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Metabolic Pathways at the Crossroads of Diabetes and Inborn Errors

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Summary

Research over the past two decades has led to advances in our understanding of the genetic and metabolic factors that underlie the pathogenesis of type 2 diabetes mellitus (T2DM). While T2DM is defined by its hallmark metabolic symptoms, the genetic risk factors for T2DM are more immune-related than metabolism-related, and the observed metabolic disease may be secondary to chronic inflammation. Regardless, these metabolic changes are not benign, as the accumulation of some metabolic intermediates serves to further drive the inflammation and cell stress, eventually leading to insulin resistance and ultimately to T2DM. Because many of the biochemical changes observed in the pre-diabetic state (i.e., ectopic lipid storage, increased acylcarnitines, increased branched-chain amino acids) are also observed in patients with rare inborn errors of fatty acid and amino acid metabolism, an interesting question is raised regarding whether isolated metabolic gene defects can confer an increased risk for T2DM. In this review, we attempt to address this question by summarizing the literature regarding the metabolic pathways at the crossroads of diabetes and inborn errors of metabolism. Studies using cell culture and animal models have revealed that, within a given pathway, disrupting some genes can lead to insulin resistance while for others there may be no effect or even improved insulin sensitivity. This differential response to ablating a single metabolic gene appears to be dependent upon the specific metabolic intermediates that accumulate and whether these intermediates subsequently activate inflammatory pathways. This highlights the need for future studies to determine whether certain inborn errors may confer increased risk for diabetes as the patients age.

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Author Contributions

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Conflicts of Interest

Eric Goetzman, Zhenwei Gong, Manuel Schiff, Yan Wang, and Radhika Muzumdar have no conflicts of interest to report related to this work.

Informed Consent/Animal Rights

This article does not contain studies with human or animal subjects performed by any of the authors.

Introduction

The boundaries between Mendelian disorders and polygenic disorders have become increasingly blurred as our understanding of human genetics has become more sophisticated. In the field of inborn errors of metabolism (IEM), this has led to some debate about exactly what constitutes an inherited metabolic disorder (Morava et al., 2015). Inherited metabolic disorders are often thought of as single-gene diseases identifiable by a specific biochemical test. In the future, as predictive genomics testing and personalized medicine become routine, polygenic metabolic diseases such as Type 2 diabetes mellitus (T2DM) may become recognized as inherited metabolic disorders. At present, we know that T2DM has a strong genetic component but we still do not understand the nature of this component. The risk of an individual developing T2DM increases greatly if one, or particularly two, parents had T2DM. Monozygotic twin studies indicate that the heritability of T2DM may exceed 75% (Carlsson et al., 2013; Lehtovirta et al., 2010) but the age of onset can vary widely within twin pairs, indicating a key triggering role for environmental factors. Many genome-wide association studies (GWAS) and a recent large-scale whole exome sequencing study have sought to identify the alleles responsible for the heritability of T2DM and have succeeded in collectively identifying about 150 gene variants (Fuchsberger et al., 2016; Scott et al., 2007; Sladek et al., 2007; Zeggini et al., 2007). These alleles are common (minor allele frequencies >5%) and largely localize to noncoding regions of immune-related genes rather than to metabolism-related genes. Subsequent studies have shown that the identified alleles only explain a small portion of the observed heritability of T2DM (Prasad and Groop, 2015) and led to the concept of “missing heritability,” the source of which is an ongoing matter of debate.

While T2DM and IEMs may not have obvious genetic overlap, they certainly have phenotypic overlap. There have been case reports of IEMs “masquerading as” or “mimicking” diabetes, particularly for methymalonic aciduria (Abramowicz et al., 1994; Boeckx and Hicks, 1982; Dejkhamron et al., 2016; Guven et al., 2012; Sharda et al., 2013). Scheuner et al mined the OMIM database and identified 37 Mendelian disorders for which adult-onset diabetes was a phenotypic component observed in two or more families (Scheuner et al., 2004). The metabolic pathways frequently disrupted in IEMs are the same pathways that become dysfunctional during the development of insulin resistance and T2DM. As mortality from IEMs has been reduced and symptomatology better controlled, IEM patients are living longer. The question then becomes whether underlying IEMs, lifestyle factors, and aging will intersect to create increased risk for insulin resistance or T2DM as IEM patients age. This review will highlight our current knowledge regarding the role of dysregulated mitochondrial function/metabolism, particularly lipid metabolism and branched-chain amino acid (BCAA) metabolism, in the etiology of T2DM. Of interest is the question of which comes first, dysregulated metabolism or insulin resistance? This question is relevant to understanding and treating T2DM as well as for assessing the T2DM risk in IEM patients who chronically contend with dysregulated metabolism from birth.

Lipid Metabolism

Carbohydrate and lipid metabolism are closely intertwined and most patients with T2DM exhibit some form of dysregulated lipid metabolism. This can include elevated blood fatty acids and acylcarnitines as well as increased intracellular lipid storage in multiple tissues such as liver, muscle, and pancreas. Recent research has refined our understanding of how these alterations in lipid metabolism can lead to downstream effects on glucose metabolism and insulin signaling. However, we still do not understand the origins of the altered lipid metabolism. In this section, we will summarize recent findings on the mechanisms by which elevated circulating or intracellular lipids disrupt glucose homeostasis (lipotoxicity), followed by an exploration of whether defects in fatty acid oxidation may play a causative role in dysregulating lipid metabolism in T2DM.

