Defining lipid mediators of insulin resistance: controversies and challenges

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Abstract

Essential elements of all cells – lipids – play important roles in energy production, signalling and as structural components. Despite these critical functions, excessive availability and intracellular accumulation of lipid is now recognised as a major factor contributing to many human diseases, including obesity and diabetes. In the context of these metabolic disorders, ectopic deposition of lipid has been proposed to have deleterious effects on insulin action. While this relationship has been recognised for some time now, there is currently no unifying mechanism to explain how lipids precipitate the development of insulin resistance. This review summarises the evidence linking specific lipid molecules to the induction of insulin resistance, describing some of the current controversies and challenges for future studies in this field.

Insulin resistance and lipid metabolism

Obesity and diabetes are metabolic conditions of increasingly widespread significance to modern populations. The global scale and gravity of their impacts on general health, life expectancy and quality of life encourage the search for new treatment options. Mechanisms which promote, instigate or maintain these disorders are complex, interrelated and certainly not simple to define. Both obesity and diabetes are, however, known to be underpinned by insulin resistance (IR), and study into this condition is hoped to elucidate pathways with therapeutic potential.

The circulating hormone insulin regulates substrate movement into tissues for either oxidation or storage. Its many activities, both stimulatory and inhibitory, are implemented via a complex signalling pathway activated by the insulin receptor (Saltiel & Kahn 2001, Taniguchi et al. 2006, Humphrey et al. 2013). Of particular relevance to diabetes onset is insulin stimulation of glucose uptake and metabolism, as well as fatty acid (FA) metabolism; the non-adipose tissues most essential in these processes are muscle and liver. Tissue desensitisation to insulin, and the resultant failure of a normal insulin dose to elicit these responses, is known as IR. Although incompletely defined, a number of different mechanisms have been proposed to promote the development of IR, including overproduction of reactive oxygen species, dysfunction of mitochondria, induction of ER stress and activation of inflammatory pathways (Donath & Shoelson 2011, Gregor & Hotamisligil 2011, Tiganis 2011, Kim et al. 2015, Montgomery & Turner 2015, Rocha et al. 2016).

Another factor that is strongly associated with IR is the accumulation of bioactive lipids in non-adipose tissues (Kraegen et al. 1991, Summers 2006, Chavez & Summers 2012, Turner et al. 2013, Bellini et al. 2015).

While unlimited access to calorie-dense foods and reductions in physical activity create an environment conducive to excessive lipid accumulation, there are various factors at the cellular level that heighten lipid
intracellular lipids and insulin resistance

Intracellular lipids and insulin resistance. In contrast, these contradictions: the overexpression of FATP1 in skeletal muscle, for example, channels lipids to oxidation and does not predispose to diet-induced IR (Holloway et al. 2011). Overexpression of the FA transporter/scavenger receptor CD36 in muscle and liver has also been shown to attenuate genetic and diet-induced IR via enhancement of oxidative capacity (Ibrahimi et al. 1999, Héron-Milhavet et al. 2004, Garbacz et al. 2016).

On the other side of the equation, involvement of mitochondrial fatty acid oxidation (FAO) in aberrant lipid accumulation is somewhat more complex. Correlative studies have shown defective mitochondrial markers in insulin-resistant skeletal muscle (Kelley et al. 2002, Mootha et al. 2003, Patti et al. 2003, Petersen et al. 2003, Ritov et al. 2005). Yet despite this seemingly plausible association, lipid excess is also seen to enhance mitochondrial oxidative capacity (Turner et al. 2007, Hancock et al. 2008, Koves et al. 2008). Genetic mitochondrial dysfunction in mice also presents contradictory evidence: long-chain acyl-CoA dehydrogenase deficiency leads to hepatic IR (Zhang et al. 2007); while defective mitochondrial substrate metabolism induced by deletion of mitochondrial transcription factor A (TFAM), apoptosis inducing factor (AIF), very long-chain acyl-CoA dehydrogenase (VLCAD) or carnitine palmitoyltransferase 2 (CPT2) protects from diet-induced obesity and IR (Wredenberg et al. 2006, Pospisilik et al. 2007, Zhang et al. 2010). These discrepancies may well reflect homeostatic compensatory mechanisms responding to either elevated lipid availability or dysfunctional mitochondria (Turner et al. 2007, Serup et al. 2016). Apparent regulatory redundancies suggest the biological relevance of mitochondrial FAO, regardless of whether its deterioration is a consequence or stimulus of lipid accumulation. The correlations between lipid accumulation and IR appear to be due not to changes in either lipid uptake or FAO alone, but likely a combination of the two, which ultimately leads to the aberrant build-up of different classes and species of lipids that have deleterious effects on insulin action.

