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Skeletal muscle insulin resistance: the interplay of local lipid excess and mitochondrial dysfunction

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1. Introduction

The prevalence of type 2 diabetes mellitus (DM2) is increasing precipitously as a consequence of the aging population and obesity epidemic. Type 2 diabetes mellitus is characterized by both pancreatic β-cell dysfunction and insulin resistance in multiple tissues, abnormalities that precede and predict the development of DM2 [1,2]. Skeletal muscle accounts for the majority of *insulin-mediated* glucose uptake in peripheral tissue [3,4]. Thus, understanding the mechanism by which insulin resistance develops in skeletal muscle may offer insight into potential therapies for the treatment or prevention of DM2.

Abnormalities in skeletal muscle lipid metabolism and mitochondrial dysfunction have been proposed as possible mechanisms for skeletal muscle insulin resistance [5]. In multiple studies of untrained subjects, elevation of intramyocellular lipid (IMCL) correlates with insulin resistance [6-8]. Lipid infusion induces insulin resistance several hours after free fatty acid (FFA) elevation [9,10]. In humans, insulin resistance has been associated with diminished mitochondrial function [8,11,12], reduced expression of oxidative metabolism genes [13,14], decreased mitochondrial size [15], and reduced mitochondrial density [15].

The purpose of this review is to explore the complex relationship between local lipid exposure, mitochondrial dysfunction, and insulin resistance at the level of human skeletal muscle. The focus will be on human studies, although selected animal and cell-based studies will be presented for more detailed insights into pathophysiology. We begin with a brief overview of the normal physiology of insulin-mediated glucose disposal and the associated abnormalities observed in insulin resistance. We will then discuss the evidence for local lipid excess contributing to skeletal muscle insulin resistance. Next, we will briefly review the relationship between skeletal muscle mitochondrial function and skeletal muscle insulin resistance with a focus on local lipid excess as a cause and/or consequence of mitochondrial dysfunction. After reviewing the role of lipid metabolism in skeletal muscle insulin resistance, we will present the concept that multiple abnormalities of nonmuscle tissues and "cross talk" between tissues also contribute to the insulin insensitivity of skeletal muscle. Our major goal is to illustrate the conflicting nature of the evidence with regard to the biochemical pathogenesis of DM2 and, thereby, to highlight the difficulties in determining a causal relationships between lipid excess, insulin resistance, and mitochondrial function in skeletal muscle.

2. Mechanism of skeletal muscle insulin resistance

Skeletal muscle is the largest site of insulin-mediated glucose disposal in the human body [3,4]. The physiology of skeletal muscle glucose uptake is shown in Fig. 1. Normally, the binding of insulin to the insulin receptor stimulates autophosphorylation of tyrosine residues [16,17] and subsequent activation of a receptor tyrosine kinase. This tyrosine kinase phosphorylates multiple intracellular substrates, including insulin receptor substrate (IRS) 1 [18] and 2 [19], which play significant roles in the insulin response [20]. The insulin response is mediated by IRS activation of phosphatidylinositol 3-kinase (PI 3-kinase), a critical player in insulin signaling [18,21-23] particularly with regard to glucose homeostasis [21,24]. Phosphatidylinositol 3-kinase facilitates the translocation of the insulin-responsive glucose transporter (GLUT4) to the plasma membrane [25-27] through a mechanism likely mediated by phosphorylation

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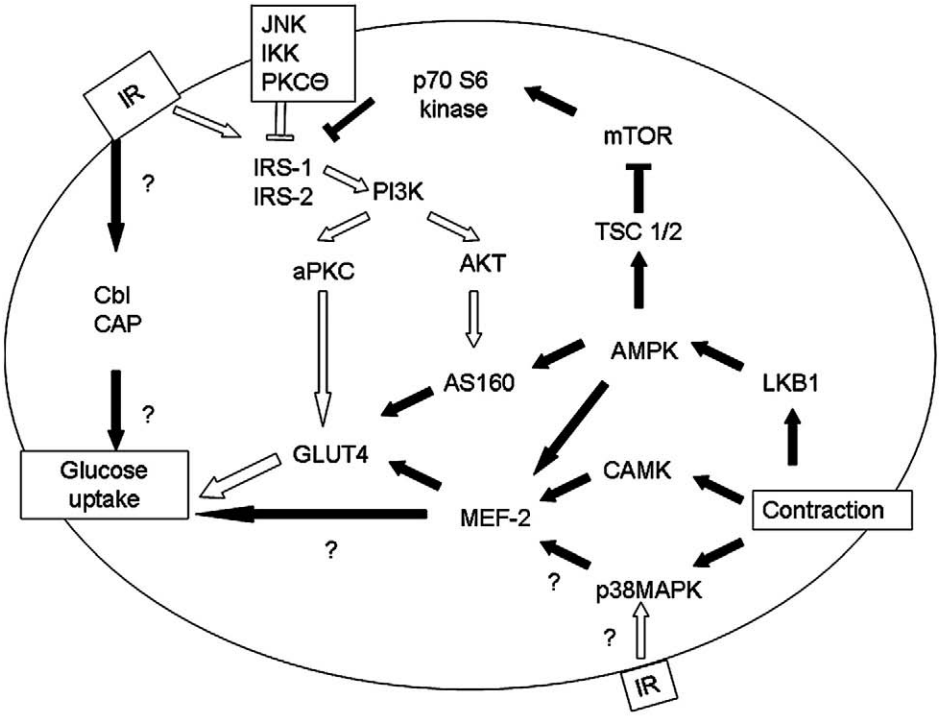

Fig. 1. Glucose uptake in skeletal muscle. The insulin receptor is phosphorylated at multiple tyrosine residues, which then activates tyrosine phosphorylation of IRS-1 and IRS-2. This allows the activation of PI 3-kinase, which proceeds through either an atypical PKC or AKT mechanism to activate GLUT4 translocation. In insulin resistance (white arrows), decreased tyrosine phosphorylation of the insulin receptor, increased serine/threonine phosphorylation of IRS-1/IRS-2, and decreased PI 3-kinase activity lead to decreased GLUT4 translocation. The IKK, c-Jun amino-terminal kinases, mTOR, and PKCΘ are serine/threonine kinases that act on IRS-1 to inhibit IRS-1 activity. Insulin resistance may also impair p38MAPK response to insulin without affecting p38MAPK response to contraction. Contraction mediates skeletal muscle glucose uptake through activation of p38MAPK, CAMK, and AMPK. Intersection of contraction-mediated glucose uptake and insulin-mediated glucose uptake includes effects at IRS-1, AS160, and p38MAPK. PI3K indicates phosphatidylinositol 3-kinase; MEF-2, myocyte enhancer factor–2, TSC, tuberous sclerosis complex; JNK, c-Jun amino-terminal kinases; CAP, Cbl-associated protein.

of protein kinase B (AKT) [28] and/or an atypical protein kinase C (aPKC) [29,30]. The translocation of GLUT4 from the intracellular pool to the plasma membrane plays a crucial role in insulin-mediated glucose entry into the skeletal muscle [31]. Current evidence suggests a minimal role for insulin stimulating the ecotropic retroviral transforming sequence homolog (Cbl) and Cbl-associated pathway in mediating skeletal muscle glucose uptake [32,33].

