

Fatty acid metabolism, energy expenditure and insulin resistance in muscle

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Abstract

Fatty acids (FAs) are essential elements of all cells and have significant roles as energy substrates, components of cellular structure and signalling molecules. The storage of excess energy intake as fat in adipose tissue is an evolutionary advantage aimed at protecting against starvation, but in much of today's world, humans are faced with an unlimited availability of food, and the excessive accumulation of fat is now a major risk for human health, especially the development of type 2 diabetes (T2D). Since the first recognition of the association between fat accumulation, reduced insulin action and increased risk of T2D, several mechanisms have been proposed to link excess FA availability to reduced insulin action, with some of them being competing or contradictory. This review summarises the evidence for these mechanisms in the context of excess dietary FAs generating insulin resistance in muscle, the major tissue involved in insulin-stimulated disposal of blood glucose. It also outlines potential problems with models and measurements that may hinder as well as help improve our understanding of the links between FAs and insulin action.

Key Words

- ▶ fatty acid metabolism
- ▶ fatty acids and energy expenditure
- ▶ muscle insulin resistance

Journal of Endocrinology
(2014) 220, T61–T79

Overview

Fatty acids (FAs) are organic acids largely defined by the length and saturation of the aliphatic side chain attached to a carboxylic acid. In animals, these side chains normally contain an even number of carbon atoms and FAs are grouped into short chain (2–6 carbon atoms), medium chain (8–12 carbon atoms), long chain (14–18 carbon atoms) and very long chain (20–26 carbon atoms). The major types of FAs in the circulation and in the tissues of mammals are the long-chain and very-long-chain FAs with varying degrees of saturation. These include palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid

(C:18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and, particularly in smaller mammals, arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3). These FAs are the major components of storage triglycerides and cellular membranes, and although C16–C18 FAs are also components of some of the FA-derived signalling molecules (diacylglycerols (DAGs) and ceramides), many of the major lipid signalling molecules (prostaglandins and leukotrienes) are synthesised from very-long-chain, unsaturated FAs (e.g. arachidonic and docosahexaenoic acids) (Kruger *et al.* 2010).

In the context of the links between excessive lipid storage (obesity) and reduced insulin action (insulin

resistance) in muscle, this article will deal with FAs as an alternative energy substrate to glucose, the relevance of this substrate competition to overall energy expenditure and an assessment of the various mechanisms by which excess FA availability is thought to reduce insulin action in muscle and predispose to metabolic diseases.

Fuel for energy production

All three of the major types of macromolecules that make up organic material (carbohydrates, proteins and fats) can be broken down and oxidised to provide energy for the maintenance, growth and reproduction of biological systems. In animals, all proteins have a cellular function (e.g. as enzymes, or with structural or carrier function), and there is no identifiable depot of proteins specifically manufactured and stored solely for future use in energy production. On the other hand, carbohydrates and fats in various forms have specific and important functional roles in cells, but are also present in animal tissues as energy storage depots of glucose polymers (glycogen) and lipid droplets (triglycerides). Although glycogen and triglyceride stores can be found in nearly all tissues, glycogen stored in the liver is critical for the maintenance of blood glucose levels when glucose is not being absorbed from the gut and triglycerides stored in adipose tissue act as an alternative, more reduced and higher energy-yielding substrate (in terms of energy per gram) for energy production in tissues with a capacity for fat oxidation. Although excess protein intake can be converted to glucose and FAs for energy storage and glucose can also be converted to fat for energy storage or amino acids for protein synthesis, it is one of the maxims of energy metabolism that fat cannot be quantitatively converted to carbohydrate or protein. Essentially, this means that FAs stored in adipose tissue can only be used as an energy source to support cellular functions or to provide specific precursors that are needed to replace or expand the structure or signalling functions of FAs.

The contribution of different tissues to fuel oxidation and energy expenditure

Some tissues have an obligatory need for glucose (brain, red blood cells and retinal cells), while most tissues have the capacity to switch between glucose and FAs. The contribution of different fuels to energy production in specific tissues and the contribution of different tissues to the overall energy production and utilisation in the whole body vary quite markedly. Because of its relative size in

man and most animals, muscle is considered to be a major tissue for the disposal of both glucose (James *et al.* 1985, Shulman *et al.* 1990) and FAs (Furler *et al.* 2000). Because of the ability of muscle to substantially increase energy expenditure during exercise (Bangsbo 2000), this tissue is also very flexible in its capacity to act as a sink for energy substrates. Other tissues such as the heart have a similar capacity to increase both the amount and type of substrate oxidation depending on demand, but because of the relative size of heart to muscle in the body, the overall contribution of the heart to whole-body substrate oxidation is only 5–10% (Rolfe & Brown 1997). The liver has a significant role in the disposal of glucose after a meal and in the provision of glucose to the circulation to maintain blood glucose levels when nutrients are not being absorbed from the gut. The liver also has the ability to take up FAs, oxidise them or package them in lipoproteins for export and storage in other tissues and is therefore central to lipid and glucose homeostasis (Postic *et al.* 2004, Moore *et al.* 2012). Adipose tissue can, particularly in obese individuals, be the tissue contributing most to whole-body mass, but per unit mass it does not have a major impact on whole-body glucose disposal (Kraegen *et al.* 1985, Ng *et al.* 2012). White adipose tissue also has little impact on the whole-body oxidation of FAs, although there is significant current research interest in investigating whether white adipocytes can acquire a more oxidative brown adipocyte phenotype with a greater contribution to whole-body substrate oxidation and energy expenditure (Wu *et al.* 2013).

The effect of fibre composition and exercise on substrate utilisation by muscle

Although the musculature as a whole is a major contributor to total body glucose and FA metabolism (Ng *et al.* 2012), individual muscles may contribute differently depending on their fibre composition. Type I red muscle fibres are considered more insulin sensitive, with a greater oxidative capacity for glucose and FAs, while type II white muscle fibres contain less mitochondria, are considered less insulin sensitive and contribute less to FA oxidation (Nyholm *et al.* 1997, Pearen *et al.* 2012). Therefore, a higher composition of type I red fibres in muscle has been reported to be associated with increased insulin responsiveness (Stuart *et al.* 2013). This view has been challenged by some recent studies where genetically manipulated mice (Izumiya *et al.* 2008, Meng *et al.* 2013) and pharmacological approaches (Akpan *et al.* 2009) suggest that altering the fibre composition of muscles towards

glycolytic type II fibres improves glucose homeostasis and insulin action in the whole animal. It does seem important to consider that the contribution of the skeletal musculature to whole-body energy metabolism and substrate oxidation should not be based on the assessment of these parameters in a single muscle type. Acute exercise and exercise training also have a significant impact on substrate preference and utilisation at a whole-body and muscle level (Spriet & Watt 2003, Kiens 2006). Some of these effects correlate with observed shifts in muscle size and fibre type that occur with training (Shaw *et al.* 2012, Stuart *et al.* 2013), but other adaptations in muscle metabolism and body organs could also contribute to changes in energy metabolism and substrate utilisation associated with exercise (Laughlin & Roseguini 2008).