The complexity of the lipidome

Lipids have well-defined roles in signalling and gene transcription, as metabolic fuels and as structural components of cells. The number of distinct chemical entities in the lipidome is not completely resolved. The combination of various backbones, headgroups and acyl chains gives rise to many thousands of lipids that are classified in distinct classes, subclasses and subgroups (Li et al. 2015, Lydic & Goo 2018). This diversity in chemical structure results in a vast spectrum of physiochemical properties across various lipids, necessitating different methods and strategies for quantification (Shevchenko & Simons 2010, Li et al. 2015, Lydic & Goo 2018). Not all lipid species have been implicated in the development of IR, but we highlight below major species where a direct or indirect association has been described (Fig. 1).
Mediators of lipid-induced IR – many culprits for the same crime

Triglyceride

The accumulation of triglyceride (TAG) content has long been associated with the insulin-resistant state (Storlien et al. 1991, Pan et al. 1997, Manco et al. 2000). TAG is the primary source of lipid storage within tissues and inappropriate TAG accumulation is thought to be indicative of a disturbance at some level in lipid metabolism pathways. While a frequent association between TAG and IR is observed, it should also be noted that a number of human and rodent studies have demonstrated overt disconnections. Intrahepatic and intramyocellular TAG accumulation have both been observed in the absence of IR (Goodpaster et al. 2001, Amaro et al. 2010, Visser et al. 2011, Gemmink et al. 2016, Ter Horst et al. 2017); hepatic IR has been observed in the absence of TAG accumulation (Semple et al. 2009). While TAGs are considered to be relatively benign with respect to directly causing IR, intracellular TAG synthesis and storage is a highly dynamic process, suggested to have a protective role by warding against accumulation of more lipotoxic species in muscle (Montell et al. 2001, Liu et al. 2007, Pickersgill et al. 2007, Schenk & Horowitz 2007, Bergman et al. 2018), adipocytes (Chavez & Summers 2003) and β-cells (Cnop et al. 2001).

Enhanced channelling of FA substrate to TAG can reduce lipotoxic pressure despite greater cellular lipid content (Listenberger et al. 2003, Coll et al. 2008, Henique et al. 2010, Capel et al. 2016). Indeed, the apparently futile, ATP-consuming cycle of FA re-esterification to TAGs during adipocyte lipolysis appears designed to protect cells from lipotoxic stress (Chitraju et al. 2017); in β-cells, this cycle may regulate insulin secretion (Corkey et al. 2000, Nolan et al. 2006, Fex & Mulder 2008). Enhanced sequestration of TAG in cytosolic lipid droplets via perilipin protein action can blunt muscle IR, such that the greatest increase in fat storage capacity is associated with lowest reduction of insulin sensitivity (Bosma et al. 2012, Billecke et al. 2015, Gemmink et al. 2016, Shepherd et al. 2017). TAGs can therefore be considered to act as a reservoir for FFA storage, on similar principles to the larger-scale role played by adipose tissue. When the protective buffer that TAG provides is exceeded, and its storage capacity is no longer able to compensate for high lipid uptake rates, the consequent formation and accumulation of more deleterious lipid metabolites results in IR.

Long-chain fatty acyl-CoA

Long-chain fatty acyl-CoA (LCA-CoA), the initial active intermediate formed during FA metabolism, has also been implicated in the development of IR. Studies have demonstrated negative correlations between LCA-CoA intracellular accumulation and insulin action in the skeletal muscle of HFD-fed rodents (Oakes et al. 1997a,b, Ellis et al. 2000, Wright et al. 2011), with acute lipid infusion-based studies in rodents and humans recapitulating these associations (Tsintzas et al. 2007, Hoy et al. 2009). Impairment of insulin signalling and glucose metabolism in this context is theorised to arise, in part, via LCA-CoA interactions with proteins including protein kinase C (Færgeman & Knudsen 1997), glycerogen synthase (Wititsuwannakul & Kim 1977), glucokinase (Tippett & Neet 1982a,b), hexokinase (Thompson & Cooney 2000), as well as LCA-CoA-induced alterations in gene transcription (Hertz et al. 1998). LCA-CoA can also impact insulin sensitivity through flow-on effects on FA metabolism and synthesis of other deleterious lipids.
Intracellular lipids and insulin resistance

The influence of LCA-CoAs on insulin action is speculated to better reflect acute changes in tissue lipid metabolism than other lipid molecules that arise through more chronic exposure to FA excess (Ellis et al. 2000).