Contraction is another critical mediator of skeletal glucose uptake. Contraction stimulates glucose uptake through activation of p38 mitogen–activated protein kinase (p38MAPK) [34,35], calmodulin-dependent kinase (CAMK) [36,37], and adenosine monophosphate–activated protein kinase (AMPK) [38] via a protein kinase (LKB1) [39]. Enhanced glucose uptake from contraction appears to be due to increased GLUT4 translocation due to several mediators: activation of myocyte enhancer factor–2 [36,37,40], activation of a Rab GTPase-activating protein (AS160) [41-43], and improved insulin signaling [44], possibly through the tuberous sclerosis complex [45] inhibitory effects on the mammalian target of rapamycin (mTOR) [46] and p70 S6 kinase [46,47].

The convergence of contraction-mediated glucose uptake and insulin-mediated glucose uptake has been investigated. Insulin has been shown to stimulate p38MAPK activation [34]. Adenosine monophosphate–activated protein kinase activation has been shown to improve insulin signaling [44]. There is significant evidence that AS160 activation regulates both contraction-mediated glucose uptake and insulin-mediated glucose uptake [42,43].

Skeletal muscle insulin resistance is characterized by impaired glucose uptake [4] resulting from impaired insulin receptor signaling [48-50]. Subjects with obesity [49] and subjects with DM2 [50] exhibit reduced IRS-1 tyrosine phosphorylation and reduced PI 3-kinase activity compared with their respective controls. The reduction in tyrosine phosphorylation of IRS-1 and IRS-2 has been related to increased serine/threonine phosphorylation of IRS-1 and IRS-2 [51]. Proposed IRS serine/threonine kinases include inhibitor κB kinase (IKK) [52], c-Jun amino-terminal kinases [53,54], mTOR [55,56], and PKC isoforms [57,58]. As a result of IRS serine/threonine phosphorylation, PI 3-kinase levels are reduced, which subsequently alters downstream effectors, that is, decreased activity of aPKC [59-61] and possibly AKT [62-65] that decreases glucose uptake, presumably by reduced GLUT4 activity/translocation [5,66,67]. A mouse model of insulin resistance (*ob*/*ob* mice) has demonstrated insulin resistance associated with

impaired p38MAPK response to insulin but preserved p38MAPK response to contraction [68] (Fig. 1).

3. Is lipotoxicity a mediator of skeletal muscle insulin resistance?

Lipotoxicity, defined as the elevation of lipids and/or lipid metabolites within blood or tissues with subsequent metabolic derangement, is a postulated mechanism for skeletal muscle insulin resistance. In humans, the normal plasma fasting FFA concentration ranges from 350 to 550 μmol/L [69,70] and increases with prolonged fasting [69,71], obesity [69,72], insulin resistance [72], and DM2 [73]. As the level of insulin resistance in untrained subjects correlates strongly with IMCL content as measured by muscle biopsy [74] or magnetic resonance imaging [6], prolonged FFA exposure was initially hypothesized to increase skeletal muscle insulin resistance by increasing IMCL content.

This hypothesis was supported by findings from human studies involving lipid infusion. Lipid infusion has been shown to increase IMCL content [75,76] and inhibit insulin-stimulated glucose uptake in subjects who are healthy [77] and in subjects with DM2 [10]. The inhibition of insulin-stimulated glucose uptake inversely correlates with the increased FFA levels produced by the lipid infusion [78]. An increase of plasma FFA levels to approximately 700 μmol/L has been shown to disrupt insulin signaling by inhibiting insulin receptor tyrosine phosphorylation, IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and AKT serine phosphorylation [78]. In healthy human subjects, the onset of lipid infusion–induced insulin resistance (steady-state FFA of approximately 1200 μmol/L) was associated with a concurrent increase in activity of PKC, an increase in the level of diacylglycerol (DAG), and a decrease in the level of IκB-α, an inhibitor of the nuclear factor κB (NF-κB) pathway [79]. Animal studies have reported that lipid infusion–induced insulin resistance is associated with activation of serine kinases such as protein kinase Θ [58,80] and inhibitor κB kinase–β), an activator of NF-κB pathway [81]. Thus, short-term exposure to a lipid substrate–rich environment (ie, lipid infusion) stimulates skeletal muscle to shift substrate use toward fatty acids, with the consequence of inducing skeletal muscle insulin resistance and reduction of glucose uptake.

Although lipid infusion is a well-documented method of producing skeletal muscle insulin resistance [10,77], the evidence is less conclusive for high-fat diets producing insulin resistance. In theory, a high-fat diet should elevate blood triglyceride (TG) and FFA levels to increase skeletal muscle fatty acid accumulation and increase insulin resistance. Although animal studies have shown that continuous high-fat feeding increases insulin resistance [82,83], the data from human studies are contradictory [76,84-88]. A portion of the uncertainty in human studies likely relates to the metabolic effects of dietary fat composition and quantity. When compared with subjects on a control diet, subjects given a Mediterranean-style diet (low in saturated fat and rich in monounsaturated and polyunsaturated fats) for 2 years reduced their weight (−5%), decreased insulin resistance (as measured by homeostasis model assessment), improved endothelial function (as measured by the L-arginine test), and lowered systemic inflammatory markers (as measured by high-sensitivity C-reactive protein, interleukin-6, interleukin-7, interleukin-18) [89]. A prospective cohort study (4.4 years' duration) of 13 380 subjects found that participants who were the most adherent to a Mediterranean diet had a lower incidence of diabetes (0.41; 95% confidence interval, 0.19-0.87) compared with participants who were least adherent to a Mediterranean diet [90]. Unfortunately, the effects of this diet on IMCL levels and muscle lipid metabolites were not measured in these studies.