Lipotoxicity

Most lipids circulating in the blood or stored within cells are in the form of triglycerides (TG). While TG by themselves are neutral lipids and are not harmful, intermediates derived from TG such as free fatty acids (FFA), ceramides, and diacylglycerol (DAG) are lipotoxic and have been associated with insulin resistance. While the concept of ectopic lipid storage and subsequent lipotoxicity have been recognized for many years, only recently has the importance of inflammatory signaling become recognized as a key link between lipids and insulin resistance (Sears and Perry, 2015). Similar mechanisms have been identified in multiple tissues including adipose tissue, hypothalamus, muscle, liver, and pancreas.

Adipose tissue is the epicenter of lipid homeostasis. Failure to properly sequester fatty acids within adipose tissue is the beginning of a cascade of events that leads to lipotoxicity. Adipose tissue is now known to be an immunologic organ, secreting dozens of factors including adipokines and inflammatory cytokines such as adiponectin, leptin, resistin, visfatin, tumor necrosis factor- α (TNF α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). Chronic overfeeding leads to enlarged adipocytes; when the adipocytes reach their expansion capacity, hypoxia occurs and hypoxia-inducible factor-1 (HIF-1) becomes upregulated and more active (Hotamisligil, 2006; Lionetti et al., 2009; Ye et al., 2007). HIF-1 is a key driver of adipose inflammation and the subsequent elevated secretion of adipokines and inflammatory cytokines which promote macrophage infiltration (Skurk et al., 2007). Macrophage infiltration and sustained inflammatory signaling can induce insulin resistance within the adipose tissue itself and beyond. Recently, Hernández and colleagues demonstrated that overwhelming the capacity of adipose tissue acutely with a 6 hour palmitate infusion decreases whole body and adipose tissue insulin sensitivity by 25% and 34% respectively in humans (Hernandez et al., 2017). When insulin resistance occurs at the level of adipose tissue, the balance of FFA storage and release is broken. FFA output from adipose tissue becomes elevated, leading to high circulating lipids, ectopic lipid storage, and lipotoxicity in other tissues. The importance of adipose tissue as a sink for lipid storage is exemplified by inborn lipodystrophies such as Berardinelli-Seip syndrome, in which the absence of adipose tissue leads to profound insulin resistance (Van Maldergem, 1993).

The liver and adipose tissue work in concert to coordinate blood lipids. If adipose tissue is the warehouse for storing lipids, then the liver is the sorting and shipping center. During fed states, the liver packages nutritional excess into VLDL particles for shipment to adipose tissue. Conversely, during fasting the liver takes up FFA released by adipose tissue and metabolizes them to ketone bodies. Under conditions of nutrient excess or adipose insulin resistance as mentioned above, delivery of FFA to the liver increases, causing fat accumulation within the liver. Increased hepatic steatosis is associated with insulin resistance and can further progress into non-alcoholic steatohepatitis (NASH) and cirrhosis. FFA-induced insulin resistance in the liver leads to increased hepatic glucose production through the upregulation of both gluconeogenesis and glycogenolysis (Boden et al., 2002; Roden et al., 2000). Thus, FFA-induced hepatic insulin resistance may compound the metabolic syndrome by further elevating blood glucose under conditions where glucose disposal is already compromised.

Lipotoxicity in pancreatic β -cells has been recognized for nearly 25 years. In a landmark 1994 study using the Zucker fatty rat model of T2DM, blood FFA rose steadily during the pre-diabetic state, causing a 10-fold increase in lipid accumulation within β -cells and a subsequent impairment of glucose-stimulated insulin secretion (Lee et al., 1994). In recent years the mechanisms of β -cell lipotoxicity have been elucidated and refined. Excessive FFA have negative effects on both β -cell survival and function through increasing oxidative stress, ER stress, inflammatory mediators, and cytokines (Sharma and Alonso, 2014). Chen et al. showed that lipid droplet associated protein perilipin 2 (PLIN2) plays a role in FFA induced lipotoxicity in β -cells through regulation of autophagy and ER stress (Chen et al., 2017). In diabetic mice, PLIN2 deficiency prevented β -cell apoptosis, increased β -cell mass, and improved insulin secretion (Chen et al., 2017). These important studies provide a causal link between neutral lipid droplet homeostasis and β -cell dysfunction/death via lipotoxicity, and provides proof of principle for the mitigation of β -cell dysfunction by preventing lipid accumulation.

Heart and muscle are major utilizers of fatty acids but chronic exposure to elevated FFA can promote insulin resistance. Recent studies point to inflammatory signaling as playing a key role. Protein kinase-c theta (PKC- θ), I κ B-kinase (IKK), and C-Jun N-terminal kinase (JNK) are activated with increased FFA; knockout of these kinases significantly protects mice from FFA-induced insulin resistance (Hirosumi et al., 2002; Kim et al., 2004; Yuan et al., 2001). Further investigation demonstrated that the activation of these inflammatory signaling pathways by FFA is mediated through direct binding to toll-like receptor-4 (TLR4), as deletion of TLR4 prevents FFA-induced impairment in insulin signaling and downstream glucose uptake in the skeletal muscle (Shi et al., 2006).