**Acylcarnitines**

Acylcarnitines are generated during an early stage of mitochondrial FAO, converted from LCA-CoAs and carnitine via carnitine palmitoyltransferase 1 (CPT1) at the outer mitochondrial membrane before reconversion to constituent parts via CPT2 in the mitochondrial matrix. Accumulation of this lipid intermediate can often therefore serve as a measure of incomplete FAO (Van Hove et al. 1993, Koves et al. 2008, Mihalik et al. 2010, Aguer et al. 2013) and thereby also of mitochondrial energy substrate overload. Given the association of diminished FAO with IR, it is perhaps unsurprising that long-term acylcarnitine accumulation in plasma and skeletal muscle has also been presented as a feature of the condition (Ukropcova et al. 2005, Mihalik et al. 2010, Wolf et al. 2013, Aguer et al. 2015, Xiang et al. 2017). Pharmacological reduction of acylcarnitine content in a mouse model of IR has been reported to recover insulin sensitivity and reduce blood glucose levels (Liepinsh et al. 2016). An accompanying study, which heightened acylcarnitine content in mice through both acute and long-term administration of palmitoylcarnitine, saw the consequential induction of muscle-specific IR (Liepinsh et al. 2017). Regulation of metabolic flexibility, production of reactive oxygen species and inhibition of insulin signalling have all been suggested as mechanisms linking acylcarnitines with IR (Muonio et al. 2012, Aguer et al. 2015, Liepinsh et al. 2017), although it is still unclear to what extent acylcarnitines have a primary role in the induction of IR.

**FA esters of hydroxy-fatty acids**

A new class of endogenous lipids identified by untargeted mass spectrometry have been linked with IR. Discovered in tissues and serum of mice overexpressing the glucose transporter protein GLUT4 in adipose tissue (AG4OX mice), branched fatty acid esters of hydroxy-fatty acids (FAHFAs) consist of a series of isomeric combinations of a fatty acid and a hydroxy-fatty acid moiety (Yore et al. 2014). FAHFA levels are higher in adipose tissue and serum of the insulin-sensitive AG4OX model and are reduced in fat and serum of insulin-resistant humans (Yore et al. 2014). Anti-inflammatory and insulin-sensitising effects of these lipids are proposed to be in part mediated by activation of the G-protein-coupled receptor GPR120 (Yore et al. 2014, Moraes-Vieira et al. 2016). In a similar vein, a recent study in humans reported that the adipose levels of another ‘non-conventional’ form of FA, monomethyl branched-chain FA (mmBCA) were responsive to weight loss and positively correlated with insulin sensitivity (Su et al. 2015).

**Phospholipids**

Cellular phospholipids comprise the key structural determinant of membrane biophysical properties, dictating facets of topology and fluidity. The principal components of the membrane lipid population, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have each been positively correlated with insulin sensitivity in human skeletal muscle, while the PC:PE ratio is negatively correlated (Newsom et al. 2016, Lee et al. 2017). Phospholipid species containing polyunsaturated fatty acids (PUFAs) are associated with improved insulin action, partly due to their effects on greater membrane fluidity and insulin receptor abundance (Yorek et al. 1989, Borkman et al. 1993, Pan et al. 1995, Janovská et al. 2010). The ω-3 PUFA-containing phospholipids in particular are reasonably well established to benefit insulin sensitivity in rodents and humans, even in conjunction with lipid excess (Popp-Snijders et al. 1987, Fasching et al. 1991, Haugaard et al. 2006, González-Pérez et al. 2009, Stephens et al. 2014). Their reduction in HFD-fed and IR conditions is seen alongside increased ω-6 PUFA content, and an overall increase in phospholipid saturation (Clore et al. 2000, Smith et al. 2010, Hoeks et al. 2011, Montgomery et al. 2017). These remodelling patterns have recently been shown to be most prominent in IR-susceptible mouse strains, while being partially resisted in the IR-resistant BALB/c strain (Montgomery et al. 2017). Phospholipid remodelling is hardly confined to the context of insulin action, however, with membrane lipid composition also implicated in skeletal muscle growth and maintenance, mitochondrial biogenesis, oxidative capacity, contractile function and exercise performance (Funai et al. 2013, Selathurai et al. 2015, Funai et al. 2016). Primary or direct causal mechanisms of phospholipids in IR aetiology are not yet fully established.