Linking substrate oxidation to energy conservation and energy expenditure

The pathways by which different fuels are oxidised to support tissue and cellular energy demands in animals are thoroughly dealt with in major textbooks and summarised in Fig. 1. Through the glycolytic pathway, pyruvate dehydrogenase (PDH) and the tricarboxylic acid (TCA) cycle, glucose can be completely oxidised to CO₂ and the energy released (as reducing equivalents) harnessed in the form of NADH+H⁺ and FADH₂. NADH+H⁺ and FADH₂ are reoxidised by the electron transport chain (ETC) and the reducing equivalents used to reduce atomic oxygen to water. The electrochemical (proton) gradient generated by the ETC then drives ATP synthesis via ATP synthase (Fig. 1). The oxidation of FAs by the mitochondrial β-oxidation pathway also produces NADH+H⁺ and FADH₂ for the ETC and acetyl CoA that can also be completely oxidised in the TCA cycle. Because FAs are chemically more reduced molecules than carbohydrates, FAs are theoretically able to produce more energy when completely oxidised than an equivalent carbohydrate molecule. In other words, the complete oxidation of six-carbon glucose consumes six oxygen molecules and produces six carbon dioxide molecules accompanied by the synthesis of 36 ATP molecules. On the other hand, the complete oxidation of six-carbon hexanoic acid consumes eight oxygen molecules and produces six carbon dioxide molecules for 44 ATP molecules. Such calculations are based on a fixed stoichiometry between NADH+H⁺ and FADH₂ oxidation and ATP synthesis and can lead to the conclusion that the oxidation of FAs produces ATP at a cost of greater oxygen consumption or lower efficiency. Therefore, a switch to the oxidation of FAs as the major

energy substrate should result in less efficient ATP production and an increase in whole-body energy expenditure that could lead to a loss of fat mass if energy intake remains constant (Clapham 2004a,b, Leverve *et al.* 2007).

Indirect calorimetry is often used in human and animal studies to determine total energy expenditure (indirectly by the measurement of oxygen consumption and carbon dioxide production), and the measurement of oxygen consumption and carbon dioxide production can also be used to calculate the relative use of glucose and FAs to support that energy expenditure (respiratory exchange ratio, RER), assuming that any contribution of protein oxidation is relatively small and constant (Ferrannini 1988, Arch *et al.* 2006). Based on the assumption that there is a direct stoichiometry between NADH+H⁺ and FADH₂ oxidation, proton translocation and ATP synthesis, calculations have been made suggesting that a complete switch from glucose to FAs as a source of energy would increase oxygen consumption by 7%. However, in practice, it is unlikely that such theoretical calculations can be applied to the regulation of energy balance with any certainty. For instance, rarely does the measured RER shift from complete glucose oxidation (1.0) to complete FA oxidation (0.7), even with prolonged exercise (Gimenez *et al.* 2013) and starvation (Hoeks *et al.* 2010) or without some change in protein oxidation. More importantly, although there is a direct stoichiometry between the oxidation of a substrate molecule and the production of NADH+H⁺ and FADH₂, NADH+H⁺ can be reoxidised in reactions other than Complex I of the ETC and the proton motive force generated by the ETC can be dissipated by processes other than ATP synthesis (e.g. counter-ion transport and uncoupling protein activity) (Mazat *et al.* 2013). The concepts of efficiency and plasticity in the coupling of substrate oxidation to energy conservation (ATP synthesis) have been expanded on in several authoritative review articles (Harper *et al.* 2008, Mazat *et al.* 2013). These review articles have highlighted the presence of a significant and variable basal proton leak in mitochondria (20–25%) of most tissues and reported that in perfused rat muscle systems futile proton cycling may contribute as much as 50% to the respiration rate (Rolfe & Brand 1996), although other methodologies have suggested that this could be as little as 10% (Marcinek *et al.* 2004, Conley *et al.* 2007).

Irrespective of the exact mechanisms of proton leak and mitochondrial coupling of substrate oxidation to ATP production and oxygen consumption, it seems clear that using strict stoichiometric relationships (three ATPs per

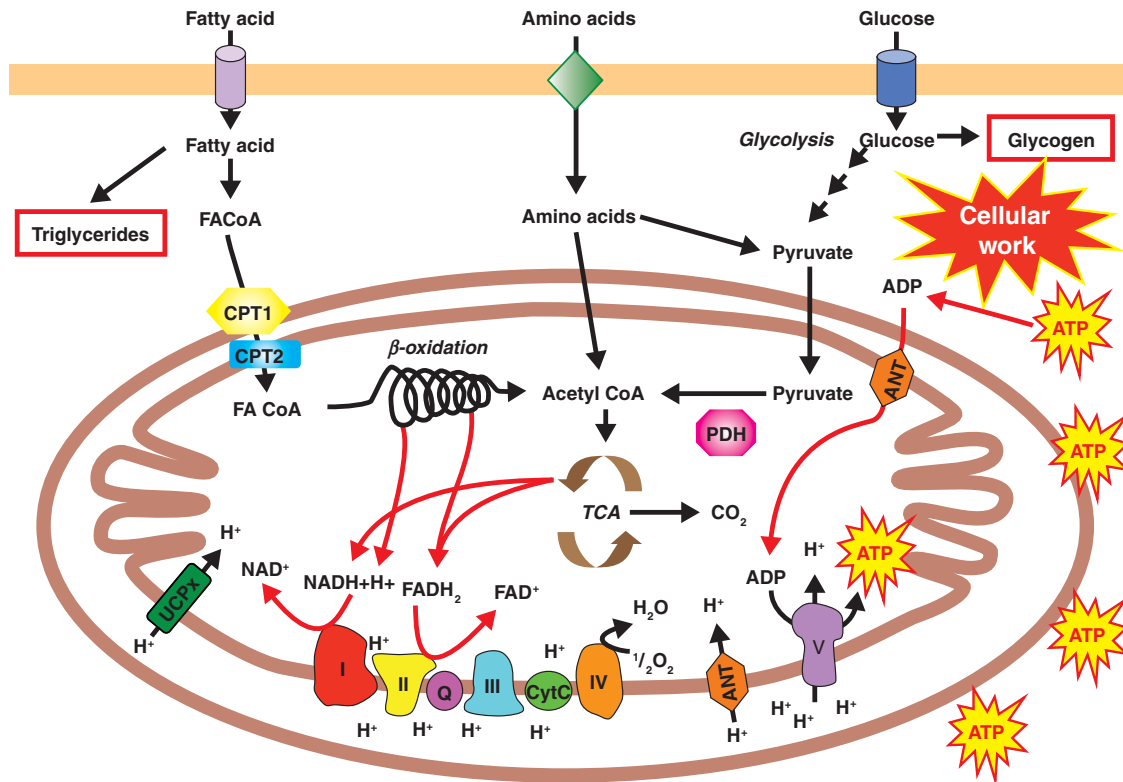


Figure 1

Pathways of substrate metabolism in muscle. Oxidation pathways of glucose, FAs and amino acids converge at the level of acetyl CoA. The energy of oxidation is conserved as $\text{NADH} + \text{H}^+$ and FADH_2 , which are used by the electron transport chain to produce a chemiosmotic gradient across the inner mitochondrial membrane. This proton motive force is dissipated

by ATP synthesis and by proton leak via the adenine nucleotide transporter (ANT) and activated uncoupling proteins (UCPx). Demand for ATP and proton leak are greater determinants of oxygen consumption and heat production than the substrate being oxidised.

