Storing up trouble: does intramyocellular triglyceride accumulation protect skeletal muscle from insulin resistance?

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Summary

1. Insulin resistance occurs when normal amounts of insulin are inadequate to produce a normal insulin response from cells. This is important in the context of whole-body glucose homeostasis because skeletal muscle is the main tissue for insulin-stimulated glucose disposal.

2. In obesity, lipid deposition in peripheral tissues such as skeletal muscle is linked to the activation of stress kinases and the development of insulin resistance. Intramyocellular triglyceride (IMTG) accumulation is positively associated with insulin resistance; however, it is unknown whether IMTG causes insulin resistance or protects cells from insulin resistance by preventing the accrual of bioactive lipid metabolites.

3. The role of IMTG in the development of insulin resistance is not resolved. Stable overexpression of the triglyceride lipase, adipose triglyceride lipase (ATGL), reduced IMTG content in myotubes but resulted in a concomitant increase in diacylglycerol (DG) and ceramide, and caused insulin resistance. Increasing TG content by muscle-specific diacylglycerol acyltransferase (DGAT) 1 overexpression protected mice from insulin resistance. Conversely, overexpression of DGAT2 in glycolytic muscle resulted in TG and ceramide accumulation, and insulin resistance in these tissues. This was sufficient to induce whole-body insulin and glucose insensitivity.

4. IMTG are unlikely to directly cause insulin resistance. Instead, it appears as though TG accumulates in skeletal muscle to sequester fatty acids and to protect from the deleterious actions of lipids such as ceramide and DG. Whether lipase inhibitors are viable therapeutics to prevent obesity-induced insulin resistance is unknown, but future studies examining tissue-specific ATGL / hormone sensitive lipase knockout will hopefully resolve this question.

Introduction

The ability to store energy in the form of triglyceride (TG) is highly conserved and is an essential process for survival. TGs are stored within lipid droplets that are composed of a core of neutral lipids (primarily TGs and cholesterol esters) surrounded by a phospholipid monolayer and lipid-droplet associated proteins.[1] Most of the body's TGs are stored in adipose tissue where they are sequentially hydrolysed by the enzymes adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase to produce fatty acids and glycerol[2] (Figure 1). These metabolites are released and transported in the circulation for delivery to other tissues. The liberated fatty acids are transported into many cell types where they undergo several fates including β-oxidation for ATP production and storage as phospholipids and other lipid types. TG is also synthesised in cells *via* the glycerolipid pathway (Figure 1).

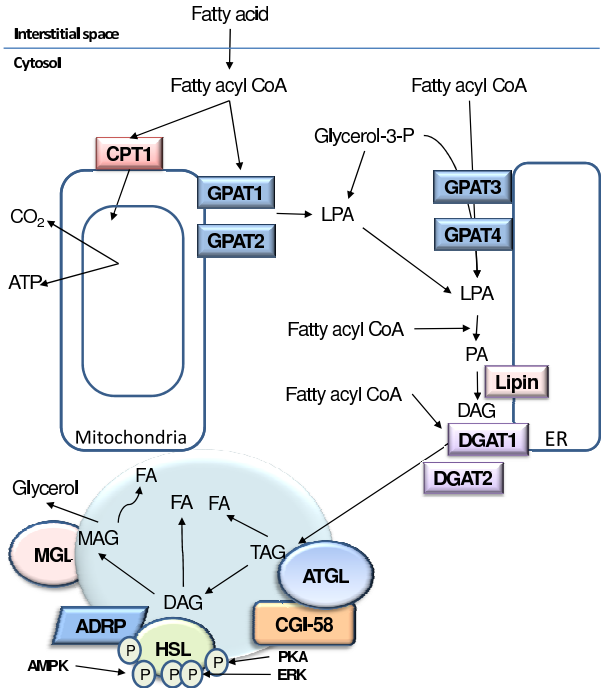

Figure 1. Control of skeletal muscle triglyceride metabolism. *Triglyceride synthesis is initiated by several glycerol-3phosphate acyltransferases (GPAT1-4) that synthesise lysophosphatidic acid (LPA) from long chain fatty acyl-CoA and glycerol-3-phosphate substrates. LPA is acylated to form phosphatidic acid (PA) and lipin converts PA to DAG. Diacylglycerol acyltransferase (DGAT) 1 and 2 acylate DAG to form TAG. Both adrenaline and contraction-mediated events mediate TG hydrolysis. Adipose triglyceride lipase (ATGL) cleaves the first fatty acid to create DAG. DAG is hydrolysed to monoacylglycerol by hormone sensitive lipase (HSL), which is regulated by several activating (*e.g. *protein kinase A, extracellular regulated kinase) and deactivating (*e.g. *5-AMP activated protein kinase) protein kinases. Monoacylglycerol lipase (MGL) cleaves the final fatty acid to produce free glycerol.*