**Diacylglycerols**

The second messenger diacylglycerol (DAG) is one of the key lipid intermediates most frequently proposed to mediate IR (Erion & Shulman 2010, Samuel & Shulman 2012). From the initial identifications of elevated DAG

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content in IR rat tissues (Turinsky et al. 1990), multiple subsequent studies have demonstrated correlations between DAG accumulation and impaired insulin action in various tissues in both rodents and humans (Samuel et al. 2004, 2007, Jornayvaz et al. 2011, Magkos et al. 2012). Conversely, decreases in DAG content have been associated with protection from IR (Neschen et al. 2005, Choi et al. 2007).

The prime attraction of DAG as a candidate for inducing IR is the existence of clearly defined mechanisms that have been put forward to explain its effects. DAGs have long been known to activate protein kinase C (PKC) (Hannun et al. 1986, Bishop & Bell 1988), which in conditions of lipid overload can result in direct interference with components of the canonical insulin signalling pathway. In muscle, DAGs activate the novel PKCβ and PKCδ isoforms (Itani et al. 2002, Szendroedi et al. 2014); in liver, the PKCε isoform (Samuel et al. 2004, 2007, Zhang et al. 2007, Petersen et al. 2016). Consequent PKC action in these tissues prevents the normal insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), promotes inhibitory serine phosphorylation, with the net effect an inhibition of phosphorylation/activation of the downstream serine/threonine kinase protein kinase B (Akt/PKB) (Szendroedi et al. 2014). Accordingly, ablation of PKCδ protects mice from lipid-induced defects in muscle insulin signalling and glucose transport (Kim et al. 2004b), while deletion/knockdown of PKCε ameliorates whole-body and hepatic IR in fat-fed animals (Samuel et al. 2007, Schmitz-Peiffer et al. 2007).

### Ceramides and sphingolipids


Mechanistically, the most convincingly demonstrated link between ceramide accumulation and the development of IR is the impairment of Akt activation. Ceramide signalling stimulates and stabilises the binding of atypical PKCζ to Akt so that the latter kinase cannot bind phosphatidylinositol (3,4,5)-triphosphate (PIP₃) and consequently cannot be activated by insulin (Bourbon et al. 2000, Stratford et al. 2001, Powell et al. 2003, Fox et al. 2007). Similarly activated by ceramide is protein phosphatase 2A (PP2A), which dephosphorylates and thus impairs Akt (Salinas et al. 2000, Teruel et al. 2001, Zinda et al. 2001, Chavez et al. 2003). These two inhibitory mechanisms are independent, targeting distinct protein domains. They appear to have cell type-dependent functional dominance (Bourbon et al. 2002, Stratford et al. 2004, Fox et al. 2007), but operate concurrently in at least some instances (Dey et al. 2007, Chen et al. 2017). Despite the direct link to insulin action, however, the fact that these mechanisms have been identified primarily in muscle has prompted some researchers to raise doubts as to their relevance in other tissues (Petersen & Shulman 2017).

Less well-reported impacts on the canonical insulin signalling pathway include the inhibition of IRS-1 action via ceramide-stimulated pathways thought to include some combination of double-stranded RNA-dependent protein kinase (PKR), mixed-lineage kinase-3 (MLK3), c-Jun N-terminal kinase (JNK), the iberikin complex (IKK) and/or protein-tyrosine phosphatase 1B (PTP1B) (Hehner et al. 2000, Sathyanarayana et al. 2002, Kim et al. 2004, Gual et al. 2005, MohammadTaghvaei et al. 2012, Hage Hassan et al. 2016). Similarly, ceramide-associated activation of JNK and IKK is suggested to block PIP₃ action via increased expression of the SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) (Gorgani-Firuzjaee et al. 2014). Somewhat more indirectly, altered ceramide and sphingolipid composition can promote the mislocalisation of membrane-associated proteins including the insulin receptor by disrupting membrane fluidity, curvature and microdomain (lipid raft) structure (Lasserre et al. 2008, Pewzner-Jung et al. 2010, Gao et al. 2011, Yurlova et al. 2011, Silva et al. 2012, Park et al. 2013). Ceramide accumulation may also impact hepatic lipid uptake via CD36 (Xia et al. 2015) or the inhibition of insulin-induced gene expression in β-cells (Poitout & Robertson 2008).
The metabolic fate of ceramides provides further potential for contribution to IR aetiology. Through (often reversible) reactions that add a variety of head-group molecules, ceramide can be converted into a diverse range of more complex sphingolipids: phosphocholine or glucose moieties, for example, giving rise to sphingomyelin or glucosylceramide respectively. Certain species of these sphingolipids have also been associated with metabolic dysfunction, particularly in studies demonstrating the beneficial effects of their deficiency. The genetic ablation of sphingomyelin synthase 2 (Sns2), for instance, has been found in HFD-fed rodents to improve whole-body insulin sensitivity, glucose tolerance and weight gain (Li et al. 2011, Sugimoto et al. 2016). It has been suggested, however, that these benefits may be due partially to elevations in very-long-chain (VLC) ceramides rather than directly from sphingomyelin deficiency (Sugimoto et al. 2016). Sarcolemmal sphingomyelin was recently reported to be negatively related to insulin sensitivity in human muscle (Perreault et al. 2018). The evidence towards a role of the glycosphingolipid species in IR is somewhat more substantive. Pharmacological inhibition of glucosylceramide synthase, which catalyses the initial step in glycosphingolipid synthesis, has likewise demonstrably improved insulin sensitivity and glucose tolerance in ob/ob mice, diet-induced obese mice and Zucker diabetic fatty rats (Aerts et al. 2007, Zhao et al. 2007). The ganglioside GM3 (ganglioside monosialo 3), which comprises a further derivation of glucosylceramide, has moreover been shown to mediate the effects of TNFα and directly interfere with the activity and membrane localisation of the insulin receptor to inhibit insulin signalling (Tagami et al. 2002, Yamashita et al. 2003, Kabayama et al. 2005, Kabayama et al. 2007). In providing the precursor to these detrimental sphingolipid species, ceramide synthesis, if perhaps not accumulation per se, can be attributed to a role in IR development.