$\text{NADH} + \text{H}^+$ and two ATPs per FADH_2) to calculate whole-body oxygen consumption and energy expenditure from the measurements of relative substrate oxidation is unlikely to reflect the actual measurements of energy expenditure. In reality, coupling efficiency can vary significantly depending on changes in proton leak or ATP demand, but in cell systems at least, changes in substrate oxidation do not appear to influence the relationship between oxygen consumption and ATP synthesis (Brand *et al.* 1993).

Can switching substrate alter energy expenditure?

The above discussion clearly leads to the conclusion that the cost of generating mitochondrial ATP in terms of ETC activity and oxygen consumption can vary significantly and is not affected to any large extent by the substrate being oxidised to provide the reducing equivalents for

electron transport. Despite this, it is not uncommon to read about studies in whole animal systems (particularly genetically modified mice) where differences in fat mass are often mechanistically related to changes in the mRNA levels of FA metabolism genes in a variety of tissues without appropriate consideration of the contribution of these tissues to whole-body energy expenditure (Abu-Elheiga *et al.* 2001, 2003, Lee *et al.* 2009, Hu *et al.* 2012, Ronis *et al.* 2013). In the context of investigations of energy balance in lean and obese mice, there are excellent recent reviews pointing out potential problems with assessing differences in food intake and energy expenditure using indirect calorimetry systems and extrapolating any differences to explain gain or loss of fat mass (Butler & Kozak 2010, Tschöp *et al.* 2012). For example, expression of oxygen consumption or heat production on a kilogram body weight basis can be misleading if animals have significantly different amounts of fat tissue, because the metabolic rate of fat per gram is much lower in tissues such

as muscle and liver (Frayn *et al.* 1995). Similarly, the difference in daily food intake needed to contribute to a significant gain of body fat over several weeks in mice can be so small as to be undetectable unless large numbers of mice (200–300) are used for the comparison (Tschop *et al.* 2012). Changes in the body weight and body fat of groups of adult mice with different genotypes on different diets should reflect cumulative differences in energy intake and energy expenditure. However, any differences might not be easily detected if animals are assessed for food intake and energy expenditure individually in indirect calorimetry systems, away from their home cage and communal environment for only a 24–48-h period of the several weeks over which body weight and fat mass have been monitored.

AMPK activation, FA oxidation and energy expenditure

AMP-activated protein kinase (AMPK) is recognised as a master regulator of energy metabolism, particularly in times of energy stress such as exercise, hypoxia and starvation (Hardie *et al.* 2012). The activation of AMPK has been shown to acutely increase FA and glucose uptake and metabolism in a variety of experimental situations including *in vitro* and *in vivo* experiments in muscle (Iglesias *et al.* 2004, Smith *et al.* 2005). The long-term effects of AMPK activation in muscle lead to the activation of gene transcription pathways that increase mitochondrial biogenesis and proteins of oxidative metabolism (Winder *et al.* 2000, Hardie *et al.* 2012). The acute regulation of FA oxidation by AMPK is largely through the phosphorylation and inactivation of the enzyme acetyl CoA carboxylase 2 (ACC2). ACC2 produces malonyl CoA, an allosteric inhibitor of the key enzyme carnitine palmitoyltransferase 1 (CPT1), which controls the entry of FAs into the mitochondria for oxidation (Hardie & Pan 2002).

The pharmacological activation of AMPK has been shown to produce changes in muscle metabolic pathway capacity similar to those produced by exercise training (O'Neill *et al.* 2013); however, there is considerable controversy as to whether AMPK activation can drive energy expenditure in the absence of exercise. A series of studies employing genetic deletion of *Acc2* (*Acacb*) have reported reduced fat depots in association with increased FA oxidation in isolated muscle (Abu-Elheiga *et al.* 2001, 2003) and have subsequently reported increased energy expenditure (although not increased FA oxidation) in *Acc2*-knockout mice with less fat and less lean mass

(Choi *et al.* 2007). These results suggest that the inhibition of ACC2 by the activation of AMPK or development of ACC2 inhibitors might promote FA oxidation and produce fat loss. Subsequent studies using independently generated *Acc2*-knockout mice did not reproduce these effects, reporting that although these mice exhibited increased FA oxidation at the whole-body and isolated muscle level, there was no measurable difference in energy expenditure, fat mass or food intake (Hoehn *et al.* 2010). However, there was an increased glycogen content in muscle, an effect of AMPK activation noted previously (Winder *et al.* 2000, Buhl *et al.* 2001), which is consistent with AMPK activation and ACC2 inhibition promoting FA oxidation and channelling glucose taken up by muscle into storage as glycogen (Vitzel *et al.* 2013). Another study using independently generated genetically manipulated mice has reported no difference in body weight, food intake or fat mass in global or muscle-specific *Acc2* gene-deleted mice (Olson *et al.* 2010), adding to the conclusion that altering FA oxidation in the absence of any change in energy expenditure or energy intake is insufficient to have a significant impact on whole-body fat mass.

Therefore, it would appear that apart from theoretical calculations suggesting that increasing fat oxidation will drive increased energy expenditure, there is little experimental evidence to support the idea that energy expenditure can be increased simply by increasing substrate availability or by switching to oxidise FAs.

Insulin regulation of energy metabolism

From an energy metabolism point of view, the flow of different substrates to tissues for oxidation or storage is largely under the control of the circulating hormone insulin. After a meal, direct stimulation of the β -cells of the islets of Langerhans of the pancreas by nutrients (glucose, FAs and amino acids) increases insulin release into the circulation. Certain gut hormones (GLP1 and G-1-P) can also augment insulin secretion, as can neural signals from the brain (Thorens 2011). Insulin has many stimulatory and inhibitory actions in different tissues mediated by a complex intracellular signalling pathway, but for the purpose of this discussion, the actions of insulin to stimulate glucose uptake and metabolism in muscle and regulate FA metabolism will be a major focus. The failure of insulin to appropriately regulate glucose and FA metabolism is termed insulin resistance, and this condition is most frequently observed in the muscle and liver of overweight or obese individuals (Eckardt *et al.* 2011). Insulin resistance is considered a significant predisposing

factor for the development of type 2 diabetes (T2D) and therefore there is considerable research effort put into determining the mechanistic relationship between excess lipid accumulation (obesity) and insulin resistance, particularly in muscle. Studies from over 20 years ago first showed that triglyceride accumulation in the muscle of high-fat diet-fed rats coincided with insulin resistance (Storlien *et al.* 1987, Kraegen *et al.* 1991), thereby establishing the hypothesis that insulin resistance is causally related to triglyceride accumulation in muscle. Since then, the relationship between muscle lipid accumulation and insulin resistance has also been established in humans, and many mechanisms have been put forward to explain how lipid accumulation could generate insulin resistance (Bosma *et al.* 2012, Samuel & Shulman 2012). Over the last decade, the major challenge has been determining whether these proposed mechanisms are universal or specific to the model of lipid-induced insulin resistance being studied. It is also possible that different mechanisms are important at different times during the development of insulin resistance and that some proposed mechanisms depend on the experimental methods used to assess insulin action.