TGs are also stored in non-adipose tissues where they can provide fatty acid substrate for metabolic processes. Skeletal muscle is an important peripheral tissue that is

central to whole-body metabolic homeostasis. Skeletal muscle accounts for ~40% of body mass, is the major tissue contributing to energy expenditure,[3] mediates a large proportion of FA uptake and oxidation[3] and in humans contributes ~80% of whole body insulin-stimulated glucose disposal.[4] The amount of IMTG found within individual muscle fibres, between different muscle fibre types (type I > type II) and between muscle groups is variable, and ranges between 2 and 10 mmol/kg mass.[5] Transmission electron microscopy reveals that lipid droplets containing TG are stored throughout the muscle fibre with ~60% in close contact with mitochondria,[6] suggesting a role for TGs in energy homeostasis. Indeed, skeletal muscle TG is an important energy substrate during physical stress, with the liberated fatty acids accounting for ~20% of the total energy expenditure during moderate intensity exercise.[7,8] Although skeletal muscle TG is an important energy substrate, excessive accumulation has been linked to systemic insulin resistance.[9,10] While the understanding of TG catabolism in adipose tissue has expanded rapidly in recent years,[11,12] the cellular and molecular mechanisms mediating skeletal muscle TG storage and breakdown, and the metabolic consequences of these processes are incompletely described.

Control of skeletal muscle triglyceride metabolism

Intramuscular lipolysis

The key lipolytic enzymes of adipose TG lipolysis, HSL and ATGL, are expressed in skeletal muscle.[13,14] Conversely, the scaffold protein, perilipin A, which is essential for regulating adipose tissue lipolysis,[11] is not expressed in skeletal muscle, highlighting a critical difference between adipose and muscle lipolysis. Other lipid droplet associated proteins from the PAT family are expressed in skeletal muscle and include adipocyte differentiation-related protein (ADRP) and tail-interacting protein of 47 kDa (TIP-47).[15] Immuno-inhibition studies indicate that HSL accounts for ~60% of total TG lipase activity in skeletal muscle at rest and almost all activity during contractions and adrenergic stimulation.[14,16,17] Adrenaline is a key activator of HSL and lipolysis. Adrenaline is elevated during exercise and results in β_2-adrenergic receptor stimulation, PKA activation, phosphorylation of Ser 563 and Ser 660 on HSL[18] and increases in HSL activity.[14,19,20] HSL is also regulated by contraction-generated signals. Protein kinase C can stimulate HSL activity *via* extracellular regulated kinase (ERK) activation during muscle contractions,[20,21] which is consistent with an activating role of ERK in adipose tissue,[22] while calcium calmodulin dependent kinase II is suggested to block HSL activation and IMTG lipolysis.[23,24] The cellular energy sensor 5′-AMP activated protein kinase (AMPK) negatively regulates HSL in skeletal muscle.[17,18,20,25] Intracellular trafficking of lipolytic enzymes in response to lipolytic stimuli is an essential regulatory control point in adipocytes.[12] Morphological analysis of single muscle fibres reveals that HSL translocates to lipid droplets within myocytes in response to adrenaline and contraction and that ADRP and TIP-47 are localised to lipid droplets.[15] HSL association with ADRP may be essential for lipolytic control in skeletal muscle given the absence of perilipin A in this tissue. The role of PAT proteins in skeletal muscle requires further study.

Studies in HSL null mice reveal the maintenance of TG lipase activity and DG accumulation in skeletal muscle, suggesting the presence of other TG lipases.[26] ATGL, is expressed in skeletal muscle and ATGL null mice have reduced TG lipase activity and accumulate TG in muscle.[13] ATGL overexpression increases TG lipase activity and reduces TG content in both cultured myotubes and rodent skeletal muscle.[27] The posttranslational regulation of ATGL is not currently understood. Collectively, these studies indicate that ATGL may be an important TG lipase at rest, but HSL appears to regulate β_2-adrenergic and contraction-induced TG mobilization *via* phosphorylation, translocation to the lipid droplet and possibly association with ADRP and TIP-47 (Figure 1).