Discrepancies in the field of lipid-induced IR

Despite the substantial literature linking aberrant accumulation of the aforementioned lipid classes with IR, there are many controversies in the field and the relative influence of any one of these lipids over another remains uncertain. The published literature is rife with studies which, when compared alongside each other, show discrepancies if not outright contradictions – with these opposing viewpoints particularly evident in DAG- and ceramide-centric research. Studies demonstrating enhanced insulin action due to decreased DAG content are countered by those which show increased DAG levels without detriment to insulin sensitivity (Chavez et al. 2003, Brown et al. 2010, Amati et al. 2011, Turpin et al. 2011, Selathurai et al. 2015). Human studies correlating ceramide accumulation with IR are similarly contradicted by studies which report dissociation between the two (Itani et al. 2002, Skovbro et al. 2008, Nowotny et al. 2013, Szendroedi et al. 2014), while inhibition of ceramide synthesis cannot always rescue insulin sensitivity (Lee et al. 2010). Another key example which demonstrates that elevated intracellular lipid may not always be an obligate inducer of IR is the so-called ‘Athlete’s Paradox’. Highly trained individuals have exquisite insulin sensitivity, despite elevated levels of multiple lipid classes including DAGs and ceramides (Goodpaster et al. 2001, Amati et al. 2011). The corresponding enhancement in the capacity for mitochondrial lipid oxidation in athletes is thought to largely underlie this phenomenon.

So why are discrepancies in this field so common? Some may be simply due to comparisons across markedly differing experimental setups and conditions, with experimental diets, housing temperatures, mouse strain and methodology for assessing insulin action (e.g. hyperinsulminemic–euglycemic clamps vs glucose tolerance tests or Akt phosphorylation) varying significantly between studies. Even the same transgenic mouse model of liver-specific DGAT2 (diacylglycerol O-acyltransferase 2) overexpression, in the hands of two different groups, produced disparate fold-changes in accumulated DAG and TAG, and entirely opposite conclusions: IR did not develop in the initial study (Monetti et al. 2007) but did in the second (Jornayvaz et al. 2011). Correlational studies could also potentially be misleading with regard to causation, much less sufficiency or necessity.

Interplay between lipid-related mechanisms is likely, and indeed at least nominally agreed upon (Meikle & Summers 2017, Petersen & Shulman 2017), as is the existence of as-yet- unidentified, non-canonical means of inducing IR. It is now becoming more broadly accepted, however, that seemingly contradictory evidence linking certain lipid species to IR is also perhaps a reflection of lipid subcellular location, the specific subspecies present and the timing of measurements relative to lipid fluxes.