Methods for assessing insulin action in muscle

All discussions of the relationship between increased fat metabolism and insulin action are dependent on the methodology used to assess insulin resistance and the assumptions associated with different methodologies. As has been mentioned previously, nearly all investigations of lipid-induced insulin resistance in rodent models utilise high-fat diet feeding to increase adiposity, but the methods of assessing insulin action can be quite varied and rely on glucose tolerance tests or insulin tolerance tests and less frequently (because of the technical difficulty) on hyperinsulinaemic–euglycaemic clamps. Various technical considerations of glucose and insulin tolerance tests must be considered when discussing the metabolic implications of these tests for muscle insulin action. The timing and route of administration of glucose and duration of fast before glucose administration influence the results of glucose tolerance tests (Andrikopoulos *et al.* 2008, McGuinness *et al.* 2009), and our recent studies suggest that changes in glucose tolerance may reflect changes in lipid content and insulin action in the liver more than insulin action in muscle, especially in the initial stages of fat accumulation after starting a high-fat diet (Montgomery *et al.* 2013, Turner *et al.* 2013). Insulin tolerance tests were devised largely to

assess the effectiveness of counter-regulatory mechanisms in response to insulin-induced hypoglycaemia and therefore the utility of these tests to assess peripheral insulin action is debatable. This is particularly the case when conclusions about insulin effectiveness are related to glucose measurements in the later half of the test (30–90 min) when the injected insulin has largely been cleared or when there is a difference in basal glycaemia and results are expressed as % basal (McGuinness *et al.* 2009). Neither glucose tolerance nor insulin tolerance tests give specific data regarding insulin effectiveness in muscle, although several methodological variations have used concurrent injection of radioactive tracers to assess glucose clearance into muscle during a glucose tolerance or insulin tolerance test (Crosson *et al.* 2003, Cooney *et al.* 2004).

The hyperinsulinaemic–euglycaemic clamp with glucose tracer administration gives the most reproducible assessment of muscle glucose clearance in response to constant insulin stimulation and constant glucose availability (Ayala *et al.* 2006, Wasserman *et al.* 2011). This technique relies on plasma insulin levels (not insulin infusion rates) during the comparison of the clamp being matched between the groups. In many studies, plasma insulin levels during the clamp are not reported, making the assessment of muscle insulin action difficult (Chapman *et al.* 2010, Laskewitz *et al.* 2010, Parlevliet *et al.* 2010). *In vitro* assessment of insulin effectiveness in isolated soleus or extensor digitorum longus muscle is also often used to demonstrate the effects of FA exposure (Thompson *et al.* 2000, Alkhateeb *et al.* 2007), and although this methodology provides reproducible comparisons between control and treatment muscle, it is subject to all the assumptions of comparing the *in vitro* situation with the *in vivo* situation (e.g. reliance on diffusion and not on perfusion). While all the above methods can give useful information about the effects of muscle lipid accumulation on insulin action, this information can be specific for the test employed. Even the data obtained from hyperinsulinaemic–euglycaemic clamp studies describe fluxes measured after at least an hour of exposure to constant insulin stimulation and constant glucose availability, a situation that is unlikely to ever exist in the normal 24-h feeding–fasting cycle. Therefore, it would seem important to consider the method used to demonstrate a difference in insulin action with lipid accumulation, when assessing the relevance of various mechanisms to reduced glucose metabolism in muscle when no restrictive experimental conditions (e.g. *in vitro* assessment, constant infusion and i.p. delivery) have been applied to the ‘free-living’ system.

Linking intramyocellular triglyceride content and insulin action

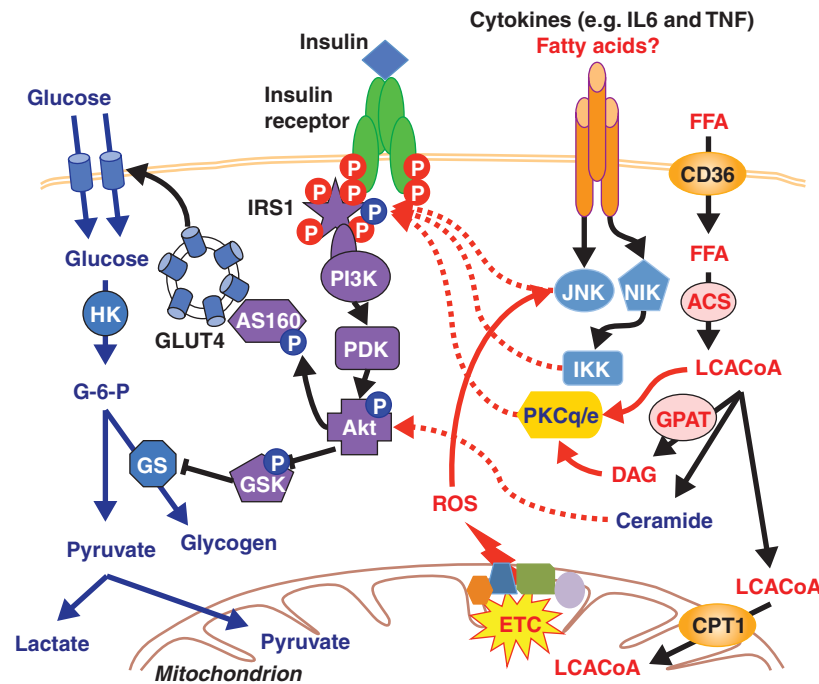
As has been mentioned above, the association between intramyocellular triglyceride (IMTG) content and insulin resistance is now well established in animals and obese humans, and most studies investigating the mechanisms of insulin resistance in muscle use high-fat diet rodent models. It has also become standard practice in assessing the phenotype of genetically manipulated mice to place them on high-fat diets to investigate whether there is any impact (favourable or detrimental) of gene manipulation on glucose and energy homeostasis. There is a reasonable assumption that, independent of genetic background in animals or humans, overconsumption of energy-dense diets plays a major role in the accumulation of fats and development of metabolic derangements in muscle. In humans, overconsumption of energy-dense diets for a few weeks is enough to increase fat mass and have detrimental effects on whole-body insulin action (Samocha-Bonet *et al.* 2010). In mice, high-fat feeding for as little as a few days can impair glucose tolerance (Turner *et al.* 2013) and 2–3 weeks of exposure to a high-fat diet is enough to observe significant insulin resistance in muscle using *in vitro* (Thompson *et al.* 2000) or *in vivo* (Turner *et al.* 2013) assessment. Apart from the well-documented ‘athlete’s paradox’ where increased IMTG content is associated with improved insulin action (Coen & Goodpaster 2012), most interventions that change insulin action are associated with reciprocal changes in IMTG content. Studies that improved insulin sensitivity by low-calorie diets in patients with T2D were accompanied by a reduction in IMTG content (Jazet *et al.* 2008, Lara-Castro *et al.* 2008). Insulin resistance associated with ageing (Nakagawa *et al.* 2007), growth hormone administration (Krag *et al.* 2007) and post-burn trauma (Cree & Wolfe 2008) has been reported to be associated with increased IMTG content. Current opinion is reasonably clear on the fact that IMTG is a useful marker of the level of cytosolic lipid accumulation, but it is more likely that active lipid metabolites such as LCACoAs, DAGs and ceramides or intermediates of FA oxidation pathways interfere with insulin action via a variety of potential mechanisms (Fig. 2). These mechanisms are largely based on the idea that insulin resistance in muscle is the result of reduced transduction of the insulin signal through the phosphorylation cascade leading to the translocation of the glucose transporter GLUT4 to the sarcolemmal membrane (Stockli *et al.* 2011). A significant body of work in the 1990s using nuclear magnetic resonance has identified glucose