Only very recently has the hypothalamus come to be recognized as a key player in lipid metabolism and regulation of whole body energy balance. It has been observed that high-fat diet induces hypothalamic inflammation within one day (Thaler et al., 2012). Hypothalamic inflammation induced by saturated fats or intermediates (palmitate and ceramides) leads to both insulin and leptin resistance (de Git and Adan, 2015; Yue and Lam, 2012), which in turn increases food intake and obesity (Thaler et al., 2013). Inflammation in the hypothalamus is mediated through TLR4 (Kalin et al., 2015), downstream IKK, and nuclear

factor kappa-light-chain-enhancer of activated B cells(NF- κ B) (Douglass et al., 2017). Blocking this pathway by intracerebroventricular (icv) injections of anti-TLR-4 and anti-TNF α antibodies improves hepatic insulin sensitivity through parasympathetic signals delivered by the vagus nerve (Milanski et al., 2012). JNK can also be activated by direct icv infusion of saturated fatty acids (Milanski et al., 2009) and chronic high-fat feeding (Belgardt et al., 2010; De Souza et al., 2005). Deletion of JNK in the CNS or icv injection of a JNK inhibitor protects mice from high-fat diet-induced obesity and alleviates glucose intolerance in diabetic mice (Benzler et al., 2013; Gao et al., 2017; Sabio et al., 2010).

Fatty Acid Oxidation (FAO) Defects

Given the negative effects of ectopic lipid accumulation as reviewed above, it would logically follow that disrupting the fatty acid oxidation (FAO) pathway that breaks down fatty acids in the mitochondria would lead to lipid accumulation, lipotoxicity, and ultimately insulin resistance. In the 1990s, with the advent of gene targeting technology in mice, it was anticipated that the first “knockout” strains in the FAO pathway would manifest with obesity and metabolic syndrome. However, the relationship between FAO insulin signaling turned out to be very complicated as evidenced by the subsequent phenotypic characterization of several FAO knockout and transgenic mouse models (highlighted in red font in Fig 1, detailed in Table 1). The rate-limiting step in the FAO pathway is transport of fatty acids into the mitochondria, conducted by carnitine palmitoyltransferases-1 and 2 (CPT1, CPT2) and carnitine acylcarnitine translocase (CACT). There are pharmacological inhibitors for CPT1 that will irreversibly block FAO. In 1992, the CPT1 inhibitor etomoxir was tested in human T2DM subjects and shown to improve insulin sensitivity (Hubinger et al., 1992), a finding which is counterintuitive to the idea that inhibiting FAO would result in lipid accumulation and lipotoxicity. In one study, exposure of cultured myotubes to fatty acids impaired insulin signaling and etomoxir blocked this effect (Koves et al., 2008). Timmers et al did parallel studies in humans, mice, and cultured myotubules and etomoxir had positive effects on glucose metabolism in all three biological systems regardless of accumulation of intracellular lipids (Timmers et al., 2012). Since etomoxir and other inhibitors could have unknown off-target effects, other groups utilized genetic approaches to block FAO in muscle. Since whole-body CPT1b (muscle isoform) deficiency is lethal in the mouse, Kim et al studied heterozygous CPT1b^{+/-} mice in the context of high-fat diet-induced diabetes. After 5 months of high-fat diet the CPT1b^{+/-} mice were leaner and more insulin sensitive than wild-type mice (Kim et al., 2014a); however, extending the high-fat feeding to 7 months led to a deterioration in insulin signaling in the muscle, but not other organs (Kim et al., 2014b). The mice remained leaner than wild-type but clearly lost any protective effect of CPT1 deficiency with longer exposures to high-fat feeding.

Wicks and colleagues improved the CPT1b mouse model by creating tissue-specific knockouts of CPT1b whereby the enzyme was absent in skeletal muscle but preserved in the heart, thereby circumventing the lethality (Wicks et al., 2015). Muscle-specific loss of CPT1 in this study led to increased circulating FFA and intramyocellular lipid accumulation. Interestingly, the mice were insulin sensitive with attenuated adiposity through the compensatory increased expression of fibroblast growth factor 21 (FGF21) and subsequently enhanced glucose utilization (Wicks et al., 2015). In further work, this group found that

knocking out Cpt-1b in the skeletal muscle caused a decreased inflammatory response and shifted macrophages from the pro-inflammatory M1 to the anti-inflammatory M2 macrophage phenotype (Warfel et al., 2016). Of note, the Wicks study did not use high-fat feeding to drive the obese/insulin resistant phenotype, perhaps making the study more physiologically relevant.