Subcellular localisation

Lipids are not static within cells and most are typically not restricted to a specific subcellular location. Because signalling events and metabolism of nutrients are generally restricted to discrete locations within cells, it
stands to reason that analysis of the ‘bulk’ lipids from an entire cell or tissue sample could obscure localised changes in key lipid species that are directly relevant to the process in question. But even though this concept is important and starting to gain traction, there is little consensus on the specific nature of these relationships. With respect to DAGs, several studies have associated IR development in muscle and liver with membrane DAG accumulation, reasoning that DAG activation of PKC at the membrane would have a major impact on the insulin signalling events that occur in this region (Bergman et al. 2012, Cantley et al. 2013, Chan et al. 2015). These studies suggest protective effects of DAG redistribution to lipid droplets and/or the lipid-associated ER (Bergman et al. 2012, Cantley et al. 2013, Chan et al. 2015). Despite this, others have found the precise reverse: that the key predictor of hepatic IR is cytosolic DAG accumulation, with membrane content only weakly related (Jornayvaz et al. 2011, Kumashiro et al. 2011, Ter Horst et al. 2017). Contradictory findings are largely unreconciled, and broadly attributed to experimental model or sample procurement and fractionation procedures (Bergman et al. 2012, Cantley et al. 2013). Moreover, recent work in human skeletal muscle indicates that potential relationships between subcellular distribution of DAG and insulin action might be more related to accumulation of specific DAG species at different intracellular sites, rather than the bulk content of DAG (Perreault et al. 2018).

The compartmentalisation of ceramide synthesis, just as for DAGs, produces localised lipid pools that are differentiated in composition and function. Ceramides are primarily generated de novo in the ER, but also from the hydrolysis of sphingomyelin at the plasma membrane and via sphingosine salvage in the mitochondria. Subcellular transport mechanisms, particularly to the Golgi where ceramides are further metabolised, show preferential targeting for specific ceramide species (Kumagai et al. 2005, Konstantynowicz-Nowicka et al. 2015, Kakazu et al. 2016, Yamaji et al. 2016), indicating the likelihood of differential function according to spatial distribution. Temporal studies have further found that ceramide species in the nucleus are differentially composed and regulated to those found in mitochondria (Aviram et al. 2016). Although relatively few studies have investigated the effects of ceramide subcellular localisation in the specific context of IR, it has been shown that ceramide content in subsarcolemmal, but not intermyofibrillar, mitochondria is related to IR parameters in postprandial humans (Chung et al. 2017a,b). More recently, an inverse relationship has been found between insulin sensitivity and ceramides of the sarcolemmal, mitochondrial/ER, and nuclear compartments in human muscle, with only the cytosolic lipids being unrelated (Perreault et al. 2018).

**Subspecies and isomer/isomer**

As described earlier, there are thousands of different lipid species, which we are now gaining a better appreciation of due to advances in analytical techniques. Using DAGs as an example, the primary de novo pathway of DAG biosynthesis occurs during TAG production, with the esterification of FAs to a phosphoglycerol backbone at sn-1 and -2, within the ER and Golgi. DAGs are also derived from the (reversible) hydrolysis of phospholipids at the plasma membrane, and of TAGs at cytosolic lipid droplets, generating sn-1,2 and sn-1,3 DAGs respectively. There is now an appreciation that the biological effects of different DAG species are related to both variations in acyl chain length and saturation, as well as isomeric differences (position of bonds on the glycerol backbone i.e. sn-1,2, sn-1,3, sn-2,3).

The influence of isomer, considered within the context provided by the established PKC-centric mechanism of DAG-mediated IR, can also perhaps suggest some clarification of the discrepancies seen regarding the relevance of DAG subcellular location. PKC activation is conventionally quantified by protein translocation from cytosol to membrane, although some lack of clarity remains regarding the relative importance of recruitment in the cytosol vs activation at the membrane (see above). Nevertheless, multiple early studies found that PKC activation is stereospecific and achieved only by sn-1,2 DAGs (Rando & Young 1984, Boni & Rando 1985, Leach et al. 1991, Wakelam 1998, Takai et al. 2012). The sn-1,3 DAGs found in lipid droplets have no effect on PKC, and indeed their accumulation in muscle has recently been shown in mice to have no negative effects on insulin signalling or glucose uptake (Serup et al. 2016, Lundsgaard et al. 2017). Likewise, differentiation in human muscle insulin sensitivity has been found to be unrelated to sn-1,3 or sn-2,3 DAGs (Perreault et al. 2018). The PKC-relevant membrane-associated sn-1,2 pool is derived from phospholipid hydrolysis and even more specifically from PIP2 (Leach et al. 1991). It is perhaps feasible that sn-1,2 DAG content can be relevant to PKC activation even when found in the cytosolic fraction, given that DAGs can be shuttled between the PM and ER (Saheki et al. 2016). A recent study in human muscle identified a positive relationship between insulin sensitivity and total sn-1,2 DAGs of the mitochondrial/ER and nuclear...
fractions, but within the mitochondrial/ER fraction, an inverse correlation was noted for disaturated sn-1,2 DAGs (Perreault et al. 2018).