transport/phosphorylation and glycogen synthesis as major defects in FA-induced insulin resistance in humans (Shulman *et al.* 1990, Roden *et al.* 1996). Since that time, research into the molecular mechanism of FA-induced insulin resistance in muscle has mainly focused on linking excess FAs to defects in the insulin signalling pathways that regulate glucose uptake. However, there are some established and some more speculative mechanisms that also link increased FA metabolism with reduced insulin action, and these are discussed in the subsequent sections.

Lipid intermediates, inflammation and insulin resistance

IMTGs are considered to be relatively benign with regard to insulin resistance (Goodpaster *et al.* 2001), largely because they are packaged into discrete lipid droplets that are located within the cytoplasm and are thus unlikely to directly interfere with proximal insulin signalling (Fujimoto & Parton 2011). However, despite the general consensus that IMTGs are metabolically inert, it is possible that the expanded IMTG pool generates intermediates of lipid metabolism that are more likely to play a mechanistic role in the development of muscle insulin resistance. In this respect, the bioactive lipid metabolites DAG and ceramide are leading candidates. The levels of both DAG (Turinsky *et al.* 1990, Turpin *et al.* 2009) and ceramide (Holland *et al.* 2007, Bruce *et al.* 2013) are elevated in the muscle of obese insulin-resistant rodents, and the earliest detectable defect in muscle insulin sensitivity in high-fat diet-fed mice is associated with the accumulation of these lipids (Turner *et al.* 2013). While less is known about the role of these lipids in humans, it has been reported that acute lipid-induced insulin resistance is associated with muscle DAG accumulation (Itani *et al.* 2002) and that ceramide levels are elevated in the muscle of obese insulin-resistant individuals (Amati *et al.* 2011). Furthermore, interventions that enhance insulin action, such as exercise training, cause reductions in muscle DAG and ceramide content (Bruce *et al.* 2006).

Mechanistically, DAG and ceramide are potent signalling molecules that may cause insulin resistance by activating a cascade of serine/threonine kinases that ultimately impinges upon insulin signalling (Summers *et al.* 1998, Ruvolo 2003, Li *et al.* 2004). Specifically, DAG accumulation is thought to impair insulin action via the activation of novel protein kinase C (PKC) isoforms, which subsequently inhibits insulin signal transduction to glucose transport via serine phosphorylation of insulin

**Figure 2**

Proposed mechanisms for the build-up of bioactive lipid species and how they interfere with insulin action in muscle to produce insulin resistance. DAG can activate lipid-sensitive kinases to serine phosphorylate and reduce tyrosine phosphorylation of IRS1. Ceramide can inhibit Akt phosphorylation and reduce transduction through the insulin signalling

pathway. Circulating cytokines or FAs themselves are reported to activate inflammatory pathway serine kinases that interfere with insulin signalling. Reduced or dysregulated FA oxidation in mitochondria could create a build-up of bioactive lipids and generate reactive oxygen species (ROS) that also activate kinases that interfere with insulin signalling.

receptor substrate 1 (IRS1; Yu *et al.* 2002, Li *et al.* 2004). Ceramide has been reported to cause insulin resistance by impairing insulin signalling at the level of Akt (Schmitz-Peiffer *et al.* 1999, Bruce *et al.* 2006, Holland *et al.* 2007). In addition, ceramide is a potent activator of inflammatory molecules, including c-Jun N-terminal kinase (JNK; Westwick *et al.* 1995) and nuclear factor κ B/inducer of κ kinase (IKK) (Wang *et al.* 1999), which have been reported to be associated with the development of muscle insulin resistance (Itani *et al.* 2002, Sriwijitkamol *et al.* 2006, Henstridge *et al.* 2012). However, while inflammation has been proposed as a critical factor causing insulin resistance, studies carried out by our group and other groups suggest that inflammation is not involved in the initiation of lipid-induced insulin resistance, but may be more important in the exacerbation and maintenance of insulin resistance once obesity is established (Lee *et al.* 2011, Turner *et al.* 2013).

Although there is mounting evidence supporting a role for DAG and ceramide in the regulation of insulin sensitivity, it is important to highlight that the accumulation of these lipids is not always associated with insulin resistance. In fact, a recent study has found that total DAG

content is actually elevated in the muscle of highly insulin-sensitive endurance-trained athletes compared with the skeletal muscle of obese individuals (Amati *et al.* 2011). Furthermore, a positive correlation between total muscle ceramide content and insulin sensitivity has been reported (Skovbro *et al.* 2008). These data suggest a more complex role for DAG and ceramide in the regulation of insulin action (Amati *et al.* 2011) and emphasise the importance of not only determining the total content of these lipids but also examining specific molecular species as well as their subcellular localisation, as these are likely to be critical factors that influence the relationship between lipids, insulin signalling and muscle insulin sensitivity (Bergman *et al.* 2012).

While the bioactive lipid hypothesis has gained strong support, an alternative concept linking the accumulation of intermediates of mitochondrial FA oxidation with muscle insulin resistance has gained attention (Koves *et al.* 2008). This model proposes that lipid oversupply drives an increase in mitochondrial β -oxidation that exceeds the capacity of the Krebs cycle, leading to the accumulation of by-products of FA oxidation (Koves *et al.* 2008). This is supported by studies demonstrating an

increase in incomplete FA oxidation and an accompanying increase in intramuscular acylcarnitine levels in obese rodents (Koves *et al.* 2008). While data in humans are currently limited, there is evidence that acylcarnitine does accumulate in the muscle of humans in response to a high-fat diet (Putman *et al.* 2003). However, it is not clear whether acylcarnitine plays a direct role in the modulation of skeletal muscle insulin sensitivity by disrupting signalling processes or whether it simply reflects a state of mitochondrial stress. Unravelling the role of acylcarnitine in muscle insulin sensitivity will no doubt be a focus of future research.

