta$  and PKC $\epsilon$ ).

PKCθ is the most frequent isoform incriminated in altering insulin signaling in SM [82,83]. Yu *et al.* demonstrated that lipid infusion in Wistar rats increased PKCθ activity and subsequently resulted in increased **insulin receptor substrate 1** (IRS-1) serine phosphorylation [84]. Similarly, Griffin and colleagues highlighted the role of PKCθ in the insulin cascade in lipid-infused rats [85]. In human muscle, Szendroedi *et al.* demonstrated that lipid infusion caused IR, increased cytosolic and membrane DAGs and temporarily increased PKCθ activity, concomitantly with increases in IRS-1 serine phosphorylation, inhibition of insulinstimulated IRS-1 tyrosine phosphorylation and Akt2 phosphorylation [58]. They also reported which DAG species had the strongest relationship with PKCθ (Table S1). Nowotny and colleagues reported increments in PKCθ activity after oral fat ingestion and after lipid infusion [57], but they observed a significant correlation between PKCθ and overall membrane DAGs, and specifically membrane di-C18:2 DAG, only when fat was ingested orally. Søgaard and colleagues found no differences in PKCθ or p-PKCθ<sup>Ser676</sup> protein expression between insulin sensitive controls and offspring of patients with T2D, neither before nor after exercise training [45].

Other PKC isoforms have also been associated with altered insulin signaling in human muscle. Itani *et al.* found that lipid infusion increases in DAG caused heightened activity of PKC $\beta$ II and PKC $\delta$  [30]. Jocken and colleagues reported data supporting a potential role of increased PKC $\delta$  activity in response to increments in saturated membrane DAGs [56]. On the one hand, Bergman and colleagues found that four specific cytosolic DAGs were negatively related to PKC $\epsilon$  but not PKC $\theta$  activity. On the other hand, they observed two membrane DAG species that explained a positive relationship with PKC $\epsilon$  but not PKC $\theta$  activity (Table S1) [55].



Here again it is important to consider intracellular localization and stereoisomers. Empirical evidence has long held the view that 1,2 DAG (as opposed to 1,3 and 2,3 DAG) is the sole stereoisomer that can activate PKC [26]. Perreault *et al.* reported a significant positive relationship between PKCε and sarcolemma 1,2 DAG C16:0-C18:2 [51] (Table 1). No other localization or DAG moiety was associated with PKCε, PKCθ, PKCδ, or PKCβII.

Further, *in vivo* and *in vitro* studies have both shown conflicting evidence regarding DAGinduced PKC activity in IR. Timmers *et al.* reported no increments in membrane PKC0 protein content in mouse muscle after etomoxir treatment [70]. Badin *et al.* reported that DAG-mediated IR development in HFD-fed mice was positively associated with higher membrane-to cytosol ratio of PKC0 and PKCc [73]. More recently, Capel and colleagues demonstrated that treating C2C12 myotubes with oleate can decrease PKC0 activity and improve IS [66].

Overall, based on the aforementioned divergent findings, it is clear that the jury is still out regarding which PKC isoforms are the most pertinent in the context of SM DAG-induced IR. Moreover, there is a clear need for lipotoxicity researchers to reach a consensus on measurement strategies for different PKC isoforms to avoid further confusion about their importance.

#### **Concluding Remarks**

Whether DAGs play a causal role in SM IR in humans remains inconclusive. In the past 6 years, lipotoxicity researchers have called into question the hypothesis that total DAG levels are the main culprit of lipid intermediate-driven IR in SM. In analyzing and summarizing the data up to 2012, it appeared that DAG levels in all experiments were measured in whole-muscle lysates, thus neglecting the importance of compartmentalization. Research since then has addressed this issue and has advanced the knowledge base regarding DAG-induced IR to a large extent. Unfortunately, given the divergent results in human and animal studies presented in this review, it is impossible to make a conclusive judgment on how much weight DAGs should receive in the potential etiology and treatment of SM IR or T2D.

Methodological reasons including study design, differences in sample collection, age of the study participants, gender differences, and genetic variation may have contributed to these divergent findings. Although advances in lipidomic technologies have greatly helped to advance our knowledge, the overall results remain inconclusive because some reports have measured levels of DAGs with two FAs, whereas others measured FAs separately from the glycerol backbone. To ascertain the real impact of DAG levels on SM IR, we believe that it is essential to adopt an integrative approach that considers different DAG stereoisomers and moieties with attention to their chain lengths, degree of saturation, and subcellular localization.

Although this review has focused on SM, it is possible that other organs such as liver or heart might provide different conclusions. Indeed, the causality of DAG-induced hepatic IR seems to be much more established, but this is beyond the scope of this review.

Among other perspectives, studies on the role of PLIN proteins in modulating DAG and IMTG levels are needed. PLIN2 and PLIN5 are considered as main promoters of IMTG storage [86,87]. Recent evidence indicates that a high IMTG synthesis rate, with lipids being partitioned into TAGs in LDs, is a protective feature [50]. Another direction for future research will be to focus on time-lapse/dynamic experiments. At present, available data describe snapshots regarding the involvement of DAGs in lipid metabolism and the insulin cascade.

#### **Outstanding Questions**

How much prominence should DAGs receive in the etiology/treatment of IR and T2D?

This review demonstrates many divergences of methods across different studies, such as total versus subcellular DAGs, and DAG moieties versus stereoisomers. Could a global consensus on the lipidomic techniques used to measure DAG-induced IR be reached? If so, what specific DAG moieties, stereoisomers, and localizations require our attention?

DAGs are thought to exert their physiological effects through various PKC isoforms. What is the most pertinent PKC isoform in the context of DAG-induced IR?

PLIN proteins regulate LD storage and use, but what is their precise role in DAG-induced IR?

Collectively, despite considerable progress in recent years, many questions remain unanswered and the exact role of DAGs in the development of IR remains to be established (see Outstanding Questions). It is possible that DAGs are not as influential in SM IR as it once was thought. Investigations encompassing different subcellular localizations, different DAG moieties as well as DAG stereoisomers are likely to open new perspectives in the field.

#### **Supplemental Information**

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