Role of skeletal muscle lipids in the pathogenesis of insulin resistance of obesity and type 2 diabetes

Marc Gilbert*
CNRS UMR 8251 Bât. Buffon, Paris Diderot University, Paris, France

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*Correspondence
Marc Gilbert
Tel: +33-1-57-27-77-92
Fax: +33-1-57-27-77-92
E-mail address: marc.gilb12@gmail.com

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ABSTRACT
Obesity predisposes individuals to the development of insulin resistance, which is a risk factor for type 2 diabetes, and muscle plays a central role in this phenomenon. Insulin resistance is associated with: (i) a metabolic inflexibility characterized by a reduced impaired switching from free fatty acid (FA) to carbohydrate substrates; and (ii) an ectopic accumulation of triglyceride in skeletal muscle, generating a cellular “lipotoxicity”, but triglyceride per se, does not contribute to insulin resistance (“athlete’s paradox”). A large body of evidence supports the idea that a decreased mitochondrial capacity to oxidize FA leads to an accretion of intracellular triglyceride and an accumulation of acyl-CoA, which are used to synthesize diacylglycerol and ceramide. These lipid derivatives activate serine kinases, leading to increase of insulin receptor substrate 1 serine phosphorylation, which impairs insulin signaling. A second model proposes that insulin resistance arises from an excessive mitochondrial FA oxidation. Studies have shown that the type of FA, unsaturated or saturated, is critical in the development of insulin resistance. It should be also stressed that FA oversupply activates inflammatory signals, induces endoplasmic reticulum stress, increases mitochondrial oxidative stress and influences the regulation of genes that contributes to impaired glucose metabolism. These cellular insults are thought to engage stress-sensitive serine kinases disrupting insulin signaling. In conclusion, reduced dietary lipid intake in association with physical exercise could be a therapeutic option to improve insulin sensitivity.

INTRODUCTION
Obesity is the most common cause of insulin resistance (IR) and type 2 diabetes. The IR state is characterized by an impairment of insulin action in insulin target tissues, such as skeletal muscle, adipocyte and liver. Given that skeletal muscle is responsible for approximately 75% of insulin-stimulated whole-body glucose disposal, any dysfunction that lowers glucose metabolism in this tissue will greatly impact the whole-body glucose homeostasis and ultimately leads to type 2 diabetes. Numerous studies have addressed the issue of how obesity contributes to the development of skeletal insulin resistance. It was observed that when obesity develops, muscle IR strongly correlates with intramyocellular lipid contents. It suggested that the adipocyte has a limited capacity to store lipids, and when the maximum of fat accumulation is reached, it results in outflow of fatty acids (FAs) from white adipose tissue, leading to a persistent elevation of circulating FA levels. Consequently, it increases their availability for other tissues, namely skeletal muscle, which uses them as a fuel source for energy production. However, this tissue is not suited for lipid storage, and from a metabolic viewpoint, when the FA flux into the muscle outpaces its energy demand, it leads to a net accretion of ectopic fat, which is a characteristic of obesity associated with insulin resistance. Therefore, the purpose of this review is to discuss several mechanisms that contribute to the development of muscle IR in response to elevated FA levels. Special emphasis is put on the respective role of lipid metabolites, mitochondria, oxidative stress/ROS, inflammation and endoplasmic reticulum (ER) stress.

FA-MEDIATED INSULIN RESISTANCE IN SKELETAL MUSCLE
In 1963, Randle et al. stated that “several abnormalities of carbohydrate metabolism in many disorders, including diabetes,
are associated with high circulating FA levels. This observation was the starting point for investigating the role of FA in the development of IR. Given that obesity is associated with elevated FA levels, it was postulated that it might cause lipid accumulation in tissues, which would be responsible for much of the IR. Numerous studies carried out in animals and humans definitively confirmed this hypothesis and reported a negative relationship between intramuscular TG content and insulin sensitivity, suggesting a functional link between FA levels and the pathogenesis of IR. To evaluate the insulin sensitivity, the glucose tolerance test and the euglycemic hyperinsulinemic clamp are commonly used. To delineate the underlying mechanisms of IR, investigators developed essentially two methodological approaches aimed at mimicking elevated circulating FA levels described in obese rodent models or in humans. One consists of an infusion of TG emulsion with heparin to activate lipoprotein lipase, which further increases plasma FA levels. When carried out in healthy subjects, it induces insulin resistance and increases the intramyocellular lipid content. The second methodological approach is based on the administration of a high-fat diet (HFD) in rodents, which results in increased intramuscular fat content, which correlates with insulin resistance. Conversely, a reduction of circulating FA levels with acipimox, an inhibitor of lipolysis, improves the insulin sensitivity and it is correlated with the reduction in intramyocellular FA-CoA content.