However, even when focusing on a high-fat diet composed of saturated fatty acids, the evidence for high-fat intake increasing insulin resistance in humans remains unclear. In one longitudinal observational cohort study, high total and saturated fat intake was associated with higher fasting insulin levels, suggesting that a high–saturated fat diet is associated with insulin resistance [91]. However, several interventional studies in healthy humans have not documented a clear correlation between a high–saturated fat diet and increased insulin resistance as measured by the hyperinsulinemic-euglycemic glucose clamp [76,84,86,87] or by the frequently sampled intravenous glucose tolerance test [85,88].

There is growing interest that increased levels of fatty acid metabolites, rather than IMCL alone, may be the mechanism responsible for lipid-induced insulin resistance. The possibility that IMCL may not be harmful has been raised by the observations in endurance athletes who have levels of IMCL comparable to those of subjects with diabetes but do not have insulin resistance [92]. In addition, elevated IMCL levels in transgenic mice do not also obligatorily correlate with insulin resistance. Transgenic mice overexpressing lipoprotein lipase in skeletal muscle have increased IMCL, fatty acyl coenzyme A (CoA), DAG, and ceramide levels in the setting of reduced muscle glucose uptake [93]. In contrast, transgenic mice overexpressing DAG acyltransferase in skeletal muscle increased skeletal muscle IMCL in the setting of reduced DAG and ceramide levels; these mice were protected from high-fat diet–induced insulin resistance [94]. Proposed fatty acid metabolites responsible for lipid-induced insulin resistance include 4-hydroxynonenal (4-HNE) [95], DAG [58,79] ceramide [94,96], and long-chain fatty acyl CoA (LC-CoA) [97].

4-Hydroxynonenal is a by-product of lipid peroxidation and an important mediator of free radical damage [98]. In humans, 4-HNE elevation has been associated with obesity [95] but not with DM2 [99] or reduced skeletal muscle mitochondrial respiration [99].

Diacylglycerol is generated by lipid hydrolysis or by de novo synthesis. Diacylglycerol is elevated in obese subjects compared with controls [100]. Much interest has been focused on DAG activation of PKC as a mechanism of lipid-induced insulin resistance and the activation of the novel PKCs (nPKCδ, Θ, ε, η) in particular. In humans, lipid infusion–induced insulin resistance has been associated with elevated skeletal muscle DAG level, elevated PKC activity (elevated PKCδ and PKCβII but not PKCΘ or ε), and decreased IκB-α protein, an inhibitor of NF-$\kappa\beta$ [79]. In rats, lipid infusion–induced insulin resistance occurs in the context of increased skeletal muscle levels of DAG, PKCΘ activation, IRS-1 serine phosphorylation, and decreased PI 3-kinase activity, suggesting that lipid infusion alters insulin signaling in skeletal muscle [58]. Because of the evidence linking DAG accumulation to abnormal insulin signaling, much effort has been made to modify DAG levels and examine the subsequent effects on insulin resistance. Reduction of DAG effects through increased fatty acid oxidation [101,102] and increased IMCL synthesis [94,101] has been associated with improvements in insulin sensitivity.

Ceramide is derived from long-chain saturated fatty acids and serves as a precursor for complex sphingolipids. Ceramides may be produced by sphingomyelin hydrolysis or de novo synthesis from palmitoyl CoA [103]. High levels of ceramides have been observed in obese insulin-resistant humans [100,104]. Exercise training is associated with improved insulin resistance and reduced ceramide levels [101,102]. In healthy humans, total ceramide levels in skeletal muscle inversely correlate with insulin resistance, as measured by a hyperinsulinemic-euglycemic clamp [105]. Lipid-induced insulin resistance (goal FFA at 1750 μmol/L) has been associated with elevated skeletal muscle total ceramide levels [105]. Lower rates of lipid infusion (goal FFA at ~800 μmol/L) do not appear to affect intramuscular ceramide levels in humans; however, this particular study did not measure the effect of the lower rate of lipid infusion on insulin resistance or DAG levels [106]. The mechanism of ceramide producing insulin resistance may be disruption of the insulin signaling cascade because ceramide-related inhibition of AKT [104,107] and tyrosine phosphorylation of IRS-1 [108] have been observed. An extensive discussion of ceramide effects on insulin resistance is presented in a review by Summers [96].

Evidence also suggests that fatty acid composition, specifically fatty acid chain length and saturation, may influence skeletal muscle insulin resistance. In humans, insulin resistance has been shown to correlate with increased saturated fatty acids in IMCL [109] and LC-CoA content in skeletal muscle [110]. Animal studies have shown high-fat feeding associated with increased LC-CoA and insulin resistance [82,111]. Myotubes (C2C12) exposed to oleate or short saturated fatty acids (laurate and myristate) generate less DAG and ceramide and have less inhibition of AKT than myotubes exposed to palmitate (16:0) and other long-chain saturated chain fatty acids (stearate, arachidate, and lignocerate) [112]. In subjects with DM2, acipimox treatment for 7 days reduced plasma FFA levels (−49%), improved insulin sensitivity (+33%), and decreased total intramuscular LC-CoA levels (−22%) [113].

4. Are mitochondrial abnormalities involved in skeletal muscle insulin resistance?

Because the mitochondria are the primary cellular site for fatty acid oxidation and utilization, there is much interest in the role of reduced mitochondrial function contributing to "toxic" lipid metabolite accumulation and consequent insulin resistance. The mitochondria generate adenosine triphosphate (ATP) through oxidative phosphorylation, which couples oxidation of reducing equivalents (ie, reduced form of nicotinamide adenine dinucleotide [NADH] and flavin adenine dinucleotide [$FADH_2$]) to the phosphorylation of adenosine diphosphate (ADP). Reducing equivalents generated from glycolysis (NADH), the tricarboxylic acid (TCA) cycle (NADH, $FADH_2$), and β-oxidation of fatty acids (NADH, $FADH_2$) are oxidized by the electron transport chain (ETC) to produce the inner mitochondrial membrane proton gradient that drives ATP synthesis.

In humans and animals, numerous measures of mitochondrial capacity have been used, ranging from quantitation of mitochondrial density and enzyme content to functional evaluation of isolated mitochondria and in situ mitochondria. In vivo mitochondrial function has also been evaluated through the use of magnetic resonance spectrometry (MRS). Table 1 provides a selected listing of mitochondrial measurements, particularly those pertinent to this review.