Intramuscular esterification

Two key proteins involved in the TG biosynthetic pathway are glycerol-3-phosphate (GPAT) and diacylglycerol acyltransferase (DGAT), which catalyse the first and last steps in TG synthesis, respectively (Figure 1). Glycerol-3-phosphate acyltransferase (GPAT) catalyses the acylation of sn-glycerol-3-phosphate at the *sn*-1 position to form lysophosphatidic acid as the initial and committed step in the *de novo* synthesis of glycerolipids. There are four GPAT isoforms, mitochondrial (GPAT 1 and 2) and microsomal (GPAT 3 and 4), and little is known about their expression and regulation. Mitochondrial GPAT protein expression and enzyme activity is higher in slow-twitch *vs* fast twitch muscle fibres, which is consistent with the increased IMTG content in this fibre type.[28] Mitochondrial GPAT does not appear to be regulated during exercise in humans.[29] Total GPAT protein expression is increased in human skeletal muscle after exercise,[30] however, GPAT activity is decreased in rodents after exercise.[30,31] This discrepancy is difficult to explain but suggests that posttranslational modification may play a role in GPAT regulation. In this regard, studies in myotubes demonstrate that AMPK decreases GPAT activity by phosphorylation,[32] which is consistent with AMPKs role as an energy sensor that promotes fatty acid oxidation for ATP production rather than fatty acid storage.

DGAT catalyses the final step of TG synthesis, a reaction that covalently links DG and fatty acyl-CoA substrates. DGAT1 and 2 bear no sequence homology to each other and do not belong to the same gene family.[33] DGAT2 is more potent[34] and specific[35] for triglyceride biosynthesis. Like GPAT, there is a paucity of data regarding DGAT expression and regulation. DGAT1 and 2 are expressed in skeletal muscle. DGAT1 mRNA[36] and protein[30] expression are increased by exercise, consistent with the increased IMTG observed in recovery from exercise. DGAT activity does not appear to be regulated by AMPK.[32]

Fatty acid-induced insulin resistance

Insulin signal transduction

Insulin resistance occurs when normal amounts of insulin are inadequate to produce a normal insulin response from cells. This is important in the context of whole-body glucose homeostasis because skeletal muscle is the main tissue for insulin-stimulated glucose disposal. Insulin resistance is largely mediated by interference of insulin signal transduction within tissues. Insulin signaling involves a complex signaling cascade with the major pathway being the phosphatidylinositol 3-kinase (PI3K)-Akt (PKB) pathway (Figure 2). Activation of the insulin receptor leads to tyrosine phosphorylation of insulin receptor substrates (IRS) 1-4, of which IRS-1 appears to be the most important in skeletal muscle. IRS-1 propagates signal transduction by stimulation of PI3K, leading to phosphorylation/activation of Akt, phosphorylation of Akt-substrate of 160 kDa (AS160) and glucose transporter (GLUT) 4 translocation. An alternate pathway for GLUT4 translocation involves insulin-mediated stimulation of atypical PKC isoforms. Indeed, muscle-specific knockout of the major murine atypical PKC, PKC-λ, is sufficient to induce local and systemic insulin resistance.[37] When IRS-1 is alternatively phosphorylated at various serine residues (*e.g.* serine 307), its downstream signaling is diminished resulting in reduced glucose uptake. Serine/threonine kinases that phosphorylate these serine residues include the stress kinases c-jun terminal amino kinase (JNK),[38] IκB kinase-β (IKK-β)[39] and conventional PKC isoforms.[40,41] Insulin signaling can also be disrupted at Akt (discussed below).