Once inside the mitochondria fatty acids are broken down by four enzymatic steps (Fig 1). The first step is catalyzed by four chain-length-specific acyl-CoA dehydrogenase (ACAD) enzymes. Two of these have largely overlapping substrate specificities *in vitro* for long-chain fatty acids C₁₂ to C₂₀—long-chain acyl-CoA dehydrogenase (LCAD) and very long-chain acyl-CoA dehydrogenase (VLCAD). The Gerald Shulman laboratory performed detailed metabolic phenotyping of both the LCAD and VLCAD knockout strains (Zhang et al., 2010; Zhang et al., 2007). LCAD^{-/-} mice accumulate triglycerides in liver and muscle even when maintained on standard rodent chow. Cholesterol levels were nearly doubled over wild-type levels, but free fatty acids and triglycerides were not increased. LCAD^{-/-} mice develop insulin resistance but only in the liver; this phenotype was mediated through reduced insulin signal transduction and aberrant diacylglycerol synthesis. VLCAD^{-/-} mice, on the other hand, were shown to be resistant to high-fat diet-induced obesity, gaining less fat mass than wild-type mice and remaining *more* insulin sensitive. Paradoxically, whole animal metabolic monitoring by indirect calorimetry revealed increased FAO in VLCAD ^{-/-} mice on a high-fat diet compared to wild-type mice on a high-fat diet. Apparently LCAD can carry the enzymatic load in the absence of VLCAD. VLCAD ^{-/-} mice show increased expression of FAO genes due to activation of the transcription factor peroxisome proliferator-activated receptor alpha (PPAR α), which regulates expression of both mitochondrial and peroxisomal FAO genes. VLCAD^{-/-} mice also have elevated serum pro-inflammatory cytokines while LCAD^{-/-} mice do not show these changes.

The first several rounds of long-chain FAO are carried out by LCAD and VLCAD working in concert with mitochondrial trifunctional protein (TFP), which catalyzes three enzymatic reactions (steps 2 to 4) to chain-shorten fatty acids. Whole-body knockout of TFP is lethal, but heterozygotes have been metabolically phenotyped. TFP^{+/-} mice have significantly impaired glucose disposal and defective insulin-mediated suppression of gluconeogenesis. Insulin signaling is altered, with increased phosphorylation of insulin receptor substrate-2 (IRS2) and reduced phosphorylation of the key glucose metabolism regulator protein kinase-B (better known as Akt). Like the LCAD ^{-/-} mice, TFP^{+/-} animals have liver-specific insulin resistance (Ibdah et al., 2005; Rector et al., 2013). Gillingham, et al has evaluated metabolic functioning in 9 patients with mutations in one of the TFP subunits (long-chain 3-hydroxyacyl-CoA dehydrogenase)(Gillingham et al., 2013). Compared to matched controls, there was a non-significant trend toward higher total body fat in patients with mutations in TFP, and higher ectopic storage of fat in muscle tissue. However, the ectopic fat in muscle was extracellular, not intracellular, and no fatty change was seen in liver. Indirect calorimetry studies revealed lower total energy expenditure in patients with mutations in TFP, but a higher respiratory quotient indicative of greater reliance upon carbohydrates for energy. Glucose tolerance testing revealed no defects in glucose disposal and insulin sensitivity was not altered.

In conclusion, blocking FAO can induce insulin resistance but it depends upon the enzyme being blocked and the tissue type. Each FAO enzyme, when blocked, results in specific patterns of substrate accumulation and/or downstream activation of other pathways that results in the observed effects, whether it be insulin resistance in LCAD^{-/-} and TFP^{+/-} liver or increased insulin sensitivity in CPT1b^{-/-} muscle. The findings of opposite insulin signaling phenotypes in LCAD and VLCAD ^{-/-} mice highlights the fact that we know very little about how the accumulation of different chain-length fatty acids might induce differential secondary effects on other pathways. Knocking out LCAD in mice results in an accumulation of C₁₄ and C₁₆ free fatty acids and acylcarnitines, while loss of VLCAD primarily increases C₁₈ species which may have totally different signaling properties in terms of activating elongases, desaturases, and transcription factors, etc (Cox et al., 2001). Inhibiting CPT1b, on the other hand, will not increase acylcarnitines, as acylcarnitines are the product of the CPT1b enzymatic reaction. As discussed in the next section, acylcarnitines may in themselves play a role in triggering inflammation and insulin resistance.

Acylcarnitine Accumulation

Acylcarnitines represent a class of lipid metabolites that become elevated in nearly all types of FAO deficiencies. This occurs because the missing or dysfunctional enzyme causes the corresponding fatty acid substrate to accumulate intra-mitochondrially, typically as a coenzyme-A (CoA) conjugate. As levels rise, CPT2 begins to run in reverse, converting acyl-CoAs to acylcarnitines which are more soluble and allow the CoA to be reclaimed for use in other reactions (Violante et al., 2013). The chain-length and species of acylcarnitines that accumulate are diagnostic for IEMs of the FAO pathway. Once thought to be benign, acylcarnitines are now known to have biological effects of their own (McCoin et al., 2015a). They are zwitterionic and will interact with cellular membranes, sometimes perturbing their functions (Yamada et al., 2000). Several studies noted that plasma acylcarnitine levels are elevated in obese and/or diabetic animals and people (Adams et al., 2009; Anderson et al., 2014; Consitt et al., 2016). This has been ascribed to a phenomenon dubbed “incomplete” FAO (Adams et al., 2009; Koves et al., 2008). The concept of incomplete FAO states that the mitochondrial FAO pathway becomes overloaded with fatty acid substrates, and further, that there is a disconnect with the TCA cycle for disposal of the FAO product acetyl-CoA. Thus, partially chain-shortened acyl-CoAs accumulate and then become subject to acylcarnitine conversion and export from the mitochondria. On the other hand, complicating our understanding of the relationship between obesity and acylcarnitines is the study of Schooneman et al who investigated blood lipids during controlled weight loss in obese humans (Schooneman et al., 2016). They showed that acylcarnitines *increased* during weight loss, even though glucose metabolism and insulin sensitivity improved. It is possible that the weight loss triggered increased export of acylcarnitines into circulation whilst improving local intracellular concentrations within the tissue itself. It was recently shown in mice that tissue acylcarnitine levels do not necessarily correlate with plasma acylcarnitines (Schooneman et al., 2014). Changes in the acylcarnitine profile in response to metabolic stress were unique within each tissue compartment. Similarly, in fasted healthy men muscle long-chain acylcarnitines did not correlate with plasma values (Soeters et al., 2009). In another human study, an acute bout of exercise had differential effects on liver and muscle