How do FAs interfere with glucose metabolism and insulin sensitivity? The mechanisms by which obesity and FAs cause insulin resistance are not clearly established, and several hypotheses have been proposed to explain how elevated FAs interfere with glucose metabolism. It was initially thought that in muscle, the “glucose–FA cycle” proposed by Randle et al. was operating in this physiological situation; that is, under increased FA availability, glucose oxidation is inhibited while FA oxidation is enhanced. This mechanism was confirmed in human skeletal muscle after 1 h of TG emulsion with heparin to increase plasma FA levels. However, subsequent studies carried out by Shulman’s group and using magnetic resonance spectroscopy, failed to observe an increase in glucose-6P, as expected from the Randle cycle, after 5 h of lipid infusion, but rather a decreased glucose-6P. This finding indicates that FA-induced insulin resistance is attributed to a defect in glucose transport or phosphorylation and not to decreased glycolysis, as Randle hypothesized. These data strongly suggested that defects in insulin signaling were likely involved in decreased glucose transport.

Given that muscle is not physiologically a lipid-storing organ, it was postulated that FA oversupply to muscle leads to altered partitioning between the lipogenic and oxidative pathways, which in turn would play a role in the development of muscle insulin resistance. Therefore, investigators evaluated: (i) intracellular TG contents and/or lipid metabolites; and (ii) mitochondrial dysfunction, including transport of long-chain fatty acid (LCFA), mitochondrial oxidation and the number of mitochondria.

**ROLE OF TRIGLYCERIDE AND LIPID METABOLITES**

According to Morino et al. and Ruderman et al., the IR is related to the accumulation of TG and lipid metabolites in skeletal muscle due to impaired mitochondrial oxidation (Figure 1). However, it was observed that endurance athletes show TG contents equal or greater than those measured in individuals with type 2 diabetes, proving that TG per se does not contribute to the pathogenesis of muscle insulin resistance and it is called “athlete’s paradox”. Similarly, activation of liver X receptor promotes lipid accumulation, but does not alter insulin action in cultured myotubes from patients with type 2 diabetes.

Other studies investigated whether some lipid metabolites that appear sequentially in the TG synthesis pathway could regulate insulin sensitivity. Initial work reported that intracellular DAG contents were increased in muscle from insulin-resistant rodents and humans, suggesting that this lipid intermediate could contribute to the development of IR. To test this hypothesis investigators created rodent models aimed at manipulating the intracellular DAG pool in skeletal muscle. When mice over-express the diacylglycerol O-acyltransferase, the enzyme that catalyzes the final step of TG synthesis, they show an increase in myocellular TG associated with a reduction in DAG contents and are protected from HFD-induced insulin resistance. This reflects the situation observed in athletes. However, mice lacking diacylglycerol O-acyltransferase are still capable of synthesizing TG and are resistant to diet-induced obesity. Similarly, the diacylglycerol kinase haploinsufficiency mice, which show a higher DAG content due to the reduced conversion to phosphatidic acid by diacylglycerol kinase, have a decreased insulin sensitivity. A series of experiments showed that DAG and one isoform among the novel protein kinase Cs (PKCδ) are likely involved in IR: (i) in high-fat-fed rats, there is a linear relationship between muscle DAG content and PKCδ translocation; (ii) a lipid infusion results in DAG accumulation, associated with a PKCδ activation, causing muscle insulin resistance; and (iii) a lipid infusion to PKCδ knockout mice prevents IR. Likewise, PKCδ overexpression causes IR in cultured myocytes.