Mitochondrial dysfunction, reactive oxygen species and insulin resistance

Another prominent theory on the aetiology of insulin resistance implicates abnormalities in mitochondrial function as a major causative factor leading to reductions in insulin sensitivity. More specifically, defects in mitochondrial metabolism have been suggested to lead to inadequate substrate oxidation, precipitating a build-up of intracellular lipid metabolites, impaired insulin signalling and the subsequent development of insulin resistance (Lowell and Shulman 2005, Kim *et al.* 2008, Turner & Heilbronn 2008, Samuel & Shulman 2012).

The initial studies that set the platform for this theory in the late 1990s showed that there was reduced mitochondrial enzyme activity and decreased fat oxidation in the skeletal muscle of obese, insulin-resistant subjects and in individuals with T2D (Kelley *et al.* 1999, Simoneau *et al.* 1999, Kelley & Mandarino 2000, Kim *et al.* 2000). Kelley *et al.* (2002) also reported that mitochondrial size, as assessed by electron microscopy, was decreased in the muscle of obese subjects with insulin resistance and/or T2D. In the following year, two prominent microarray studies were published, describing a coordinated down-regulation of genes involved in mitochondrial biogenesis and oxidative phosphorylation in subjects with T2D and, importantly, also in non-diabetic individuals with a family history of T2D (Mootha *et al.* 2003, Patti *et al.* 2003).

In the ensuing decade since the publication of these landmark studies, many groups have reported defects in different mitochondrial parameters in the skeletal muscle of a range of different insulin-resistant populations (obese, T2D and PCOS). These include decreased mRNA and/or protein expression of mitochondrial genes/proteins (Morino *et al.* 2005, Heilbronn *et al.* 2007, Skov *et al.* 2007, Hwang *et al.* 2010), reductions in mitochondrial DNA (mtDNA) levels (Ritov *et al.* 2005, Boushel *et al.*

2007), lower oxidative enzyme activity (Ritov *et al.* 2005, 2010, Heilbronn *et al.* 2007) and a reduction in mitochondrial content measured by electron microscopy (Kelley *et al.* 2002, Morino *et al.* 2005, Ritov *et al.* 2005). Functional studies in muscle biopsy samples or *in vivo* using magnetic resonance spectroscopy have also reported decreases in mitochondrial oxidative capacity in insulin-resistant individuals (Petersen *et al.* 2003, 2004, Befroy *et al.* 2007, Mogensen *et al.* 2007, Phielix *et al.* 2008). Collectively, all these studies suggest that at some level, mitochondria in insulin-resistant individuals are not as effective at burning fuel substrates in muscle and this compromises insulin action.

Despite the large body of evidence described above, this area is controversial, as many studies report a dissociation between insulin resistance and mitochondrial dysfunction. For example, providing rodents with excess fat in their diet leads to an enhancement of mitochondrial oxidative capacity in muscle while at the same time inducing insulin resistance (Turner *et al.* 2007, Hancock *et al.* 2008, Stephenson *et al.* 2012). Several lines of mice with genetic manipulations that cause compromised mitochondrial function in muscle do not exhibit insulin resistance (Vianna *et al.* 2006, Wredenberg *et al.* 2006, Handschin *et al.* 2007, Pospisilik *et al.* 2007). Conversely, two separate lines of muscle-specific *Pgc1 α* (*Ppargc1a*) transgenic mice displayed a significant enhancement in the markers of mitochondrial content and yet were insulin resistant due to excessive FA delivery and reduced GLUT4 (SLC2A4) expression in muscle (Miura *et al.* 2003, Choi *et al.* 2008). A growing number of studies in humans have also reported intact mitochondrial function in various insulin-resistant populations (De Feyter *et al.* 2008, Trenell *et al.* 2008, Lefort *et al.* 2010, van Tienen *et al.* 2012, Fisher-Wellman *et al.* 2013). Collectively, these studies suggest that mitochondrial dysfunction in muscle is not an obligatory factor required for the accumulation of intramuscular lipids and the development of insulin resistance. Furthermore, it has also been argued that as muscle has such a high amount of 'spare' capacity to elevate substrate oxidation over basal levels (Bangsbo 2000), it is questionable whether mitochondrial deficiencies of the magnitude reported in some insulin-resistant subjects would have any impact on the rate of FA oxidation (and lipid accumulation) when energy requirements are relatively low (e.g. normal free-living conditions) (Hancock *et al.* 2008).

In addition to their role as major sites for energy transduction, mitochondria are also known to be a major source of reactive oxygen species (ROS), which are

produced as a by-product of normal metabolic reactions (Andreyev *et al.* 2005). ROS have the capacity to damage macromolecules, and when the production of these reactive species is in excess of the antioxidant defences, a state of oxidative stress results. FA catabolism is known to promote mitochondrial ROS production (St-Pierre *et al.* 2002, Anderson *et al.* 2009, Seifert *et al.* 2010), and studies carried out by several groups have shown that in cultured cell models, genetic or diet-induced obese rodents, and in human subjects fed a high-fat diet, there is increased mitochondrial ROS production in muscle in association with insulin resistance (Houstis *et al.* 2006, Anderson *et al.* 2009, Hoehn *et al.* 2009, Hey-Mogensen *et al.* 2012, Fisher-Wellman *et al.* 2013). Importantly, many studies have shown that insulin action is improved when mitochondrial ROS production is attenuated (Houstis *et al.* 2006, Anderson *et al.* 2009, Hoehn *et al.* 2009, Boden *et al.* 2012), indicating a potentially important role for reactive species generation in this organelle in insulin resistance. While the exact mechanism linking mitochondrial ROS with insulin resistance is not resolved, it has been proposed that insulin resistance may be caused by ROS-dependent changes in stress-sensitive Ser/Thr kinases, leading to perturbed insulin signalling, although this requires verification (Fisher-Wellman & Neuffer 2012).

Substrate competition and reduced insulin action

Before the elucidation of the insulin signalling pathway and recognition of the complex processes involved in the translocation of GLUT4 from intracellular vesicles to sarcolemmal membrane, there was a large amount of experimental data pointing to significant FA regulation of glucose metabolism at the level of PDH (Randle *et al.* 1963, Randle 1998). If humans, animals or *in vitro* preparations of muscle are exposed to an increased availability of FAs in the presence of glucose, the oxidation of FAs increases and the oxidation and uptake of glucose decrease (Boden *et al.* 1994, Vaag *et al.* 1994). On the other hand, reduction of the availability of FAs by inhibiting lipolysis (Vaag *et al.* 1991, Lim *et al.* 2011) and blocking FA entry into the mitochondria reduces FA oxidation and increases glucose uptake and oxidation (Oakes *et al.* 1997, Timmers *et al.* 2012, Keung *et al.* 2013), although there are some reports that prolonged inhibition of FA oxidation can lead to reduced glucose uptake (Dobbins *et al.* 2001). Although the initial observations of Randle and colleagues on the reciprocal relationship between glucose and FA metabolism were made 50 years ago, the idea that increasing or

reducing FA availability will reciprocally affect glucose utilisation is no less valid today. Therefore, in the context FA-induced insulin resistance, a role for substrate competition and regulation at the level of PDH should not be overlooked.