with regards to acylcarnitine uptake/release (Xu et al., 2016). Acylcarnitine profiles measured in plasma represent an amalgamation of these changes across the body and must therefore be interpreted with caution.

Given the recent findings that acylcarnitines can induce oxidative stress and drive a pro-inflammatory state (Adams et al., 2009; McCoin et al., 2015b; Rutkowski et al., 2014), question arises as to whether an intracellular accumulation of acylcarnitines may play a direct role in the etiology of diabetes or whether they are just a symptom or secondary effect of insulin resistance. To answer this question Aguer et al applied physiological concentrations of acylcarnitines to cultured myotubes (Aguer et al., 2015). They observed a disruption of insulin signaling in the presence of acylcarnitines with all chain lengths tested (4, 14, and 16-carbons). Oxidative stress was also increased, and the negative effects of acylcarnitines on insulin sensitivity could be partially reversed by treating cells with antioxidants. *In vivo*, a pharmacological approach to reduce circulating acylcarnitines improved glucose tolerance in mice maintained on high-fat diet (Liepinsh et al., 2016). Together these studies support the idea that acylcarnitines from dysfunctional FAO may disrupt insulin signaling, and may form a source of increased risk for diabetes in patients with IEMs in the FAO pathway.

ACAD10—an FAO Enzyme Linked to Diabetes?

As described in the section above on FAO, the ACADs conduct the first step in mitochondrial FAO. Additionally, there are other ACAD family members involved in BCAA catabolism. There are nine characterized ACAD family members thus far that are all capable of dehydrogenating acyl-CoA substrates and passing electrons to ETF. In addition, there are two genes bearing ACAD domains that have been dubbed acyl-CoA dehydrogenase 10 (ACAD10) and acyl-CoA dehydrogenase 11 (ACAD11) (He et al., 2011). However, to date these enzymes have not been definitively shown to either dehydrogenate acyl-CoAs or pass electrons to ETF. Both contain multiple other conserved protein domains and their function remains unknown. Of the two new ACAD genes, more is known about ACAD10 in that it has been linked to metabolic phenotypes and is known to localize to mitochondria. In addition to having a conserved ACAD domain at the C-terminus, ACAD10 contains an N-terminal hydrolase domain and a serine/threonine protein kinase catalytic domain. The first link between ACAD10 and diabetes came when a genome wide association study (GWAS) identified an association between two SNPs, rs601663 and rs659964, and T2DM in the Pima Indian population of the southwestern United States (Bian et al., 2010). Bloom et al recently published a study using ACAD10 knockout mice (Bloom et al., 2017). ACAD10 knockout animals become obese with age and exhibit fasting-induced rhabdomyolysis and impaired glucose tolerance. Interestingly, a recent study revealed that ACAD10 is required for the effects of the anti-diabetic drug metformin in *c. elegans* (Wu et al., 2016). Metformin increased ACAD10 expression in cultured cells. Likewise, inhibitors and uncouplers of the respiratory chain such as rotenone and 2,4 dinitrophenol increased ACAD10 expression. Future studies will be needed to determine what biochemical function ACAD10 plays, how it relates to insulin signaling, and whether patients exist with ACAD10 deficiency.

Branched-Chain Amino Acid Metabolism

Branched-chain amino acids (BCAA), namely leucine, isoleucine, and valine, represent another major node of cellular metabolism that lies at the crossroads of inborn errors and insulin resistance (Figure 2). Like FAO described above, BCAA catabolism takes place in the mitochondria and requires integration with the TCA cycle and the electron transport chain for full oxidation of the breakdown products of the BCAA machinery (acetoacetate, acetyl-CoA, succinyl-CoA). The downstream portion of BCAA catabolic pathways involves small branched-chain acyl-CoAs which can become acylcarnitines in the face of metabolic bottlenecks; these acylcarnitines have not been studied in detail for biological effects. Metabolism of valine, isoleucine, and leucine further require acyl-CoA dehydrogenases (ACADs) from the same family of flavoproteins that catalyze the first step of FAO, and these ACADs pass electrons to the same acceptor (ETF) which then go to Complex III through ETF dehydrogenase. These interconnections suggest that, as with the FAO pathway, there can be secondary perturbations in BCAA metabolism due to primary defects in other mitochondrial pathways. This was demonstrated by Corkey et al who showed that the presence of oleate or palmitoylcarnitine can suppress the oxidation of the leucine metabolite α -ketoisocaproic acid (Corkey et al., 1982).