Mechanisms linking the activation of PKCδ to the insulin pathway are incompletely elucidated, because activation of other PKCs also occurs during lipid infusion. Furthermore, increased intracellular level of LCFA-CoAs, which also occur during lipid infusion, are also strong activators of PKCs. However, numerous studies carried out in lipid-infused rodents have reported that activation of PKCδ is associated with decreased insulin signaling at the level of insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation. A similar sequence of events was observed in human skeletal muscle during lipid infusion, and PKCδ activation increased IRS-1 Ser phosphorylation. As a result, there was decreased insulin activation of v-akt murine thymoma viral oncogene homolog 2, leading to reduced glucose transporter type 4 translocation, which ultimately decreases
glucose uptake. Although the regulation of specific serine phosphorylation sites of IRS-1 is largely unknown, it is well established that it reduces the IRS-1 tyrosine phosphorylation by the insulin receptor, which then leads to an attenuation of the signaling cascade initiated by the interaction of IRS-1 with phosphoinositide 3-kinase. Another member of the serine/threonine-protein kinase family, PKCβ, is markedly upregulated in the skeletal muscle of obese and diabetes patients. PKCβ knockout mice show resistance to HFD-induced obesity and show improvement in insulin sensitivity.

Evidence suggests that fatty acid composition, specifically fatty acid chain length and saturation influence skeletal muscle IR. In humans, LCFA-CoA content and saturated FA in intramyocellular lipid correlate positively with insulin resistance. Mice receiving a HFD are associated with increased LCFA-CoAs and IR. Increased availability of saturated LCFAs, such as palmitate (which is the most common FA in human diet) gives rise to ceramides. In a large number of studies, a positive correlation between ceramides and IR was observed in skeletal muscle, suggesting a potential role in the disease.

The biosynthesis of ceramides starts with the condensation of palmitoyl-CoA (CoA) and serine, and the reaction is catalyzed by the serine, palmitoyl transferase. To delineate the role of this class of sphingolipid, studies were carried out with an inhibitor (myriocin) of their synthesis, thus lowering ceramide levels. As a result, it markedly improves IR in HFD-fed mice without any change in LCFA-CoA, and palmitate infusion prevents acute muscle insulin resistance in rats. Ceramide synthesis occurs mainly from the C16 and C18 acyl chains, and the degree of saturation of FA correlates with insulin resistance. In cultured muscle, palmitate causes insulin resistance and oleate improves insulin sensitivity; when they are combined, insulin resistance is relieved. Ceramides were found to be potent inhibitors of protein kinase B (Akt) translocation/activation, thereby attenuating the insulin signaling. One can consider that DAG and ceramide are key players in the development of muscle insulin resistance.

Figure 1 | Schematic representation of the impact of increased supply of fatty acids (FA) to skeletal muscle. It enhances intracellular FA levels resulting in altered partitioning of long-chain fatty acid CoAs (LCFA-CoA) between the oxidative and lipogenic pathways. Hence, a non-oxidized fraction is channeled toward the triglyceride synthesis (TG), but its accumulation does not play a role in the development of muscle insulin resistance (IR). It was postulated that diacylglycerol (DAG) would be a molecular player in IR, and it was shown that elevated DAG content activates PKCθ, which phosphorylates Ser/Thr residues of insulin receptor substrate (IRS), leading to a downregulation of insulin signaling. Excess of saturated FAs, such as palmitate, promote ceramide synthesis, whose role is to prevent protein kinase B (Akt) translocation/activation, thereby attenuating the insulin signaling. One can consider that DAG and ceramide are key players in the development of muscle insulin resistance.

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Ceramides show numerous actions on mitochondria, leading to diminished electron transport chain activity and increased mitochondrial outer membrane permeability. These actions lead to decreased mitochondrial respiratory capacity and increased tissue apoptosis, respectively.