Techniques for quantifying mitochondria range from electron microscopy–based morphometric measures of size, morphology, and tissue density [15]; to measurements of mitochondrial DNA (mtDNA) copy number [114]; to quantification of inner mitochondrial membrane area [115]. Measures of mitochondrial function include measures of fatty acid oxidation and oxidative phosphorylation. Indicators of fatty acid oxidation potential include measurements of enzymes associated with fatty acid transport protein into the cell (fatty acid transport protein [FAT/CD36]) [116], fatty acid transport into the mitochondria (carnitine palmitoyltransferase I [CPT1]) [117], and fatty acid β-oxidation (β hydroxyacyl-CoA dehydrogenase [HADH]) [118]. Estimates of oxidative phosphorylation potential include measurement of enzymes associated with the TCA cycle (pyruvate dehydrogenase, citrate synthase, succinate dehydrogenase) and ETC (NADH dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase) [119]. Active measures of mitochondria function include extraction of mitochondria to measure O_2 consumption capacity with varying substrates [99,120], CO_2 production with lipid substrates [121,122], and maximal ATP production with

Table 1
Measures of mitochondrial function

Mitochondrial measurements	Examples	Description
Mitochondrial structure		
	1. Histology	Measurement of mitochondrial size and morphology on electron microscopy
	2. Mitochondrial DNA copy number	Measurement of mtDNA content
	3. cardiolipin	Measurement of mitochondrial inner membrane area
Markers of mitochondrial function		
Fatty acid oxidation		
	1. CPT1	Enzyme located in outer mitochondrial membrane and facilitates transport of long-chain fatty acids across the membrane
	2. HADH	Enzyme involved in formation of acetyl CoA from fatty acid oxidation
	3. Fatty acid translocase	Long-chain transporter of fatty acids found in plasma and mitochondrial membranes
Oxidative phosphorylation		
	1. Pyruvate dehydrogenase	Enzyme involved in the conversion of pyruvate to acetyl CoA
	2. Citrate synthase	Enzyme in the 1st step of the TCA cycle (encoded from nuclear DNA)
	3. Succinate dehydrogenase	Enzyme in TCA cycle and ETC (complex 2, encoded from nuclear DNA)
	4. NADH dehydrogenase	Enzyme in ETC (complex 1, encoded from nuclear and mtDNA)
	5. Cytochrome *c* oxidase	Enzyme in ETC (complex 4, encoded from nuclear and mtDNA)
UCPs		
	UCP1-3	Dissipates proton gradient across inner mitochondrial membrane to separate electron transport from oxidative phosphorylation
Measures of mitochondrial function		
	1. O_2 consumption	Measurement of O_2 consumption under various scenarios of substrate/ADP/ATP inhibitor availability
	2. Fatty acid oxidation	
	a. Lipid utilization	Extraction of mitochondrial and measurement of mitochondrial CO_2 production after exposure to lipid
	b. Fatty acid transport	Extraction of mitochondria and measurement of lipid uptake
	3. In vitro ATP production	Extraction of mitochondria and measurement of ATP production with various substrates
	4. In vivo ATP production	MRS-based measurement of TCA cycle rate using ^{13}C
		MRS-based measurement of ATP synthesis or phosphocreatinine resynthesis using ^{31}P
Mitochondrial biogenesis markers		
	1. PGC-1a	Master regulator of mitochondrial function
	2. AMPK	Enhances oxidative (instead of glycolytic) energy production; objects of activation include PGC-1a and TFAM
	3. TFAM	Activator of mitochondrial transcription and mitochondrial genome replication

various substrates supporting oxidative phosphorylation [12]. Recently, MRS has been used to measure mitochondrial function in vivo by measuring the rate of ATP phosphorylation [123], the rate of postischemic phosphocreatinine recovery [124,125], and TCA cycle flux [126,127]. Markers of mitochondrial biogenesis include peroxisome proliferator–activated receptor γ coactivator–1α (PGC-1a) [128,129], mitochondrial transcription factor A (TFAM) [130], and AMPK [131,132].

Uncoupling proteins (UCPs) play a key role in mitochondrial function. Uncoupling proteins ameliorate the generation of reactive oxygen radicals by allowing protons to leak across the inner mitochondrial membrane, reducing mitochondrial inner membrane potential [133]. An adverse effect of UCP action is reduction of the efficiency of ATP synthesis in relation to the mitochondrial respiratory rate. Uncoupling protein 1 appears to be limited to the brown adipose tissue [134], UCP2 is expressed in a wide variety of tissues (including adipose tissue and skeletal muscle) [135], and UCP3 in humans appears to be restricted to skeletal muscle [136].

5. Does mitochondrial dysfunction correlate with skeletal muscle insulin resistance?

Evidence for reduced mitochondrial function has been seen both in the context of DM2 [11-15,99,124,137] and in insulin-resistant subjects without DM2 [8,14,127]. Specific measures of reduced mitochondrial function in such subjects include smaller mitochondrial size [15], decreased expression of oxidative phosphorylation genes [13,14], lower levels of mitochondrial enzyme activity [11,12], lower mitochondrial ATP production [11,12], lower ATP synthetic flux rates [123,137], slower TCA cycle flux rates [127], and slower postischemic phosphocreatinine recovery [124].

Reduced mitochondrial function can also take the form of "metabolic inflexibility," a reduced ability to switch from predominant fat utilization during fasting to predominant glucose utilization during fed (insulin-stimulated) conditions [138]. The severity of metabolic inflexibility (assessed by in vivo and in vitro measures) has been associated with the degree of insulin insensitivity in healthy subjects [122] and in subjects with a family history of DM2 [139].

The existence of multiple types of mitochondrial functional capacity measurements, however, has led to discrepant and often contradictory observations between individual studies examining mitochondrial function and insulin resistance. When insulin-resistant subjects were compared with control subjects, reductions in insulin signaling, mitochondrial density, and cytochrome *c* oxidase activity were observed; but the anticipated concordant changes in levels of succinate dehydrogenase (protein), pyruvate dehydrogenase (protein), mtDNA copy number, PGC-1a (messenger RNA [mRNA] and protein) and PGC-1β (mRNA and protein), and TFAM (mRNA and protein) were not observed [140]. In subjects with DM2, a reduction in mitochondrial respiration in the absence of expected changes in citrate synthase activity and fatty acid oxidation (HADH levels) was observed [99]. In another study in subjects with DM2, reductions in skeletal muscle mitochondrial respiration were not observed when mitochondria respiration was normalized to mtDNA content or citrate synthase activity [120].