Newgard et al first reported the association between circulating BCAA levels and insulin resistance in 2009 (Newgard et al., 2009). Since that time, numerous other studies have reported similar findings (Fiehn et al., 2010; Haufe et al., 2016; Hu et al., 2016; Lee et al., 2016; Tulipani et al., 2016). A recent meta-analysis concluded that while circulating BCAA levels vary substantially by race, gender, and diet, they remain an important biomarker and predictor of insulin resistance (Zhao et al., 2016). As with acylcarnitines, the major question then becomes whether BCAA are part of the etiology of insulin resistance or just a symptom of general mitochondrial dysfunction.

Paradoxically, administering BCAA to either mice or humans has been associated with positive effects on health and metabolism in several studies. Dietary BCAA supplementation (leucine in particular) can reduce fat mass, body weight, and even improve glucose metabolism (Arrieta-Cruz et al., 2016; Binder et al., 2013; English et al., 2016; Heimann et al., 2016). Leucine activates mTOR, which has been proposed as a potential mechanism by which elevated circulating BCAA might contribute to T2DM (Blagosklonny, 2013; Macotela et al., 2011). However, recent studies provide data that contradicts this notion. First, long-term supplementation studies in rodents have not observed insulin resistance despite chronic activation of mTOR (D'Antona et al., 2010). Rather, long-term BCAA supplementation led to greater lifespan and enhanced physical endurance at advanced ages. Mitochondrial biogenesis was stimulated in several tissues, and this was associated with decreased oxidative stress (D'Antona et al., 2010).

Another particularly informative study involved knockout mice lacking the mitochondrial isoform of branched-chain aminotransferase (BCAT)(She et al., 2007). BCAT catalyzes the first step of BCAA catabolism. Despite a more than 10-fold accumulation of BCAA over wild-type, along with signs of mTOR activation, the BCAT $-/-$ mice demonstrated improved glucose tolerance and insulin sensitivity. The mice were lean and showed resistance to high-

fat diet-induced obesity. On the other hand, disrupting the BCAA catabolic pathway further downstream does not cause this phenotype. Knocking out components of the mitochondrial branched-chain- α -ketoacid dehydrogenase (BCKDH) complex or its regulators leads to a more severe phenotype resembling human BCKDH deficiency, also known as maple syrup urine disease (MSUD) (Skvorak, 2009). Knocking out PPM1K, a phosphatase required for activation of the BCKDH complex, causes oxidative stress and apoptosis in cultured cells (Lu et al., 2009). The ketoacids that accumulate when BCKDH is disrupted have been shown to cause mitochondrial dysfunction and respiratory chain inhibition (Jouvet et al., 2000; Moghei et al., 2016; Oyarzabal et al., 2016; Sun et al., 2016). Jang et al recently showed that 3-hydroxisobutyrate, a downstream metabolite in valine metabolism, can increase lipid flux into muscle cells and cause insulin resistance in mice (Jang et al., 2016). Expression levels of BCKDH and other downstream BCAA enzymes has been shown to correlate with insulin sensitivity in humans (Lerin et al., 2016). Therefore, while still requiring much more investigation to completely discern the metabolic role of BCAA in T2DM, at present it seems most likely that elevated BCAA are themselves not pathologic, but accumulation of downstream intermediates in the pathway may be. The presence of higher levels of BCAA in the blood of those with or at risk for T2DM most likely is a biomarker that reflects underlying mitochondrial dysfunction. At the same time, the accumulation of intermediates in the BCAA pathway such as ketoacids or 3-HIB might serve to exacerbate mitochondrial dysfunction in a vicious cycle, thereby promoting insulin resistance, but this postulate remains to be fully evaluated experimentally.

Mitochondrial Lysine Acylation

Lysine acylation is the process by which lysine residues on cellular proteins become modified by acyl groups of varying length and composition. Lysine acylation is reversible via the action of a family of seven NAD⁺-dependent enzymes known as the sirtuins (SIRT6). The past decade of research on reversible acylation has led to the conclusion that in the mitochondria, acylation of lysine residues occurs non-enzymatically due to high concentrations of acyl-CoAs reacting with deprotonated lysine residues (Wagner et al., 2017; Wagner and Hirschey, 2014). In other words, reversible acylation is a one-sided mechanism in which the SIRT6 remove deleterious modifications that accumulate as inevitable byproducts of mitochondrial energy metabolism.

In general, higher levels of mitochondrial protein acylation are associated with metabolic dysfunction, due to direct effects on the enzymes being modified, disrupted protein: protein interactions, disrupted protein: lipid interactions, etc. Ultimately, any condition that perturbs mitochondrial acyl-CoA levels has the potential to alter the mitochondrial lysine acylome and thereby trigger secondary mitochondrial dysfunction. Many IEMs are associated with intra-mitochondrial accumulation of acyl-CoA species that can subsequently alter the mitochondrial protein acylome. Indeed, lysine butyrylation, malonylation, and propionylation are increased in short-chain acyl-CoA dehydrogenase (SCAD), malonyl-CoA decarboxylase (MCD), and propionyl-CoA carboxylase (PCC) deficient patient cells, respectively (Pougovkina et al., 2014b). Glutaryl-CoA dehydrogenase (GCDH) knockout mice have increased glutaryl-CoA and elevated lysine glutarylation (Tan et al., 2014). Similarly, paucity of an acyl-CoA metabolite can lead to reduced lysine acylation. The best

example of this is lysine acetylation, derived from acetyl-CoA. FAO, by producing acetyl-CoA, is the major driver of protein acetylation in both mitochondria and peroxisomes (Pougovkina et al., 2014a). Consequently, mice with an FAO defect have lower protein acetylation. FAO-derived acetyl-CoA has also been shown to be the primary source of acetyl groups for nuclear histone acetylation (McDonnell et al., 2016). An interesting question thus arises as to whether FAO defects may induce global changes in gene expression via shifts in the epigenetic landscape. This could potentially also occur in T2DM muscle experiencing incomplete FAO as discussed above.