Reassessment of the role of insulin signalling in FA-induced insulin resistance

As outlined in other sections of this review, the current dogma suggests that the major mechanisms for FA-induced insulin resistance in muscle involve active lipid species interfering with insulin signalling via the activation of various serine kinases (Fig. 2). The canonical insulin signalling cascade comprises scaffolding proteins (e.g. IRS1) and enzymes (e.g. PI3 kinase, Akt and GSK3), and the activity of these proteins is modulated by tyrosine and/or serine phosphorylation. DAG via the activation of PKC and inflammatory factors via the activation of the serine kinases JNK and IKK are thought to serine phosphorylate and reduce the insulin receptor-mediated tyrosine phosphorylation of IRS1 (Samuel & Shulman 2012). Mitochondrial insufficiency and ROS are also thought to feedback and impinge on the efficiency of insulin signalling via the activation of regulatory kinases. While there are many studies showing clear differences in the phosphorylation status of various insulin signalling proteins after insulin stimulation in control and FA-exposed or obese or high-fat diet-fed muscle, these changes are not always consistent. For example, a change in Akt phosphorylation is not always accompanied by a detectable change in downstream GSK3 or AS160 phosphorylation or upstream changes in IRS1 phosphorylation (Frangioudakis & Cooney 2008, Hoehn *et al.* 2008, Tonks *et al.* 2013). There are a number of studies reporting that insulin-stimulated Akt activation is in fact not impaired in the muscle of obese individuals with insulin resistance, of glucose-intolerant first-degree relatives of patients with T2D and of patients with T2D (Kim *et al.* 1999, Storgaard *et al.* 2004). Furthermore, in rats made insulin resistant by 5 h of hyperlipidemia/hyperinsulinaemia (Hoy *et al.* 2009) or in isolated soleus muscle made insulin resistant by palmitate incubation (Alkhateeb *et al.* 2007), no defect in insulin-stimulated Akt phosphorylation was reported. Finally, reduction of IRS1 levels in muscle by 60% by direct *in vivo* genetic manipulation did not result in impaired insulin action (Cleasby *et al.* 2007).

This dissociation between measured changes in insulin-stimulated glucose flux and insulin effects on signalling proteins has a number of implications. First, it

might highlight the technical difficulties of obtaining reliable, quantitative data on protein modification using the essentially non-quantitative technique of immunoblotting. The ability to detect differences with this methodology can also depend on the affinity of individual antibodies, and the amount of phosphorylation does not necessarily correlate linearly with the activity of the signalling protein. A good example of this is provided by two studies showing that in adipocytes maximal insulin-stimulated glucose transport and GLUT4 translocation are achieved when only 10–20% of the total IRS1 and Akt is phosphorylated (Whitehead *et al.* 2001, Hoehn *et al.* 2008). If a similar situation exists in muscle, the physiological importance of statistically significant differences of 10–20% in the phosphorylation of signalling intermediates could be difficult to assess. The introduction of mass spectrometry techniques to analyse changes in global protein phosphorylation in response to insulin, as has been applied in adipocytes (Humphrey *et al.* 2013), could be helpful in this regard. Another possibility is that phosphorylation is not the only post-translational modification of proteins involved in the generation of lipid-induced insulin resistance. Recently, the emergence of nitrosative modifications (White *et al.* 2010), reversible acetylation, malonylation and succinylation of proteins in central metabolic pathways has revealed new possibilities by which increased FA metabolism could influence metabolic fluxes (Newman *et al.* 2012, Park *et al.* 2013). Similarly, reversible modification of proteins by O-linked N-acetylglucosamine has been proposed to have a significant impact on metabolism in response to nutrient levels (Bond & Hanover 2013, Ruan *et al.* 2013).

Circadian metabolism and insulin resistance

Another area of research that is increasingly realised to have a significant impact on metabolic disease is circadian biology. Daily patterns of activity and rest are historically aligned with feeding and fasting and changes in energy metabolism are intrinsically linked to the light/dark cycle (Bass 2012). The suprachiasmatic nucleus in the brain is considered to be the master regulator of circadian behaviour because of its ability to coordinate inputs from the environment (light, food, exercise and temperature), but it is now clear that every tissue has the molecular components that comprise the clock, raising the possibility that circadian processes in tissues could be regulated directly by some inputs. Some mouse models with genetic manipulations of core clock genes have altered circadian rhythms and are more prone to developing obesity (Turek

et al. 2005, Kennaway *et al.* 2007, Paschos *et al.* 2012), and manipulation of feeding schedules in mice and rats has been shown to have significant effects on adiposity, energy expenditure and glucose homeostasis (Bray *et al.* 2013, Coomans *et al.* 2013, Reznick *et al.* 2013). If there is an underlying rhythm to metabolism in muscle driven by the molecular clock (Lefta *et al.* 2011), the timing of experiments over the normal 24-h period might be critical to a proper understanding of how repeated daily exposure to a high-fat diet leads to lipid accumulation and insulin resistance in muscle. In fact, a recent report has suggested that the time of day can have a significant effect on the data obtained from euglycaemic-hyperinsulinaemic clamps in mice (Shi *et al.* 2013), and as rodents have an phase opposite to that of humans with regard to activity and sleep and feeding and fasting, the relevance of daylight experiments in nocturnal animals to human physiology requires renewed debate.

Summary and perspective

The correlation between increased FA availability and reduced insulin-stimulated glucose metabolism is well established. Despite this clear relationship, to date, there has been no unifying mechanism that explains lipid-induced reductions in insulin action under all circumstances. The most described mechanisms are that toxic lipid intermediates and/or activation of inflammatory and stress signalling pathways act to decrease the phosphorylation and function of proteins in the insulin signalling pathway, and this explains the decreased insulin-stimulated glucose uptake observed with lipid accumulation. However, there are an increasing number of experimental situations where reduced effects of insulin in muscle have been observed without significant changes in the phosphorylation of signalling proteins or where differences in phosphorylation are only observed with stimulation by supraphysiological insulin concentrations. This suggests that other control mechanisms or other forms of protein modification may predominate depending on the exact experimental conditions used to examine insulin resistance (e.g. bolus insulin injections, hyperinsulinaemic clamps and glucose or lipid infusion).