In contrast to a previous study [124], another study using in vivo ^{31}P MRS estimates of mitochondrial capacity from postexercise measures of phosphocreatinine and ADP recovery times found no differences between subjects with long-standing insulin-treated DM2 (>5 years), subjects with prediabetes, subjects with recently diagnosed DM2 (<1 month), and sedentary normal controls [125]. In further support of a possible disconnect between mitochondrial ATP synthetic capacity and insulin resistance, comparison between Northern European subjects with DM2 and Asian Indian subjects with DM2 found that Asian Indians with DM2 had higher levels of insulin resistance despite the presence of higher skeletal muscle mitochondrial capacity, as measured by mtDNA copy number, mRNA of oxidative phosphorylation genes, citrate synthase enzyme activity, and maximal ATP production rate [141]. Thus, in humans, the relationship between mitochondrial function and insulin resistance remains indeterminate, with varying degrees of correlation observed depending on mitochondrial function measurement and patient selection. Given the recent findings in Asian Indians with DM2 [141], ethnicity may play a significant role in the etiology of insulin resistance; and the mitochondrial abnormalities reported in non-Asian subjects [11-15,99,124,137] are likely less relevant to the development of insulin resistance in Asian Indians.

Data from animal studies have also questioned the role of mitochondrial dysfunction in causing insulin resistance. A recent longitudinal study comparing Zucker diabetic fatty rats vs lean heterozygote littermates showed Zucker diabetic fatty rats developing diabetes in association with increasing IMCL content, whereas skeletal muscle mitochondrial function (measured by ^{31}P MRS, succinate dehydrogenase activity, citrate synthase activity) remained comparable to the lean littermates and mitochondrial fatty acid oxidation (measured by very long chain acyl CoA dehydrogenase activity) was increased compared with lean littermates [142]. Several animal models of mitochondrial dysfunction including whole-body LC-CoA dehydrogenase knockout mice [143], skeletal muscle–specific PGC-1a knockout mice [144], and mice with targeted dysfunction of oxidative phosphorylation [145] have not demonstrated skeletal muscle insulin resistance.

6. Does lipid exposure affect skeletal muscle mitochondrial function?

Not surprisingly, the aforementioned effects of lipid excess on insulin sensitivity have generated interest in defining the role of excess lipid exposure on the development of skeletal muscle mitochondrial dysfunction. Although IMCL levels have been shown to be inversely correlated with MRS-based measurement of in vivo mitochondrial function [123], this finding has not been consistently observed in other in vivo MRS studies [124,137]. Exposure of isolated human and mice mitochondria to doses of FFA metabolites (palmitoyl-L-carnitine, palmitoyl CoA, oleoyl CoA) similar to intramyocellular concentrations (0.5-2 μmol/L) has been shown to stimulate ATP synthesis, whereas exposure to high levels of FFA metabolites (>5 μmol/L) showed a dose-dependent inhibition of ATP synthesis [146]. This evidence raises the possibility that exposure to high levels of FFA may cause mitochondrial dysfunction and lead to insulin resistance.

6.1. What is the role of lipid infusion on mitochondrial function?

In 1963, Randle et al [147] proposed that excess lipid exposure inhibited glucose oxidation by increasing intramitochondrial CoA levels with subsequent inhibition of pyruvate dehydrogenase and phosphofructokinase, 2 critical enzymes in the glycolytic pathway. As lipid infusion is a well-established model for lipid insulin resistance in humans [10,75-77], the effects of lipid infusion on skeletal muscle mitochondrial function has been studied. In humans, lipid infusion (6 hours; plateau FFA, 2300 ± 300 μmol/L) has been shown to decrease activity of pyruvate dehydrogenase and increase mRNA levels of pyruvate dehydrogenase kinase isoform 4, an inhibitor of pyruvate dehydrogenase, suggesting that skeletal muscle preferentially oxidizes lipid in the setting of lipid infusion [148]. In humans, lipid infusion (6 hours; plateau FFA, 1037 ± 29 μmol/L) decreased mitochondrial function in vivo as measured by ^{31}P MRS, with a decline in insulin sensitivity that correlated with declines in intramyocellular glucose-6-phosphate and insulin stimulated Pi → ATP flux [149]. This decline in glucose-6-phosphate suggests that a major effect of lipid infusion is to decrease glucose entry into the cell, thus limiting glycolysis and glucose oxidation.

Lipid infusion effects on mitochondrial parameters have been measured. The effects of short-term (6 hours or less) lipid infusion on mitochondrial gene expression have been

mixed. A crossover study of lipid (6 hours; plateau FFA, 1475 ± 88 μmol/L) vs glycerol (plateau FFA, 129 ± 14 μmol/L) infusion in healthy humans demonstrated lipid infusion increasing IMCL, insulin resistance, and UCP3 mRNA levels while decreasing PGC-1a mRNA and PGC-1b mRNA levels [150]. In contrast, another crossover study comparing lipid (5 hours; plateau FFA, 1670 ± 130 μmol/L) vs saline (plateau FFA, 490 ± 87 μmol/L) vs saline + heparin (plateau FFA, 670 ± 90 μmol/L) infusion showed no changes in PGC-1a or UCP3 mRNA levels [151]. The discrepancy between these 2 studies, particularly with regard to PGC-1 and UCP3 measurements, may be related to several factors, including use of different controls (glycerol [150] vs saline [151]), shorter duration of infusion (6 [150] vs 5 hours [151]), use of heparin (no use [150] vs use [151]), concurrent hyperinsulinemic-euglycemic clamp (present [150] or absent [151]), and reference for reporting mRNA changes (reference gene 36B4 [150], reference gene β-actin [151], fold change from baseline levels [151]). In particular, the effects of the hyperinsulinemic-euglycemic clamp on mitochondrial parameters must be considered, as the hyperinsulinemic-euglycemic clamp has been shown to increase PGC-1a mRNA levels [152].This issue was clarified by a crossover study of prolonged lipid infusion (48 hours; plateau FFA, 1730 ± 430 μmol/L) vs saline (48 hours; plateau FFA, 480 ± 20 μmol/L) in healthy subjects that demonstrated that lipid infusion increased insulin resistance (+24%) and decreased mRNA expression of numerous nuclear encoded mitochondrial genes including PGC-1 [153]. In comparison with the studies described earlier, this study did not have a concurrent heparin infusion [151]; and the muscle biopsy for evaluation of mitochondrial gene expression was performed after the lipid/saline infusion and before the hyperinsulinemic-euglycemic clamp [150]. In contrast to the earlier studies [150,151], microarray analysis of muscle gene expression was used, with specific confirmation of mRNA changes (in particular PGC-1) by polymerase chain reaction analysis (reference gene 18S ribosomal RNA) [153].