SIRT3, SIRT4, and SIRT5 localize to mitochondria to counter mitochondrial lysine acylation. SIRT3 has been linked to obesity and insulin resistance in both animal models and humans (Hirschey et al., 2011; Jing et al., 2011; Lantier et al., 2015; Yang et al., 2016). Obese people have lower vascular expression of SIRT3, which in endothelial cells *in vitro* is associated with disrupted insulin signaling (Yang et al., 2016). Lantier et al showed that SIRT3 knockout mice have normal muscle insulin signaling when maintained on regular chow, but after high-fat feeding it becomes deranged (Lantier et al., 2015). Muscle from high-fat fed SIRT3 knockout mice show impaired insulin-stimulated glucose uptake and a compensatory shift to fatty acids as a source of energy. Mitochondrial respiration is decreased while oxidative stress is increased (Jing et al., 2011; Lantier et al., 2015). In humans, reduced SIRT3 expression in obesity may cause a similar effect. Hirschey et al identified a single nucleotide polymorphism (SNP) in the SIRT3 gene that is associated with metabolic syndrome (Hirschey et al., 2011). This SNP results in an amino acid substitution in the catalytic domain of SIRT3 that decreases its activity. SIRT4 and SIRT5 are less well studied compared to SIRT3. Both SIRT4 and SIRT5 regulate aspects of FAO (Laurent et al., 2013a; Laurent et al., 2013b; Rardin et al., 2013; Zhang et al., 2015). SIRT5 has further been shown to regulate the function of enzymes in the urea cycle and electron transport chain (Nakagawa and Guarente, 2009; Zhang et al., 2017), but has not been well-investigated in the context of diabetes. Regarding SIRT4, the Hirschey lab recently demonstrated a role for Sirt4 in deacylating the enzymes needed for BCAA catabolism (Anderson et al., 2017). Sirt4 knockout mice showed elevated leucine-stimulated insulin secretion, which as the mice age develops into glucose intolerance (Anderson et al., 2017; Huynh et al., 2017).

In summary, like fatty acid metabolism and BCAA metabolism, the SIRTs and reversible protein acylation lie at the crossroads of IEMs and T2DM. Chronically altered levels of acyl-CoAs in both diseases might translate into secondary defects of mitochondrial function and possibly epigenetic changes with widespread cellular consequences. It has been proposed that SIRT agonist drugs may be a therapeutic avenue for treating the symptoms of both IEMs and T2DM (Bleeker and Houtkooper, 2016; Huynh et al., 2013). Several SIRT activators have been reported in the literature, most notably resveratrol, a SIRT1 activator. Several clinical trials have tested resveratrol as an anti-diabetic agent with mixed results (Novelle et al., 2015). At present, the consensus appears to be that resveratrol is beneficial when used to supplement other therapies (Frendo-Cumbo et al., 2016; Hausenblas et al., 2015). Activators of the mitochondrial SIRTs have been more elusive. Intriguingly, since all SIRTs are NAD⁺-dependent, one promising strategy is to provide synthetic precursors of NAD⁺ such as nicotinamide mononucleotide (NMN) or nicotinamide riboside (NR). Both have been shown to have anti-diabetic potential and are in early phase clinical trials

(Bonkowski and Sinclair, 2016). With regards to IEMs, NR was shown to improve mitochondrial function in Complex I-deficient patient fibroblasts (Felici et al., 2015). *In vivo*, NR increased respiratory chain activity and treadmill performance in mice lacking the Complex IV assembly factor Sco2 (Cerutti et al., 2014). Finally, NMN administration to mice activated SIRT3 and increased the activity of mitochondrial enzymes including medium-chain acyl-CoA dehydrogenase (MCAD) and ornithine transcarbamylase (OTC) (Peek et al., 2013).