PKC substrate preferences are further delimited by the length and degree of (un)saturation of DAG acyl chains, with the different isoforms displaying a variety of sensitivities. The IR-associated novel PKCs δ, θ and ε are for example preferentially activated by DAG species containing longer and polyunsaturated acyl chains (Marignani et al. 1996, Madani et al. 2001, Kamiya et al. 2016). Although measures of specific DAG acyl species are neither unanimously reported nor uniformly associated across the literature, the relationship has been at least partially corroborated on a whole-body scale (Holloway et al. 2014). Rodent studies taking a more interventionist approach have altered cellular DAG pool composition and abundance by, for example, targeting specific degradation or metabolism pathways, which also often possess specific preferences for DAG substrate species. Diaclylglycerol kinases (DGKs) catalyse the conversion of DAGs to phosphatidic acid. The intracellular type II isoform DGKδ targets palmitic acid-containing DAGs and its decreased expression is closely related to the development of IR (Chibalin et al. 2008, Sakai et al. 2014). In contrast, the ER- and plasma membrane-bound type III isoform DGKε, which is instrumental in PI-cycling, has particularly high specificity for PKC-relevant 18:0/20:4 DAG (Lung et al. 2009, Shulga et al. 2011, Nakano et al. 2016). Its ablation, despite increasing DAG levels, demonstrably increases whole-body glucose tolerance by means other than improving muscle insulin sensitivity (Mannerås-Holm et al. 2017).

As a determinant in dissecting conflicting results, however, DAG acyl species may not wholly apply in isolation: di-C18:0 DAG accumulation in muscle has been identified as the greatest contributor to DAG-mediated IR (Van Hees et al. 2011, Bergman et al. 2012, Holloway et al. 2014), but also as preferentially correlated with the enhanced insulin sensitivity displayed by athletes (Amati et al. 2011) and, more recently, via calcitriol supplementation (Jefferson et al. 2017). There is some indication that chain length, too, can depend on subcellular location for its relevance. Ter Horst et al. (2017) have found accumulated cytosolic DAGs of C18:1–C16:0, C16:0–C16:0 and C18:1–C18:1 to coincide with hepatic suppression of endogenous glucose production; membrane DAG of C20:4–C20:5 was meanwhile positively correlated with insulin sensitivity.

In contrast to the dual effect of acyl chain length and position on DAG function, the primary structural determinant differentiating ceramide subspecies and function is acyl chain length. Acylation is catalysed by the six ceramide synthase isoforms (CerS1-6), which are essential to two of the three ceramide synthesis pathways and display isoform-specific preferences for FA-CoA chain length (Tidhar et al. 2012, Tidhar & Futerman 2013). Variable acylation grants ceramides correspondingly differentiated biophysical properties, and consequently differential interactions. These in some cases fulfil entirely opposing functions: C16-ceramide promotes cell apoptosis, whereas C24-ceramide is anti-apoptotic and promotes proliferation (Karahatay et al. 2007, Mesicek et al. 2010, Hartmann et al. 2012, Stiban & Perera 2015). In the context of IR, long-chain (LC) and VLC ceramides have similarly divergent roles. C16-ceramide is identified as the critical species mediating impairment of glucose tolerance and hepatic insulin sensitivity (Raichur et al. 2014, Turpin et al. 2014). Mice deficient in this species were described to exhibit a favourable metabolic profile without significant pathologies (Turpin et al. 2014, Gosejacob et al. 2016). C18-ceramides, predominant in muscle and strongly correlated with whole-body glucose metabolism, are implicated as mediators of muscle IR and offer an additional target for beneficial reductions (Bergman et al. 2016, Blachnio-Zabielska et al. 2016, Tonks et al. 2016, Perreault et al. 2018).

The VLC ceramides (C>22), in contrast, are observed to promote favourable metabolic processes. With respect to insulin action, recent work has shown correlations between murine glucose homeostasis and C24-ceramide content in liver and that directly increasing C24-ceramide content improves insulin signalling (Montgomery et al. 2016). These findings are consistent with the glucose intolerance/IR observed in livers of CerS2-knockout mice and obese HFD-fed CerS2 haploinsufficient mice (Park et al. 2013, Raichur et al. 2014). In addition, elevations in C24-ceramide content have recently been associated with increased insulin sensitivity in skeletal and in cardiac muscle, both in vivo and in vitro (Xie et al. 2015, Chung et al. 2017b, Jefferson et al. 2017). Role differentiation mandates that, rather than total accumulation or deficit per se, cell health is underlain by ceramide equilibrium.