6.2. What is the role of high-fat diet on mitochondrial function?

Although lipid infusion appears to reduce measures of mitochondrial function, the consequences of a high-fat diet on mitochondrial function have been mixed. Based on the aforementioned lipid infusion data, a high-fat diet would be predicted to increase FFA availability and to decrease glucose uptake by skeletal muscle. Therefore, the accumulation of IMCL and lipid metabolites expected to occur with the ingestion of a high-fat diet could promote mitochondrial damage. Conversely, intrinsic mitochondrial dysfunction might facilitate the development of insulin resistance, as ingestion of a high-fat diet in the setting of mitochondrial dysfunction may facilitate accumulation of IMCL and lipid metabolites. However, there are conflicting animal and human reports showing that a high-fat diet may decrease [154,155], not affect [118,156,157], or increase [158-160] measures of skeletal muscle mitochondrial performance.

6.3. Human studies of high-fat feeding on mitochondrial function

A short-term high-fat diet has been shown to reduce markers of skeletal muscle mitochondrial function. Compared with insulin-sensitive subjects without a family history of DM2, insulin-sensitive subjects with a family history of DM2 exhibited a decline in mRNA expression of PGC-1a and FAT/CD36 3 hours after ingesting a single high-fat meal (76% fat), suggesting that impaired ability to oxidize fat upon exposure to a high-fat meal precedes the development of insulin resistance in subjects with a family history of DM2 [154]. In another study, healthy young men who completed a 3-day high-fat diet (50% fat) experienced reductions in the gene expression of PGC-1a (decline of 20%, $P < .01$), PGC-1β (decline of 25%, $P < .05$), and 6 genes involved in oxidative phosphorylation [155].

Compared with the short-term (3 hours to 3 days) high-fat diets in which skeletal muscle mitochondrial function was found to be reduced, longer duration of high-fat feeding (3-7 weeks) in humans has had equivocal effects on measures of mitochondrial function, with alterations of β-oxidative capacity but not oxidative phosphorylation capacity. Comparing athletes feed a high-fat (69% fat) vs usual diet (30% fat) for 15 days, skeletal muscle CPT1 activity increased without alterations of citrate synthase activity or HADH activity, suggesting that high-fat diet in this study increased capacity for transfer of fatty acids into mitochondria but did not increase mitochondrial oxidative capacity [156]. Similarly, athletes fed a high-fat (53%) vs low-fat (17%) diet for 5 weeks increased skeletal muscle IMCL levels but did not alter mitochondrial density [157]. In untrained humans receiving a high-fat (62%) diet for 7 weeks, increased HADH activity but no change in citrate synthase enzyme activity was observed [118].

6.4. Animal studies of high-fat feeding on mitochondrial function

Short-term high-fat diets in animals have been shown to down-regulate measures of mitochondrial function. A 3-day high-fat diet (33% fat) in rats (age not stated; average weight, 320 g) down-regulates gene expression of several oxidative phosphorylation enzymes (malate dehydrogenase, ATP synthase, NADH dehydrogenase, cytochrome *c* oxidase) in skeletal muscle [161]. A high-fat diet in mice (age, 5 weeks; 45% high-fat diet for 3 weeks) was associated with declines in skeletal muscle mRNA expression of numerous mitochondrial genes compared with control mice (10% fat diet for 3 weeks) as well as PGC-1a (decline of 90%, $P < .01$) and PGC-1β (decline of 90%, $P < .05$); yet the activity of citrate synthase, cytochrome *c* oxidase, and HADH was not significantly changed [155]. A high-fat, high-sucrose diet (36% fat, 35% carbohydrate, 50% sucrose) in mice (age,

4 weeks) produced glucose intolerance at 4 weeks and diabetes at 16 weeks [162]. Yet, these mice did not have evidence of mitochondrial dysfunction at 4 weeks as determined by measures of mitochondrial function such as mitochondrial density (mtDNA and electron microscopy), PGC-1a, and oxygen consumption of isolated muscle fibers; however, there was evidence of mitochondrial dysfunction by 16 weeks, as demonstrated by reduced mitochondrial density (mtDNA and electron microscopy), reduced mitochondrial gene expression (Cox 1, Cox 3, PGC-1a), and reduced oxygen consumption of isolated fibers [162]. Because of the lack of normalization relative to mitochondrial content, mitochondrial dysfunction at the level of the individual mitochondria could not be assessed; nevertheless, the authors concluded that mitochondrial dysfunction at the level of skeletal muscle does not precede the development of insulin resistance [162].

Although prolonged high-fat feeding in animals has been shown to reduce skeletal muscle mitochondrial function [155,162], other studies have shown prolonged high-fat feeding in animals to have no effect [163,164] or may even increase measures of mitochondrial function [158-160,165,166]. Rats (age not stated; weight, 245-300 g) fed a 5-week high-fat diet (78% fat) as compared with a control diet (11% fat) had higher citrate synthase activity in skeletal muscle after 1 week of feeding, which was not maintained after 5 weeks of feeding, whereas HADH rose after 1 week of a high-fat diet and continued to remain high after 5 weeks of a high-fat diet [163]. In a study of more prolonged feeding, rats (starting age 10 weeks) fed a high-fat (60% fat) diet compared with rats fed a control diet (25% fat) for 36 weeks had no change in mitochondrial ATP production, citrate synthase activity, or cytochrome *c* oxidase mRNA levels [164]. Insulin resistance in these rats was not specifically reported; however, the high-fat diet–fed rats were heavier (~730-770 g) compared with the control diet–fed rats (626 g) [164].

Increases in mitochondrial function measures have also been observed in high-fat feeding studies. Compared with a 15-day low-fat diet (10.6%), recently weaned rats (age, 25 days) subjected to a 15-day high-fat diet (50%) had comparable succinate dehydrogenase and citrate synthase activity in skeletal muscle; yet the isolated skeletal muscle mitochondria from rats fed the high-fat diet had higher rates of mitochondrial respiration and had higher rates of uncoupling with palmitate exposure [158]. Mice (8 weeks old) fed a high-fat diet (45% fat) vs a control diet (8% from fat) had higher rates of palmitate oxidation to CO_2 production in muscle homogenates (at 5 and 20 weeks); increased oxygen consumption in isolated mitochondria (at 20 weeks) given sufficient substrate; and increased activity (at 5 and 20 weeks) of citrate synthase, HADH, medium-chain acyl CoA dehydrogenase, and CPT1 [159]. Similar to the previous study [158], a high-fat diet may increase mitochondrial uncoupling [159] as evidenced by elevated protein expression of UCP3 (at 5 and 20 weeks) in mice fed the high-fat compared with the low-fat diet. In another study, rats (age, 6 weeks; personal communication with Dr Holloszy) fed a high-fat diet specifically designed to raise plasma FFAs (4-week diet, 60% fat + daily heparin injection for activation of lipoprotein lipase) demonstrated increased mtDNA copy number, increased palmitate oxidation to CO_2 from muscle homogenate, increased enzyme levels of citrate synthase, increased oxidative phosphorylation enzyme levels (COX1, COX4, ATP synthase subunit α), and increased fatty acid oxidation enzyme levels (medium-chain acyl CoA dehydrogenase, LC-CoA dehydrogenase, very long chain acyl CoA dehydrogenase) [166]. In contrast with previous studies, older rats (3 months old) subjected to a high-fat diet (45% fat vs 13.8% fat for 40 days) initially had increased in vitro muscle fiber mitochondrial ATP synthesis (days 14-20) and mitochondrial respiration (day 14), which were not sustained and actually declined (compared with the low-fat group) with continued feeding (day 40) [165]. Mechanistically, this decline in in vitro muscle fiber muscle ATP synthesis and mitochondrial respiration observed at the conclusion of the high-fat diet (day 40) was associated with increased fractional synthesis rate of mitochondrial and mixed muscle proteins, suggesting possible effects from protein turnover rates or disassociation of in vitro muscle fiber muscle ATP synthesis and mitochondrial respiration measurements from muscle/mitochondrial protein synthesis [160].