Linking fatty acids to insulin resistance

Intramuscular lipid accumulation is associated with insulin resistance[9,10] and experimentally increasing intramuscular lipids with lipid infusions[40,42] or genetic modifications such as muscle specific overexpression of lipoprotein lipase and fatty acid translocase/CD36[43,44] causes insulin resistance. The metabolic events connecting fatty acid oversupply to stress kinase activation (*e.g.* JNK, IKK-β) and insulin resistance are incompletely defined; however, several lipid metabolites appear to be involved (Figure 2). DG accumulates in skeletal muscle *via* shuttling of fatty acids through the glycerolipid synthesis pathway and from TG degradation. DG accumulates in the skeletal muscle of obese rodents[45,46] and humans[47] and many studies have shown temporal associations between acute elevation of plasma free fatty acids, DG accumulation and insulin resistance.[40] DG is thought to induce serine/threonine phosphorylation of IRS-1 at Ser307 due to activation of PKCθ and IKKβ,[48,49] however, others have shown that genetic disruption of IKKβ or PKCθ does not protect against obesity-induced insulin resistance.[50,51] Experimental approaches that selectively manipulate intracellular DG levels are required to determine whether DGs are in fact a critical regulator of fatty acid-induced insulin resistance.

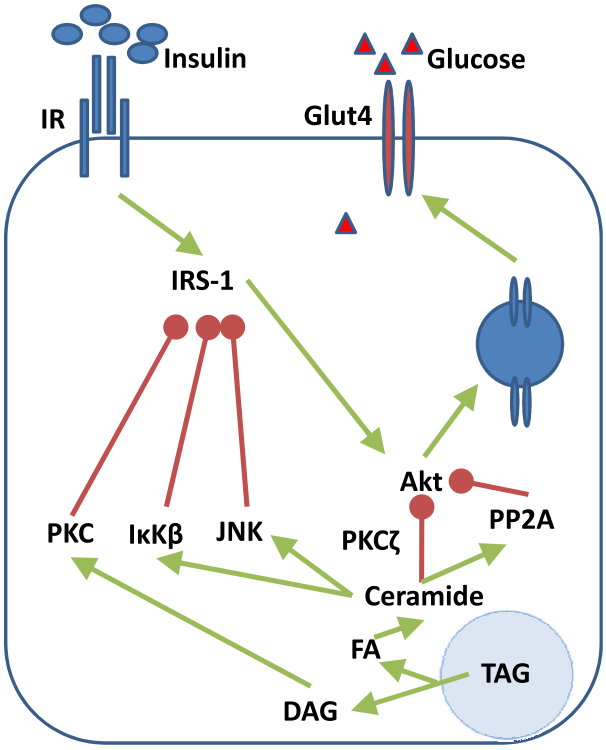

Figure 2. Fatty acid mediators on insulin resistance. *Several fatty acids interfere with insulin signal transduction. DAG results in activation of several PKC isoforms that may induce serine phosphorylation of insulin receptor substrate (IRS)-1. The fatty acids liberated from TAG breakdown can be used for* de novo *ceramide synthesis. Ceramide activates protein phosphatase 2A (PP2A) that in turn dephosphorylates (T308, S472) and deactivates Akt. By activating protein kinase Cζ, ceramide induces phosphorylation of Akt (T43) that prevents its translocation. Ceramide also activates JNK and IκKβ, which inhibits insulin signal transduction by serine phosphorylation of IRS-1. These events ultimately lead to reduced glucose transporter 4 (Glut4) translocation to the plasma membrane and less glucose transport.*

Ceramides are composed of a sphingosine backbone and fatty acid side chain, and their synthesis is driven by increased levels of the saturated fatty acid palmitate. They accumulate in skeletal muscle of obese, insulin-resistant humans[52] and rodents,[46] and are negatively correlated with insulin sensitivity.[53] Ceramides are linked to insulin resistance induced by saturated, but not unsaturated fatty acids, and chemical inhibition of ceramide synthesis *in vivo* improves glucose tolerance.[54] Ceramides inhibit insulin signaling at Akt and possibly at IRS-1. The principal site of inhibition involves Akt: first by dephosphorylation mediated by protein phosphatase 2A[55] and second by preventing translocation of Akt, possibly by PKCζ mediated phosphorylation at Thr-34.[56] Inhibition of IRS-1 tyrosine phosphorylation occurs when cultured cells are incubated with short-chain ceramide analogues.[57] Mixed linage kinase (MLK) 3 is a putative link in this process as it

is activated by ceramides and is an upstream kinase of JNK,[58,59] however, several studies do not report inhibition of IRS-1 by ceramides.[54] Ceramide derivatives such as sphinosine and ganglioside GM3 may be involved in the pathogenesis of lipid-induced insulin resistance, but this awaits further experimental clarification.[60]

