REVIEW

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.

Key Words

- ▶ insulin resistance
- ▶ lipids
- ▶ lipidomics
- ▶ fatty acid metabolism

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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

accumulation in non-adipose tissues, with alterations to adipose tissue function/metabolism that occur during obesity thought to be intimately involved. Hypertrophic and hyperplastic responses both expand adipose storage capacity in apparent direct compensation upon lipid accumulation. Such increases in adipocyte size and number are also proven, however, to independently predict the development of IR. Controlled for BMI, insulin-resistant individuals have been found to possess a greater proportion of small adipose cells than do insulin-sensitive individuals, while their large adipose cells are fewer but larger (Weyer *et al.* 2000, McLaughlin *et al.* 2007, Johannsen *et al.* 2014, McLaughlin *et al.* 2014, Kim *et al.* 2015). Moreover, adipogenesis is impaired with IR (Deurenberg *et al.* 1991, Yang *et al.* 2004, McLaughlin *et al.* 2007, 2014) and promotion of adipocyte differentiation improves insulin sensitivity (De Souza *et al.* 2001, McLaughlin *et al.* 2010, Eliasson *et al.* 2014). Taken together, such findings tie IR to stunted expandability of adipose tissue, and a corresponding overspill of lipids from overloaded adipose depots into insulin-sensitive peripheral tissues such as skeletal muscle and liver.

At the level of tissues such as muscle and liver, intracellular lipid accumulation is logically caused by either heightened uptake, diminished utilisation and for some tissues enhanced lipogenesis. Rodent models have demonstrated that muscle- and liver-specific overexpression of the lipid hydrolysis enzyme, lipoprotein lipase (LPL) leads to selective lipid accumulation and IR in the respective tissues (Kim *et al.* 2001). In contrast muscle-specific deletion of LPL results in improved insulin sensitivity in muscle, but impaired insulin action in liver and other tissues (Wang *et al.* 2009). Whole-body deletion of fatty acid transport protein FATP1 protects against the development of IR in skeletal muscle (where it is highly expressed) (Kim *et al.* 2004a), while viral-mediated hepatic deletion of FATP2 or FATP5 protects against hepatic steatosis and glucose intolerance, although how modulating these transporters in liver influenced insulin action in other tissues was not assessed (Doege *et al.* 2008, Falcon *et al.* 2010). These findings are not without their 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).

Figure 1

Lipid classes and metabolites linked with insulin resistance. Example structures of lipids within each class are shown, along with proposed mechanisms by which accumulation or depletion of these lipids is thought to induce insulin resistance. Grey lines highlight that LCA-CoAs provide fatty acyl chains for all lipid species, as well as other key conversions between lipid classes. FAHFA, branched fatty acid esters of hydroxy fatty acids; ROS, reactive oxygen species.

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, Henrique *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), glycogen synthase (Wititsuwannakul & Kim 1977), glucokinase (Tippet & 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

(Fig. 1). 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 (Muoio *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

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 PKC θ and PKC δ isoforms (Itani *et al.* 2002, Szendroedi *et al.* 2014); in liver, the PKC ϵ 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 PKC θ protects mice from lipid-induced defects in muscle insulin signalling and glucose transport (Kim *et al.* 2004b), while deletion/knockdown of PKC ϵ ameliorates whole-body and hepatic IR in fat-fed animals (Samuel *et al.* 2007, Schmitz-Peiffer *et al.* 2007).

Ceramides and sphingolipids

Ceramide, the basic unit of all sphingolipids, is situated at a nexus of sphingolipid metabolism: converted to a myriad of other more complex sphingolipid species, which are in turn recycled back to ceramide. Lipidomic profiling suggests that ceramide accumulation in plasma, muscle, liver, adipose and/or brain strongly corresponds with development of IR (Adams *et al.* 2004, Strackowski *et al.* 2004, Holland *et al.* 2007, Haus *et al.* 2009, Coen *et al.* 2010, Frangioudakis *et al.* 2010, Ussher *et al.* 2010, Longato *et al.* 2012, Xia *et al.* 2015, Luukkonen *et al.* 2016, Campana *et al.* 2018, Perreault *et al.* 2018). Exercise and/or weight loss interventions, improving insulin sensitivity, have also been shown to reduce ceramide content in skeletal muscle and in plasma (Dube *et al.* 2008, 2011, Amati *et al.* 2011, Bergman *et al.* 2015, Warshauer *et al.* 2015). Additionally, pharmacological and genetic interventions

to reduce (whole-body) ceramide content, by inhibiting its synthesis or enhancing its downstream metabolism, improve insulin sensitivity in both humans and rodents (Holland *et al.* 2007, Yang *et al.* 2009, Ussher *et al.* 2010, Dekker *et al.* 2013, Turpin *et al.* 2014, Holland *et al.* 2017).

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 PKC ζ 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 I κ B kinase 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 (*Sms2*), 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 TNF α 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. hyperinsulinemic–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 isoform/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 isozymes 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. Diacylglycerol kinases (DGKs) catalyse the conversion of DAGs to phosphatidic acid. The intracellular type II isoform DGK δ 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 DGK ϵ , 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 vitro* and *in vivo* (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 isoform specific (Wegner *et al.* 2016). Interaction with ELOVL1 (elongation of VLC FAs protein 1) or ACBP (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, Roomp *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 β -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—however 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, Triebel *et al.* 2017), but in brief, they offer increasingly sensitive lipid profiling techniques to better examine discrete spatial compartmentation and complex biological mixtures (Gethings *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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