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The Biochemistry and Physiology of Mitochondrial Fatty Acid β-Oxidation and Its Genetic Disorders

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Abstract

Mitochondrial fatty acid β-oxidation (FAO) is the major pathway for the degradation of fatty acids and is essential for maintaining energy homeostasis in the human body. Fatty acids are a crucial energy source in the postabsorptive and fasted states when glucose supply is limiting. But even when glucose is abundantly available, FAO is a main energy source for the heart, skeletal muscle, and kidney. A series of enzymes, transporters, and other facilitating proteins are involved in FAO. Recessively inherited defects are known for most of the genes encoding these proteins. The clinical presentation of these disorders may include hypoketotic hypoglycemia, (cardio)myopathy, arrhythmia, and rhabdomyolysis and illustrates the importance of FAO during fasting and in hepatic and (cardio)muscular function. In this review, we present the current state of knowledge on the biochemistry and physiological functions of FAO and discuss the pathophysiological processes associated with FAO disorders.

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INTRODUCTION

Fatty acids are important nutrients, and their storage as triglycerides in adipose tissue allows humans to tolerate extended periods of starvation or fasting and other metabolically challenging conditions such as febrile illness and exercise. The major pathway for the degradation of long-chain fatty acids is mitochondrial fatty acid β-oxidation (FAO). FAO not only fuels the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, but also stimulates hepatic synthesis of the ketone bodies (*R*)-3-hydroxybutyrate and acetoacetate. The importance of FAO for human physiology is highlighted by patients with inherited disorders caused by defects in the FAO pathway. Such patients typically have low fasting tolerance presenting as hypoketotic hypoglycemia that may progress into life-threatening Reye-like syndrome. Patients with FAO disorders may also have skeletal muscle and cardiac disease.

In the 1900s, the German chemist Georg Franz Knoop discovered the mechanism underlying FAO (1). His classic experiments using odd- and even-chain ω-phenyl fatty acids illustrated that the metabolism of fatty acids proceeds by the successive removal of two carbon fragments. The history of research on FAO continues along the lines of major scientific developments in the twentieth century with the elucidation of the individual steps in FAO and their intermediates, followed by the purification, characterization, and crystallization of the individual enzymes performing these steps (2). In the 1970s, the first inherited diseases of FAO were described. Two decades later, developments in molecular biology led to the cloning of the genes encoding the enzymes, transporters, and other facilitating proteins involved in FAO and to the subsequent identification of mutations in these genes causing FAO disorders (3). Since the last review on FAO in this journal in 2002 (4), much progress has been made in the understanding of pathophysiological processes in FAO disorders through in vivo studies involving patients and mouse models, as well as in vitro studies using cell lines. These studies led to the development of triheptanoin and bezafibrate as new treatment options (5, 6). In addition, FAO was also shown to play significant roles in the pathophysiology of common disorders such as insulin resistance, diabetes, obesity, kidney fibrosis, and heart failure.

In this review, we present the current state of knowledge on the biochemistry and physiological functions of FAO in humans and mice. We discuss the pathophysiology in patients with FAO disorders and in corresponding mouse models.

BIOCHEMICAL ASPECTS OF MITOCHONDRIAL FATTY ACID β-OXIDATION

Approximately 20 different proteins play specific roles in FAO. **Table 1** shows these proteins, their encoding genes, and associated disease phenotypes. **Figure 1** shows a schematic representation of their enzymatic or transport function and subcellular localization.

Fatty Acid Uptake and the Carnitine Cycle

Although fatty acid uptake into cells was long thought to be the result of simple diffusion, it is now well established that specific proteins such as CD36 and plasma membrane fatty acid binding protein ($FABP_{pm}$, also known as GOT2) facilitate this process. This topic was recently extensively reviewed (7) and is not further discussed. In the cytosol, fatty acids are activated to acyl-coenzyme A (CoA) esters by acyl-CoA synthetases before they can be directed into several different metabolic pathways, such as lipid synthesis and FAO (8); the latter requires mitochondrial import of acyl-CoA. Because the mitochondrial membrane is impermeable to acyl-CoAs, the carnitine cycle is