LCFACoA accumulates in skeletal muscle with obesity[61] and with acute fatty acid-induced insulin resistance.[40] LCFACoAs effect glucose utilization by directly inhibiting hexokinase activity and through indirect mechanisms involving PKC activity and by acting as a substrate for DG and ceramide generation.[62] Recent studies indicate that specific phosphatidic acid species (*i.e.* dilinoleoyl) contribute to insulin resistance in muscle cells.[60]

Role of triglycerides in muscle metabolism and insulin resistance: Recent advances

Increased TG deposition in skeletal muscle is positively associated with insulin resistance,[9,10,42,63-65] however, it is not currently known whether IMTG causes insulin resistance. This uncertainly results from two observations. Firstly, in contrast to the positive association between IMTG and insulin resistance in obesity and type 2 diabetic patients, endurance trained athletes are highly insulin sensitive despite elevated IMTG deposition.[66,67] The reason for this "athletes paradox" is unknown but may be due to an increase in lipid droplet-to-mitochondria contact[6] and an enhanced oxidative capacity of athletes to oxidize TG-derived fatty acids[66] and thereby prevent the accumulation of insulin resistance-inducing fatty acid metabolites. Secondly, studies that have altered IMTG levels through various perturbations (*e.g.* diet, lipid and heparin infusion, exercise) have concomitantly changed other lipid metabolites such as DG, ceramides and long chain acyl-CoAs, making it impossible to discern specific IMTG effects. There is an alternative view that IMTG provides a protective role in skeletal muscle by preventing the accumulation of so-called bioactive lipid species such as ceramides and DG, factors known to cause cellular "lipotoxicity"[68] and insulin resistance. The cellular localisation of TGs within lipid droplets supports this view. Moreover, the reversal of fatty acid-induced insulin resistance in humans after exercise is accompanied by an enhanced lipogenic capacity of skeletal muscle, increased IMTG synthesis and concomitant reductions in lipid metabolites and proinflammatory signalling.[30] To overcome these methodological issues, recent studies have implemented several experimental designs in mice to investigate the role of IMTG content on skeletal muscle insulin resistance.

ATGL overexpression

ATGL was recently cloned and was demonstrated to be essential for efficient TG metabolism in adipose tissue.[69-72] ATGL shares several common motifs with other known TG lipases including a GXSXG motif with an active serine at position 47, an α/β hydrolase fold and an N-terminal "patatin" homology domain, which is common to plant and mammalian proteins with acyl-hydrolase activity on phospholipid, monoglyceride and DG substrates.[72,73] It exerts a key role in lipid droplet degradation in adipocytes and non-adipocyte cells[70,74,75] and ATGL null mice are obese due to defective lipolysis.[13] ATGL exhibits high substrate specificity for TGs[69,72] and full activation appears to require interaction with the regulator CGI-58.[76] Given the substrate specificity of ATGL, we reasoned that one approach for examining the effect of TG on insulin sensitivity was to overexpress ATGL in skeletal muscle. We anticipated that DG would not accumulate because HSL has a high substrate affinity for DG.[77]

ATGL protein expression is reduced in skeletal muscle of obese insulin resistant mice, and is negatively associated with IMTG content, indicating a potential role of ATGL in IMTG metabolism. The effects of ATGL were tested in two models: retroviral overexpression in L6 myotubes and acute electroporation-mediated overexpression in rat tibialis anterior *in vivo*.[27] Both approaches increased ATGL protein expression, TG lipase activity and reduced IMTG content. Surprisingly, DG accumulated in muscle suggesting that the catalytic activity of HSL could not match the increased supply of DG substrate produced with ATGL overexpression. Ceramides also accumulated indicating that the increased flux of TG-derived fatty acids was facilitating *de novo* ceramide synthesis. There was no effect of ATGL overexpression on *in vivo* insulin sensitivity as assessed by euglycemic-hyperinsulinemic clamp. While these studies showed that ATGL is an important TG lipase in muscle that alters fatty acid storage and oxidation, reducing TG content by ~50% was unable to prevent insulin resistance, indicating that TG *per se* is not an important factor in directly mediating insulin resistance. Rather, excessive TG degradation may promote insulin resistance when fatty acid oxidation cannot match intracellular production, resulting in the accumulation of bioactive fatty acid metabolites.