**MITOCHONDRIAL DYSFUNCTION AND INSULIN SENSITIVITY**

The existence of mitochondrial dysfunction was first reported in skeletal muscle of obese individuals with type 2 diabetes\(^29\). This finding raised the possibility of impaired FA oxidation as an additional aspect of the pathogenesis of insulin resistance. However, it is still unclear whether changes in mitochondrial function are a cause or consequence of insulin resistance. Several potential defects of mitochondrial function have been investigated, such as: LCFA-CoA transport into the mitochondria, metabolic flux through the tricarboxylic acid cycle (TCA) cycle, oxidative phosphorylation activity, adenosine triphosphate synthesis and biogenesis.

**LCFA-CoA transport into the mitochondria**

The transport of LCFA-CoA into the mitochondria represents the first step of β-oxidation, and to cross the inner membrane of mitochondria, the carnitine palmitoyl transferase I (CPT1) enzyme converts them to their corresponding LC acylcarnitines. It was suggested that the transport of LCFA into the mitochondria could be the rate-limiting step in the flux of FA oxidation, as there is an inverse relationship between CPT1 activity and body mass index\(^30\). To test this hypothesis, two experimental approaches were designed to increase the flux of LCFA-CoA. Overexpressing CPT1 (≈30%) in the muscle of mice with the electrophoresis procedure results in stimulated rates of FA oxidation (≈30%) associated with reduced FA incorporation into the muscle TG pool. It highlights that CPT1 is a regulator of FA oxidation. A second strategy was proposed to increase FA influx in the mitochondria of mice lacking acetyl-CoA carboxylase 2 (ACC2). The mutant mice show markedly low malonyl-CoA levels, which thereby attenuates its inhibitory effect on CPT1\(^31\). These mice lacking acetyl-CoA carboxylase 2, fed HFD, showed higher FA oxidation rate in the soleus muscle, and they were protected against obesity and diabetes. They also showed a high glucose clearance rate during a glucose tolerance test, likely associated with an increased rate of glucose oxidation, which is not in line with the Randle hypothesis, stating that fat oxidation and carbohydrate oxidation are mutually inhibitory (Figure 2).

**Deficient β-oxidation**

In healthy individuals, skeletal muscle chiefly relies on fat oxidation, and this capacity of oxidation is augmented in individuals with increased insulin sensitivity, leanness and aerobic fitness. Furthermore, these individuals enhance their fat oxidation and glucose oxidation in response to fasting and insulin, respectively. This ability to switch easily between these fuels is termed “metabolic flexibility”. This flexibility is lost in type 2 diabetes patients and in obese individuals compared with lean individuals, despite similar FA levels\(^32\). It was proposed that muscle’s impaired capacity to increase reliance on fat oxidation might be an underlying mechanism explaining the pathogenesis of insulin resistance.

Increased myocellular long-chain acylcarnitines (chain length >10 C) reflecting incomplete FA oxidation was reported in insulin-resistant Zucker diabetic fatty rats, and it was associated with decreased TCA cycle metabolites. These observations were interpreted as reflecting fatty acid oxidation mismatched to TCA flux, causing mitochondrial stress. The question that then arises is whether acylcarnitine mediates IR. Other studies showed that expression of many glycolysis and TCA genes, and multiple components of the mitochondrial respiratory chain are also reduced in insulin-resistant individuals.

Electron microscopic studies have reported a reduction in both mitochondrial content and size in muscle of obese non-diabetic individuals and type 2 diabetes patients\(^33\), and a decrease in oxidative enzyme activity\(^34\). This is consistent with reduced expression of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) gene, which is a strong activator of mitochondrial biogenesis and oxidative metabolism. However, there is no relationship between expression of PGC1α and abnormal glucose metabolism\(^35\). To further elucidate a potential role of PGC1α, a skeletal muscle-specific PGC1α knockout mouse was generated and they displayed a reduction in oxidative phosphorylation genes, but it did not cause systemic IR despite impaired glucose tolerance\(^35\). However, mildly elevated muscle levels of PGC1α in mice leads to resistant to age-related obesity and diabetes.

Using magnetic resonance spectroscopy, the mitochondrial rates of adenosine triphosphate production are reduced by approximately 30% in the muscle of the insulin-resistant individuals, as compared with the insulin-sensitive control individuals\(^36\).