The inconsistency between high-fat feeding and markers of mitochondrial function is likely related to inconsistency between the individual studies. A high-fat diet appears to increase mitochondrial measures of β-oxidation. Whether it may affect mitochondrial oxygen consumption or ATP generation capacity may be related to contributing factors such as differences in active vs passive measures of mitochondrial function, species, age at initiation of diet, diet composition, diet duration, diet effects on plasma fatty acid levels, and sampling timing during the dietary program. Most notably, the age of the animal at the time of exposure to the high-fat diet may influence the effects of the high-fat diet on mitochondrial function. Because enhanced mitochondrial function with high-fat feeding has generally been observed in younger animals, this suggests an adaptive mechanism to high-fat feeding that is more prominent in younger animals [158,159,166].

7. Does mitochondrial dysfunction facilitate accumulation of toxic lipid species?

The available evidence demonstrates that lipid exposure may cause mitochondrial dysfunction; however, it has also been theorized that mitochondrial dysfunction may lead to accumulation of toxic lipid species with subsequent effects on insulin resistance [167]. Mitochondria from untrained rats had higher rates of incomplete fatty acid oxidation compared with mitochondria from trained (4 weeks of treadmill

running) rats [168]. In rats, prolonged blockage (4 weeks) of fatty acid β-oxidation by inhibition of CPT1 increased IMCL content and increased high-fat diet–induced insulin resistance [169]. However, it appears that the effect of mitochondrial dysfunction on lipid metabolite accumulation and insulin resistance may be particularly dependent on the mechanism used to cause mitochondrial dysfunction and the extent of metabolite accumulation. Mice with blockage of fatty acid β-oxidation by a whole-body knockout of LC-CoA dehydrogenase had increased skeletal muscle long-chain Co A levels compared with control mice, yet maintained similar muscle glucose uptake and insulin signaling; this observation was attributed to comparable skeletal muscle DAG levels between the knockout and the control mice [143]. Mice with partial CPT1 inhibition through a whole-body knockout of malonyl-CoA decarboxylase, an enzyme that degrades malonyl-CoA, a natural inhibitor of CPT1, had lower rates of incomplete fatty acid catabolism and were protected from developing insulin resistance after a 12-week high-fat diet [170]. It has been hypothesized that increased β-oxidation in the setting of reduced TCA cycling or oxidative phosphorylation may lead to increased rates of incomplete fatty acid catabolism and reduced insulin signaling [170].

There is also interest in skeletal muscle insulin resistance arising from mitochondrial generation of reactive oxygen species (ROS) [171]. In the process of generating ATP by oxidative phosphorylation, the mitochondrial ETC produces ROS [172]. Consequently, mitochondria have evolved several mechanisms to mitigate the effects of ROS including oxygen radical scavengers (ie, mitochondrial superoxide dismutase [SOD2]) [173] and UCPs [174].

Studies have examined the physiologic effects of ROS by disrupting the protective mechanisms found in mitochondria. Compared with wild-type mice, homozygous mutant mice lacking SOD2 have reduced succinate dehydrogenase activity and normal cytochrome *c* oxidase activity in the skeletal muscle and heart. These mice die approximately 10 days after birth from a dilated cardiomyopathy [175]. Follow-up preliminary studies of heterozygote mice with reduced levels of SOD2 (50%) demonstrated no difference in skeletal muscle insulin resistance (as measured by hyperinsulinemic clamp or in vitro glucose uptake at level of vastus lateralis muscle) compared with wild-type mice [176].

In mitochondria, UCPs antagonize the generation of ROS by allowing protons to leak across the inner mitochondrial membrane and reduce the mitochondrial inner membrane potential [133]. This process decreases the efficiency of oxidative phosphorylation by partially uncoupling respiration from ATP synthesis [133]. Because generation of mitochondrial ROS depends on the mitochondrial inner membrane potential, reduction of the inner membrane potential through uncoupling reduces the rate of generation of ROS. Several types of UCPs (UCP 1-5) have been described [177], of which UCP3 has generated particular interest because of its expression in skeletal muscle [136,178] and its possible role in exporting fatty acids out of mitochondria [179,180] or transporting fatty acid peroxide anions across the inner mitochondrial membrane [181].

Uncoupling protein 3 was discovered in 1997 by screening a human skeletal muscle complementary DNA library [136]. Increased function [133] and expression [162] of UCP3 have been observed with exposure to 4-HNE [182] and ROS [133,162]. In humans, skeletal muscle UCP3 mRNA levels rise with fasting [183], lipid infusion [151], and saline infusion [151]. Unlike adipose tissue UCP2 mRNA levels, skeletal muscle UCP2 and UCP3 mRNA levels do not correlate with body mass index [183]. In humans, the correlation between UCP3 expression and insulin resistance has been inconsistent. Increased skeletal muscle UCP3 mRNA levels has been observed in obesity [184] and DM2 [184,185]. Yet, reduced skeletal muscle UCP3 mRNA levels in DM2 [186] and reduced UCP3 protein levels in DM2 [187] have been observed as well. Although more studies are necessary to reconcile the differences, the discrepancy between UCP3 mRNA expression level in DM2 may be influenced by differences in withholding diabetes treatment before the biopsy (held overnight [186] vs more than 1 week [184,185]) as well as use of a prebiopsy standardized diet (not used [186] vs 2 days [184,185]). Thus, the human studies relating alterations in UCP3 to DM2 have remained inconclusive.