Conclusions

At the time of this writing, a *Pubmed* search with the terms “energy metabolism” and “diabetes” turns up over 19,000 results. Yet, it is still uncertain whether changes in energy metabolism are a symptom or the cause of diabetes. Here we have reviewed some of the major metabolic pathways implicated in the pathogenesis of T2DM. T2DM has a substantial genetic component but this component does not localize to the metabolic pathways affected in T2DM, but rather, primarily to inflammatory genes. While there are several monogenic forms of diabetes, again, the great majority of these genes are involved in insulin production/secretion rather than energy metabolism or mitochondrial function. It therefore seems likely that for most individuals with T2DM, the observed changes in energy metabolism are a symptom—albeit a symptom that can trigger a downstream vicious cycle of declining mitochondrial function leading to irreversible loss of the ability to integrate glucose, lipid, and amino acid metabolism across the pancreas, liver, muscle, and adipose tissue. The current literature does not support the notion that interfering with a metabolic pathway by itself is sufficient to induce insulin resistance or T2DM. In both the FAO and BCAA pathways, for example, different effects are seen depending upon which enzyme is “knocked out” and in which tissue type. The most logical explanation for the highly variable effects, such as insulin resistance in LCAD and TFP knockout liver but insulin sensitivity in VLCAD knockout liver, is that the important factor is which metabolites accumulate and how these metabolites alter secondary cellular processes or signaling pathways. In isolation, the study on LCAD mice would lead one to conclude that blocking FAO induces insulin resistance; however, this is clearly an over-simplification, as studies in other animal models of FAO disorders unveil the complexity of responses to disrupted FAO and highlight our woefully inadequate understanding of metabolic crosstalk across pathways, organelles, cells, and organs. For individuals with IEMs, the biology will certainly be even more complex than that seen in cell culture or mice; while these IEMs are likely not sufficient to induce T2DM in isolation, over time they may interact with diet, activity levels, weight gain, and inflammation to produce increased risk. Continuing research is needed to understand the complexities of metabolic disruption and compensatory responses in IEMs and how these responses overlap with T2DM.

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Figure 1. Genes in the mitochondrial fatty acid oxidation (FAO) pathway that have been studied for links to T2DM.

Fatty acids are carried across the mitochondrial membrane by the carnitine system consisting of carnitine palmitoyltransferase-1 (isoforms a and b) and 2 (CPT1a, CPT1b, CPT2) working with carnitine acylcarnitine translocase (CACT). Under pathologic conditions transport can be reversed to release acylcarnitines from mitochondria. Beta-oxidation consists of four enzymatic steps. Step one is conducted by short-chain, medium-chain, long-chain, and very long-chain acyl-CoA dehydrogenases (SCAD, MCAD, LCAD, VLCAD). For long-chain substrates steps 2–4 are conducted by mitochondrial trifunctional protein (TFP) while for shorter substrates these steps are catalyzed by separate enzymes, including the enoyl-CoA hydratases (ECH, ECHS1), the hydroxyacyl-CoA dehydrogenases

(HCDH, HCDH2), and finally, the thiolases (MKT, ACAT1). The genes indicated in red font have been studied for loss-of-function or gain-of-function relationships to insulin resistance and T2DM phenotypes. See Table 1 and text for details.

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Figure 2. Summary of links between branched-chain amino acid (BCAA) catabolism and T2DM. BCAAs are widely considered an early blood biomarker of T2DM. Exogenous administration of BCAAs themselves (Val, Ile, Leu) to mice or humans has generally been associated with positive metabolic effects. Similarly, mice lacking branched-chain aminotransferase (BCAT) accumulate BCAAs and are resistant to T2DM. Disrupting the pathway downstream of BCAT, or exogenous administration of intermediate metabolites in the pathway, has been associated with negative consequences such as respiratory chain inhibition, oxidative stress, apoptosis, and insulin resistance.

Table 1:

Insulin signaling/T2DM-related phenotypes in mice and humans with FAO disorders.

FAO Enzyme/genotype	Organism	Diet Used	Body Weight/ Adiposity (Vs. Control)	Insulin-signaling/T2DM Phenotype	Refs.
Very long-chain acyl-CoA dehydrogenase (–/–)	Mice	3 wk High Fat	Reduced	Greater hepatic insulin sensitivity	(Zhang et al., 2010)
Long-chain acyl-CoA dehydrogenase (–/–)	Mice	Chow	Increased/ Same *	Hepatic insulin resistance	(Zhang et al., 2007)
Mitochondrial trifunctional protein (+/–)	Mice	Chow	Reduced/Same	Defective insulin suppression of gluconeogenesis; impaired glucose tolerance; hepatic insulin resistance	(Ibdah et al., 2005; Rector et al., 2013)
Mitochondrial trifunctional protein (various mutations)	Human	--	Same	Muscle extracellular fat accumulation; normal glucose tolerance and insulin sensitivity	(Gillingham et al., 2013)
Carnitine palmitoyltransferase-1b (+/–)	Mice	5 mo. High Fat	Reduced	Protected against T2DM; increased muscle reliance upon glucose	(Kim et al., 2014a)
Carnitine palmitoyltransferase-1b (+/–)	Mice	7 mo. High Fat	Reduced	insulin resistant in muscle	(Kim et al., 2014b)
Carnitine palmitoyltransferase-1b (muscle over-expression)	Rats	High Fat	N/A (acute over-expression study)	Increased muscle FAO; improved muscle insulin signaling and reduced muscle fat deposition	(Bruce et al., 2009)
Short/medium-chain hydroxyacyl-CoA dehydrogenase (–/–)	Mice	High Fat	Reduced	Enhanced insulin secretion in response to glucose, amino acids, and fatty acids	(Li et al., 2010; Molven et al., 2016)
Short/medium-chain hydroxyacyl-CoA dehydrogenase (various mutations)	Human	--	N/A	Hyperinsulinism; hypoglycemia in response to leucine load	(Heslegrave and Hussain, 2013; Molven et al., 2004)

* Zhang et al reported increased adiposity of LCAD–/– mice on normal chow, but we have not observed this in our laboratory (unpublished results).
N/A=not applicable.