Table 1 Human mitochondrial fatty acid β-oxidation enzymes and transporters

Name	Most common alias	Gene	EC number	Phenotype MIM number
Carnitine shuttle				
Carnitine palmitoyltransferase 1A (liver)	CPT1A	*CPT1A*	2.3.1.21	255120
Carnitine palmitoyltransferase 1B (muscle)	CPT1B	*CPT1B*	2.3.1.21	Not reported
Carnitine acylcarnitine translocase	CACT	*SLC25A20*	NA	212138
Carnitine palmitoyltransferase 2	CPT2	*CPT2*	2.3.1.21	600649; 608836; 255110; 614212
Organic cation/carnitine transporter	OCTN2	*SLC22A5*	NA	212140
Fatty acid β-oxidation cycle				
Very long chain acyl-CoA dehydrogenase	VLCAD	*ACADVL*	1.3.8.9	201475
Medium-chain acyl-CoA dehydrogenase	MCAD	*ACADM*	1.3.8.7	201450
Short-chain acyl-CoA dehydrogenase	SCAD	*ACADS*	1.3.8.1	201470
Mitochondrial trifunctional protein, alpha subunit	MTPα	*HADHA*	4.2.1.74; 1.1.1.211	609015; 609016
Mitochondrial trifunctional protein, beta subunit	MTPβ	*HADHB*	2.3.1.16	609015
Short-chain enoyl-CoA hydratase	Crotonase	*ECHS1*	4.2.1.150	616277
Short-chain (*S*)-3-hydroxyacyl-CoA dehydrogenase	SCHAD	*HADH*	1.1.1.35	231530; 609975
Medium-chain 3-ketoacyl-CoA thiolase	MCKAT	*ACAA2*	2.3.1.16	Not reported
Acetoacetyl-CoA thiolase	T2	*ACAT1*	2.3.1.9	203750
Long-chain acyl-CoA dehydrogenase	LCAD	*ACADL*	1.3.8.8	Not reported
Acyl-CoA dehydrogenase 9	ACAD9	*ACAD9*	1.3.8.9	611126
Auxiliary enzymes				
Δ3,Δ2-Enoyl-CoA isomerase 1	DCI	*ECI1*	5.3.3.8	Not reported
Δ3,Δ2-Enoyl-CoA isomerase 2	PECI	*ECI2*	5.3.3.8	Not reported
2,4-Dienoyl-CoA reductase	DECR	*DECR1*	1.3.1.34	Not reported
Δ3,5-Δ2,4-Dienoyl-CoA isomerase	ECH1	*ECH1*	Not assigned	Not reported

Abbreviations: EC number, enzyme commission number; NA, not applicable. The MIM number refers to the numbering in the Online Mendelian Inheritance in Man (OMIM) database.

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needed for import into the mitochondria. This system requires L-carnitine and is composed of two acyltransferases, carnitine palmitoyltransferases 1 and 2 (CPT1 and CPT2), and carnitine acylcarnitine translocase (CACT), which is a member of the mitochondrial carrier family of proteins (SLC25A20). CPT1, an integral outer-mitochondrial-membrane protein, catalyzes the transesterification of the acyl-CoA to acylcarnitine. CACT carries out the transport of acylcarnitines across the inner mitochondrial membrane in exchange for a free carnitine molecule. Inside the mitochondria, CPT2, which is a peripheral inner-mitochondrial-membrane protein, completes the cycle by reconverting the acylcarnitine into an acyl-CoA. Although some tissues can synthesize carnitine, most carnitine is of dietary origin and is transported across the plasma membrane by the organic cation transporter OCTN2 (SLC22A5) (9).

The β-Oxidation Cycle

Inside the mitochondrion, acyl-CoAs are degraded via β-oxidation, a cyclic process consisting of four enzymatic steps (**Figure 2**). Each cycle shortens the acyl-CoA by releasing the two carboxy-terminal carbon atoms as acetyl-CoA. The cycle is initiated by dehydrogenation of