DGAT overexpression

An alternative strategy for examining the role of TG on insulin resistance is to enhance its storage. Two recent studies have described the phenotypes of mice where TG accumulation was driven by similar transgenic approaches. DGAT catalyses the formation of TG from DG and fatty acyl-CoA. Two DGAT isoforms exist, with DGAT2 being more potent[34] and specific[35] than DGAT1. In the first study by Liu *et al.*,[78] transgenic overexpression of DGAT1 in mouse skeletal muscle increased IMTG by ~30% and protected mice from high fat diet-induced insulin resistance. The heightened insulin sensitivity was associated with a reduction in DG and ceramide contents, and reduced serine kinase activation. This channelling of fatty acids in myocytes *in vivo* was consistent with previous work demonstrating that DGAT1 promotes the conversion of saturated fatty acids to TG and protects mammalian cells from lipid-induced-apoptosis.[79] In this regard, ATGL null mice that are unable to efficiently hydrolyse TG exhibit

whole body insulin sensitivity despite marked IMTG accumulation.[13] Together, these studies indicate that driving fatty acids into TG and/or preventing their release protects from obesity-induced insulin resistance.

We recently completed similar experiments, but instead focussed on the role of DGAT2 when it was overexpressed in glycolytic (type II) muscle fibres.[80] Skeletal muscle is composed mainly of two different fibre types: oxidative (type I, slow-twitch, red) and glycolytic (white, type II, fast-twitch, white) fibres. It is unknown whether the type of muscle fibre is an important consideration in TG associated insulin resistance. Oxidative muscle stores more TG[81] and is more insulin-sensitive than glycolytic muscle.[82] Moreover, endurance training increases both TG content and insulin sensitivity in oxidative muscle.[66] This suggests that increased lipid content in oxidative muscle may not be detrimental. On the other hand, glycolytic muscle stores less TG and is less insulin sensitive than oxidative muscle. Accordingly, we hypothesised that TG accumulation in glycolytic muscle would induce insulin resistance. DGAT2 was overexpressed in glycolytic muscle by using the muscle creatine kinase (MCK) promoter. MCK-DGAT2 mice stored more TG in glycolytic muscle and less DG compared with wild-type control mice. Surprisingly, MCK-DGAT2 mice accumulated ceramide and LCFACoA in glycolytic muscle and this was associated with inhibition of IRS-1 / PI3K and PKC-λ activity and insulin-stimulated glucose disposal. Significantly, reducing insulin sensitivity in glycolytic muscle was sufficient to induce whole-body glucose intolerance and insulin resistance. While these studies show that DGAT2 overexpression in glycolytic muscle caused insulin resistance, a definitive role for TG could not be ascertained due to concomitant increases in muscle ceramide and LCFACoA.

The discordant findings between the DGAT1 and DGAT2 overexpression studies support the premise that oxidative capacity, or the ability to efficiently oxidise fatty acids (*i.e.*, type I > type II muscle fibres), may be an important factor determining whether a muscle cell is likely to become insulin resistant. In other words, storing TG in muscle that is not programmed to oxidize it may be problematic. Alternatively, there may be intrinsic differences between the myocellular TG pool that results from DGAT2 *vs* DGAT1 overexpression, with the former promoting signaling programs that interfere with insulin's effects.

Summary and future directions

Muscle TG are implicated in the development of insulin resistance; however, they are unlikely to directly cause insulin resistance primarily because they are located within lipid droplets. Instead, it appears as though TG accumulates in skeletal muscle to protect muscle cells from the deleterious actions of fatty acid species such as ceramide, DG and LCFACoA. Whether lipase inhibitors are viable therapeutics to prevent obesity-induced insulin resistance is unresolved, but future studies examining tissue-specific ATGL / HSL knockout will hopefully resolve this question. However, the consequences of continued TG storage may present unexpected complications, especially in sedentary individuals where the capacity to clear excess TG-derived fatty acids is impaired and lipotoxicity may result. Clarifying the cellular and molecular factors modulating the expression and posttranslational control of key proteins in TG metabolism may unmask novel approaches to safely manipulate TG content in health and disease.

Acknowledgements

I would like to acknowledge members of the lab and the collaborators that conducted much of this research, with particular thanks to Drs. Malin Levin, Mara Monetti and Robert Farese from the Gladstone Institute, USA. MJW is supported by a R Douglas Wright Fellowship from the National Health and Medical Research Council of Australia and is a Monash Fellow.

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Received 17 March 2008, in revised form 9 July 2008.
Accepted 11 July 2008.

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