Of interest, several UCP3 knockout mouse models have been created. One mouse knockout model showed increased respiratory coupling in skeletal muscle mitochondria associated with increased generation of ROS; however, these mice did not display any differences in weight, exercise tolerance, fatty acid oxidation, fasting glucose, or glucose oxidation compared with wild-type mice [188]. Another mouse UCP3 knockout model displayed fasting glucose levels comparable to wild-type mice [189]. Magnetic resonance spectroscopy has been used to measure skeletal muscle mitochondrial function in UCP3 knockout mice with contradictory results. Increased Pi → ATP flux observed with ^{31}P nuclear magnetic resonance (NMR) suggested elevated ATP synthesis, yet ^{13}C NMR measurements from ^{13}C acetate infusion demonstrated no change in TCA cycle flux rate [190]. Thus, although UCP3 underexpression is associated with increased oxidative damage [191], the effects of the UCP3 underexpression on mitochondrial performance and insulin resistance remain uncertain.

Several mouse models of UCP3 overexpression have also been created. In one model, UCP3 was overexpressed using the rat myosin light-chain 2 promoter [192]. Compared with wild-type mice, the transgenic mice had higher skeletal muscle oxygen consumption, lower fasting glucose levels, and improved glucose tolerance and were resistant to diet-induced obesity and diet-induced insulin resistance [192]. In another mouse model of UCP3 overexpression in skeletal muscle, the human α-skeletal actin promoter was used [193]. These mice were hyperphagic, yet were leaner and more insulin sensitive than wild-type controls [193]. Further studies of these mice demonstrated maintenance of insulin

signaling in the setting of a high-fat diet. This appeared to result from lower DAG levels, reduced PKCΘ activity, and preserved PI 3-kinase activity [194]. The effect of UCP3 overexpression on ROS remains unclear. Overexpression of UCP3 in L6 myotubes decreased the production of mitochondrial ROS and increased fatty acid oxidation [195]. However, when L6 myotubes overexpressing UCP3 were deliberately exposed to palmitate, an increase in intracellular ROS was observed compared with control L6 myotubes [196]. This discrepancy highlights the complex role of UCP3. Although UCP3 overexpression may reduce coupling and, thereby, reduce ROS production, perhaps the increased fatty acid oxidation arising from UCP3 overexpression may overwhelm the uncoupling effects. Thus, although UCP3 is the primary UCP in skeletal muscle, UCP3 overexpression appears to have a more significant physiologic impact than its absence in diet-induced obesity and insulin resistance. The mechanism of this protection remains unclear, but may be related to decreased generation of ROS or increased ability to oxidize lipids.

8. A broader view of the etiology of skeletal muscle insulin resistance

Although the skeletal muscle is the largest site of insulin-mediated glucose disposal [4], skeletal muscle–specific alterations may not necessarily improve overall insulin resistance. A mouse with skeletal muscle–specific deletion of lipoprotein lipase exhibited improved insulin signaling and glucose uptake in skeletal muscle yet exhibited increased insulin resistance in the heart, white adipose tissue, and liver [197]. As plasma FFA, TG, and cytokine (interleukin-1β and interleukin-6) were not significantly different between the knockout and wild-type mice, the authors proposed that the observed effects may be due to the heart and liver shifting to lipid oxidation in compensation for the reduced skeletal muscle lipid oxidation [197]. Mice with skeletal muscle–specific knockout of PGC-1a demonstrated impaired glucose tolerance, in the surprising context of reduced β-cell function and normal skeletal muscle insulin sensitivity [144]. Further investigation demonstrated increased expression of proinflammatory genes in the skeletal muscle (ie, suppressor of cytokine signaling 1 [SOCS1] and suppressor of cytokine signaling 3 [SOCS3] tumor necrosis factor–α, interleukin-6, CD68) as well as increased serum interleukin-6 levels [144]. Exposure of β-cells to interleukin-6 in vitro suppressed insulin secretion in response to hyperglycemia, providing evidence for cross talk between skeletal muscle and β-cells [144].

Likewise, changes at the level of the liver and adipose tissue may influence skeletal muscle insulin resistance. In mice with adipose-specific knockout of GLUT4, muscle glucose uptake was impaired in vivo but preserved in vitro [198], suggesting the presence of circulatory factor (such as serum retinol binding protein 4, cytokines, etc) affecting skeletal muscle insulin sensitivity [199]. Stress at the level of the endoplasmic reticulum in liver and adipose tissue (but not skeletal muscle) is associated with obesity and insulin resistance [200]. Yet in mice, treatment of endoplasmic reticulum stress with orally administered ursodeoxycholic acid improved peripheral insulin sensitivity and muscle glucose uptake [201]. These observations suggest that skeletal muscle–specific changes must be interpreted in the context of cross talk with other organs involved in glucose homeostasis. These observations suggest that the causes of alterations of skeletal muscle mitochondrial function and insulin sensitivity may be related to exogenous and endogenous factors.

9. Summary

This review explores the complex relationship between excess lipid exposure, mitochondrial dysfunction, and insulin resistance at the level of human skeletal muscle. Lipotoxicity, that is, the elevation of lipids and/or associated lipid metabolites within blood and tissues with subsequent metabolic derangement, has been proposed as a possible mechanism of skeletal muscle insulin resistance. Intravenous lipid infusion is a well-documented method of inducing insulin resistance. Although IMCL content has been correlated with insulin resistance, there is increasing evidence that lipid metabolites such as 4-HNE, DAG, ceramide, and LC-CoA may play a more significant role than TGs in producing skeletal muscle insulin resistance.

The association between mitochondrial dysfunction and insulin resistance is unclear, particularly because of the varied options for measuring mitochondrial function. The effect of acute lipid exposure producing skeletal muscle insulin resistance in humans is well documented. The effects of sustained lipid exposure from dietary ingestion on skeletal muscle insulin resistance and skeletal muscle mitochondrial function remain disputed. The effects of skeletal muscle mitochondrial dysfunction on accumulation of lipotoxic species and skeletal muscle insulin resistance also remain uncertain. Certainly, pursuit of the role of lipid metabolites and of their roles in the generation of skeletal muscle insulin resistance remains an exciting area for future research.

Moreover, alteration in skeletal muscle insulin resistance does not occur in isolation, as functional perturbations of any component of the glucose homeostatic system may initiate development of insulin resistance in skeletal muscle through cross talk between tissues. Nevertheless, understanding pathophysiology of skeletal muscle insulin resistance remains critically important because of its role in preceding and facilitating the development of DM2.

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