Shifts in ceramide acyl chain profile may occur naturally as an adaptive chronic cellular response to FA excess. Certainly, an apparent compensatory mechanism has been evident in human and murine models, in vitro and in vivo, adjusting ceramide ratio to maintain steady total levels following CerS deficiency (Pewzner-Jung et al. 2010, Mullen et al. 2011). The identified range of epigenetic, transcriptional, post-transcriptional and post-translational CerS regulatory mechanisms moreover...
appear to be isof orm specific (Wegner et al. 2016). Interaction with ELOVL1 (elongation of VLC FAs protein 1) or ACPB (acyl-CoA-binding protein), for example, stimulates specifically CerS2 and CerS3 (Sassa et al. 2013, Ferreira et al. 2017). Less is known, however, regarding regulatory impairment.

The effect of fatty acyl side chains extends beyond DAGs and ceramides, with specific acyl species of phospholipids, acylcarnitines and LCA-CoAs also implicated in IR (Borkman et al. 1993, Mihalik et al. 2010, Montgomery et al. 2017, Stöckli et al. 2017). As a final note, there may also be some further relevance to be found in acyl chain lengths when their associations with IR are compared across lipid species. The aforementioned divergence between LC- and VLC-Cer acyl species with regard to IR has recently been paralleled in a study relating LC-DAGs to IR and VLC-DAGs to insulin sensitivity (Ter Horst et al. 2017). Skeletal muscle IR has been associated with changes in C18-acyl chain species for both DAGs and ceramides alike (Bergman et al. 2012, Holloway et al. 2014, Szendroedi et al. 2014, Zabielski et al. 2017, Bergman et al. 2018, Perreault et al. 2018).

**Lipid fluxes – timing is everything**

Progression of IR is overtly and inherently time dependent. Any associated alterations in lipid content, whether stimulus or consequence, likewise develop over time, as has been clearly demonstrated on the whole-body scale (Kraegen et al. 1991, Turner et al. 2013). Both in vitro and in vivo studies have shown that the consequences of lipid oversupply are not only time dependent, but in some cases, also transient (Yu et al. 2002, Szendroedi et al. 2014, Rooomp et al. 2017). Such transient shifts could well be missed if measurements are taken outside what can comprise a relatively narrow window: glucose-stimulated DAG turnover in ß-cells, for example, produces effects that are typically <10s in duration (Wuttke et al. 2013). Even changes with genuine (patho)physiological relevance could also be masked by the inherent dynamism of natural cellular lipid metabolism. Flux rates through the elaborately interconnected metabolic pathways, within cells and between tissues, can appear disconnected from simple metabolite quantification and expression profiles (Turner et al. 2007, You et al. 2014, Harding et al. 2015). Futile cycles, converting substrate back and forth between reversible reactions, exacerbate the disconnection.

Conventionally, however, lipid analyses are with reference to the single time point at which the sample was taken and thus lose any contextual data on natural flux dynamics. Chosen time points may not accurately reflect transient responses in lipid metabolism and are frequently distinct from the timepoint where measures of insulin action/glucose homeostasis are taken (e.g. a glucose or insulin tolerance test are performed several days prior to the collection of tissue for biochemical analyses). While experimental design often dictates this requirement, the disconnect in timeframe may create ostensible but misleading correlations. The analysis of lipid flux, subspecies and subcellular compartmentation—howsoever beneficial to understanding—is also simply not always feasible to perform in combination.

**Perspective**

There is a large body of evidence linking disordered lipid metabolism with IR; yet, there is still significant controversy regarding the principal lipid(s) that impair insulin action. Advances in lipidomic techniques have revealed a new layer of complexity and other relevant techniques in this area are being developed (and adopted). Stable isotope studies permit labelled compounds to be tracked through metabolic pathways in either in vivo or in vitro setups (Magkos & Mittendorfer 2009, Umpleby 2015). Recent investigation into lipid droplets has developed methodology for more accurate measurements of (instantaneous alterations in) lipogenic activity in living cells via time-course label-free stimulated Raman scattering (SRS) imaging (Zhang et al. 2017). Current advances in mass spectrometry have been more comprehensively reviewed elsewhere (Gross 2017, Triebl et al. 2017), but in brief, they offer increasingly sensitive lipid profiling techniques to better examine discrete spatial compartmentation and complex biological mixtures (Gething et al. 2017, Gulin et al. 2017, Kaya et al. 2017, Xiang et al. 2017). Although hardly yet standard practice, these exciting new methodologies will increase our capacity to understand the finer details of lipid intermediate accumulation in IR and potentially reveal targets with therapeutic utility for treating obesity and type 2 diabetes.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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