**Excessive β-oxidation**

As aforementioned, it was proposed that IR develops secondary to diminished FA oxidation. Other studies have challenged this theory, because increased FA oxidation with PPARβ agonists does not improve IR\(^37\), and it was proposed that IR might arise from excessive rather than reduced β-oxidation\(^38\). This is in keeping with other reports showing that HFD or obesity results in increased expression of several β-oxidative and glucose-sparing enzymes that are targeted by the PPAR family of lipid-activated transcription factors\(^39\). The concept of excessive β-oxidation was evaluated with knockout mice lacking malonyl-CoA decarboxylase (mcdf⁻/⁻), which showed elevated malonyl-CoA levels and marked inhibition of CPT1\(^40\). This genetic manipulation reduces mitochondrial FA import and protects against lipid-induced insulin resistance, despite increased intramuscular accumulation of both LCFA-CoAs and TG (Figure 2). Interestingly, this model prevents the depletion of TCA cycle intermediates, which occurs in HFD wild-type mice. The
flux of LCFA-CoAs into the mitochondria is not accompanied by complete β-oxidation because of the inability of the TCA cycle to cope with the increase in the demand on FA metabolism. Finally, the changes in mitochondrial function could be due to chronic oversupply of a diet enriched in both carbohydrate and lipid, leading to a progressive decrease of oxidative mitochondrial capacities.

Role of oxidative stress, ER stress and inflammation in the development of IR

In both rodents and humans, HFD intake for a prolonged period of time increases mitochondrial reactive oxygen species generation in skeletal muscle. Attenuation of hydrogen peroxide production by genetically engineering the overexpression of catalase in mitochondria of mouse muscle preserves insulin sensitivity despite HFD, showing that the degree of insulin sensitivity is functionally linked to the cellular redox state. Furthermore, the shift to a more oxidized redox environment activates various intracellular signaling pathways. Among the potential effectors, there are various kinases, such as inhibitor of NF-κB kinase-β, c-Jun N-terminal kinase, PKCs and p38 mitogen-activated protein kinase, that have been postulated to catalyze the phosphorylation of serine residues of IRS-1, leading to a disruption of insulin signaling.

Recently, ER stress has gained major interest, as it has been proposed that in states of chronic overnutrition it could contribute to insulin resistance. Several lines of evidence support this hypothesis: (i) activation of ER stress is associated with the development of insulin resistance and (ii) inhibition of its activation with a chemical chaperone prevents IR in obese and diabetic mice. Other studies also report that high-palm diet fed mice increased the expression of ER stress markers in association with a reduction of insulin signaling. Emerging data suggest that the pseudokinase, tribbles 3, could mediate ER stress-induced insulin resistance, as it is markedly increased in the skeletal muscle of db/db mice, and in obese and type 2 diabetes patients. Tribbles 3 knockout mice are protected from HFD-induced insulin resistance.

Obesity and type 2 diabetes are often also associated with low-grade inflammation. Mice receiving a HFD have been shown to have increases in Toll-like receptors and inflammatory cytokines. This increase in inflammation occurs partially...
through ER stress mechanisms. This is demonstrated in a study showing that mice lacking Toll-like receptor 4 have reduced ER stress after a HFD50.

Activation of PPARα through the agonist, fenofibrate, improves ER stress associated with inflammation and insulin resistance in skeletal muscle. Mice on a HFD that received fenofibrate improved ER stress, and inflammation and insulin sensitivity.

In conclusion, there is no unifying theory for understanding how an oversupply of FAs to skeletal muscle can elicit a wide spectrum of defects that underlie muscle insulin resistance. One hypothesis that has been discussed for a long time proposes that insulin resistance results from a decreased mitochondrial oxidation of FAs and the unoxidized fraction is rerouted toward DAG and ceramide synthesis, and these lipid metabolites impair insulin signaling. The question is whether these molecular players are causative or markers of decreased insulin sensitivity. Last but not least, we are still searching for the initial mechanisms resulting in insulin resistance (Figure 3).

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