Figure 3 summarises some of the key control points other than insulin signalling for GLUT4 translocation that could alter the balance between glucose and FA metabolism and affect insulin-stimulated glucose disposal. For example, utilisation of glucose and FAs is dependent on their availability in the circulation and delivery to the muscle tissue, and changes in microvasculature occur with

obesity and contribute to muscle insulin resistance (St-Pierre *et al.* 2010, Premilovac *et al.* 2013). Other work (Furler *et al.* 1991, Wasserman 2009) has established that glucose transport into muscle is not rate limiting for glucose metabolism under all conditions. The phosphorylation of glucose by hexokinase and the pathway for conversion of glucose-6-phosphate to glycogen are subject to regulation by glucose-6-phosphate and glycogen respectively, and decreased glucose phosphorylation and glycogen synthesis will affect glucose uptake (Fueger *et al.* 2007, Bouskila *et al.* 2010). Another well-documented node regulating the metabolism of glucose is centred on the activity of PDH. The activity of this enzyme complex is inhibited by phosphorylation via PDH kinase 4 (PDK4). Interestingly, the amount of PDK4 in muscle is significantly increased in high-fat diet-fed, insulin-resistant animals and PDK4 is activated by acetyl CoA, providing evidence that this regulatory node could significantly

affect glucose metabolism in muscle as hypothesised by Newsholme and Randle many years ago (Randle *et al.* 1963) and many others since (Holness & Sugden 2003, Hue & Taegtmeyer 2009).

FA metabolism in muscle can also be regulated at the membrane by transporter proteins (such as CD36), and at activation to acyl CoA by acyl CoA synthase (Glatz *et al.* 2010). The partitioning of FAs towards triglyceride storage or mitochondrial oxidation may depend on the activity of key enzymes such as glycerol phosphate acyltransferase and adipose triglyceride lipase (Greenberg *et al.* 2011, Watt & Hoy 2012). The entry of long-chain FAs into the mitochondria for oxidation is thought to be largely regulated by the activity of CPT1. The activity of CPT1 is modulated allosterically by malonyl CoA, and numerous studies, including our recently published papers using genetic and pharmacological interventions (Bruce *et al.* 2009, Hoehn *et al.* 2010), have manipulated CPT1B, AMPK

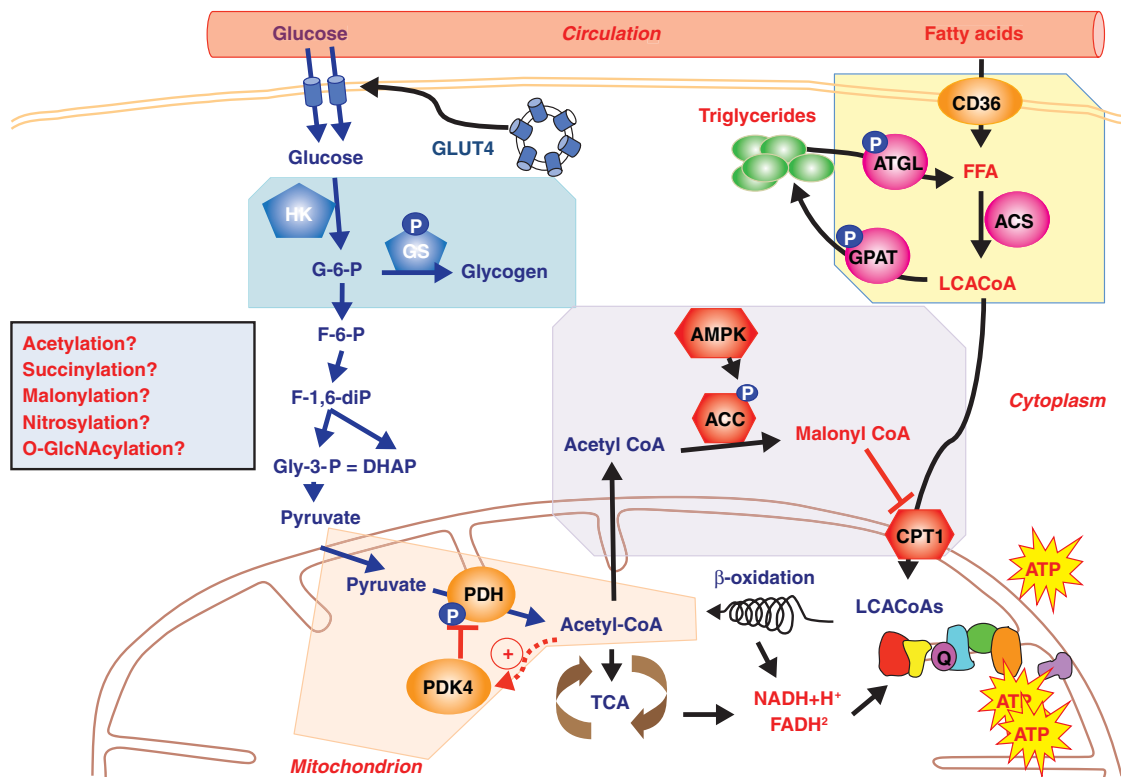


Figure 3

Nodes of control of glucose metabolism other than insulin-stimulated translocation of GLUT4 that could be influenced by the excess availability of FAs. Utilisation of glucose and FAs is dependent on their availability in the circulation and delivery to the muscle tissue. The phosphorylation of glucose and conversion to glycogen are regulated by substrate availability and G-6-P concentration. PDH is a critical regulator balancing glucose use and FA oxidation to support energy requirements. The regulation of FA

sequestration in, or release from, muscle fat droplets can control the level of bioactive lipid species. The regulation of FA metabolism at the AMPK–ACC2–malonyl CoA–CPT1 axis also has a significant impact on the balance between FA and glucose metabolism. There are a number of newly recognised post-translational modifications that can occur on key metabolic or signalling proteins and would be expected to be influenced by changes in the availability and metabolism of FAs.

and ACC activity to increase FA oxidation. Depending on the experimental design used, acutely increasing fatty oxidation in muscle can decrease glucose utilisation (Hoehn *et al.* 2010), while chronically increasing FA oxidation in muscle via CPT1 (CPT1B) overexpression can subsequently improve insulin-stimulated glucose uptake in fat-fed animals (Bruce *et al.* 2009). Interestingly, acute blockade of FA oxidation increases insulin-stimulated glucose uptake (Oakes *et al.* 1997), while chronic blockade of FA oxidation has been shown to be associated with decreased insulin sensitivity (Dobbins *et al.* 2001). These differences in acute and chronic responses when substrate metabolism is manipulated may be reconciled by considering the fact that energy metabolism is not constant in animals and humans, but has a substantial diurnal variation that is highly relevant to designing appropriate experiments to investigate lipid-induced insulin resistance.

In conclusion, it may be unrealistic to expect that a unifying mechanism may explain all situations where there is reduced glucose metabolism in muscle in response to insulin, as multiple factors may contribute to the establishment and long-term maintenance of insulin resistance in this tissue. With the emergence of powerful techniques for determining global changes in gene expression, protein modifications and metabolite profiles, it will hopefully become possible to gain a more comprehensive idea of the factors and pathways that may contribute to the aetiology of lipid-induced insulin resistance in muscle.

Declaration of interest

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

Funding

The work carried out in the laboratories of the authors is supported by Program and Project grant funding from the National Health and Medical Research Council of Australia (NHMRC), the Australian Research Council (ARC) and the Diabetes Australia Research Trust. NT is supported by an ARC Future Fellowship. G J C and E W K hold research fellowships from the NHMRC and C R B has received a career development award from the NHMRC.

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Received in final form 27 November 2013

Accepted 9 December 2013

Accepted Preprint published online 9 December 2013