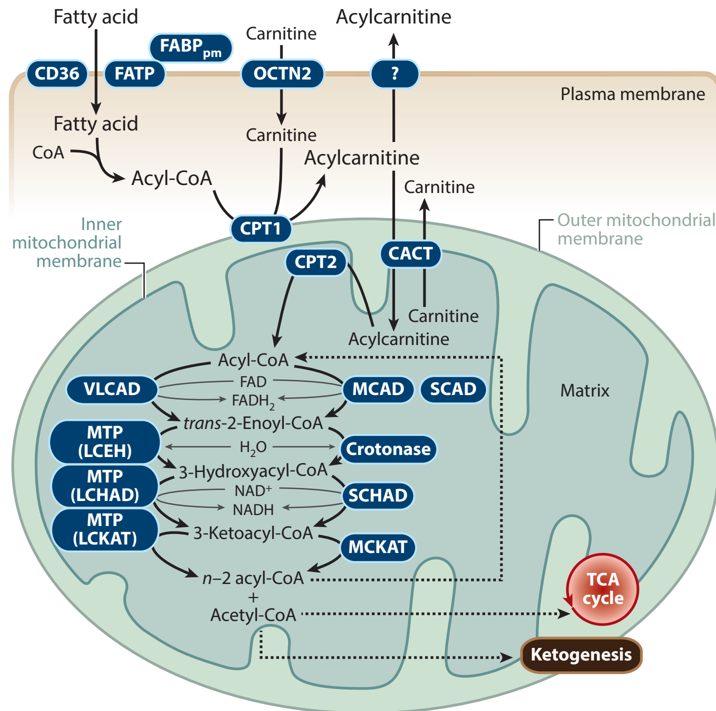

Figure 1

A schematic representation of mitochondrial fatty acid β-oxidation. The subcellular location and the (enzymatic) role of the different proteins are displayed. The auxiliary enzymes, which are involved in the oxidation of unsaturated fatty acids, are not displayed. Although involved mainly in mitochondrial acyl-CoA import, CPT2 and CACT can operate in the reverse direction when intramitochondrial acyl-CoA levels rise. This mechanism facilitates the export of these intermediates as acylcarnitines. CRAT, which is involved mainly in the formation of short-chain acylcarnitines, is not displayed. The plasmalemmal acylcarnitine exporter remains to be identified. Abbreviations: CACT, carnitine acylcarnitine translocase; CPT1/2, carnitine palmitoyltransferases 1 and 2; $FABP_{pm}$, plasma membrane fatty acid binding protein; FATP, fatty acid transport protein; LCEH, long-chain enoyl-CoA hydratase; LCHAD, long-chain (*S*)-3-hydroxyacyl-CoA dehydrogenase; LCKAT, long-chain 3-ketoacyl-CoA thiolase; MCAD, medium-chain acyl-CoA dehydrogenase; MCKAT, medium-chain 3-ketoacyl-CoA thiolase; MTP, mitochondrial trifunctional protein; SCAD, short-chain acyl-CoA dehydrogenase; SCHAD, short-chain (*S*)-3-hydroxyacyl-CoA dehydrogenase; VLCAD, very long chain acyl-CoA dehydrogenase.

the acyl-CoA to *trans*-2-enoyl-CoA by an acyl-CoA dehydrogenase. This step is followed by a hydration catalyzed by an enoyl-CoA hydratase, generating (*S*)-3-hydroxyacyl-CoA, which is subsequently dehydrogenated to 3-ketoacyl-CoA in a reaction performed by (*S*)-3-hydroxyacyl-CoA dehydrogenase. Finally, a thiolase cleaves the 3-ketoacyl-CoA into a two-carbon chain–shortened acyl-CoA and an acetyl-CoA. FAO not only produces acetyl-CoA to fuel the TCA cycle and ketogenesis, but also reduces flavin adenine dinucleotide (to $FADH_2$) and nicotinamide adenine dinucleotide (to NADH), and these reduced products directly feed into the respiratory chain. As the acyl-CoA gets shorter, its physicochemical properties change. To be able to fully degrade fatty acids, the β-oxidation machinery harbors different chain length–specific enzymes.

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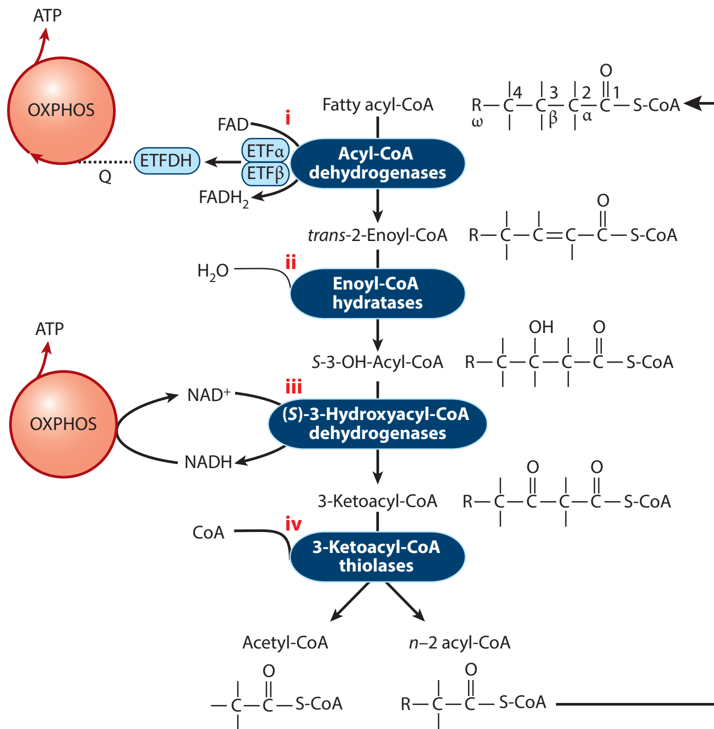

Figure 2

The four steps (*i*–*iv*) of the fatty acid β-oxidation cycle with the structures of the different intermediates. The acyl-CoA dehydrogenases use enzyme-bound FAD as the electron acceptor. The resulting $FADH_2$ needs to be oxidized on the enzyme, which is performed by the electron transfer flavoprotein (a heterodimer of ETFα and ETFβ, which are encoded by *ETFA* and *ETFB*, respectively). Via ETF dehydrogenase (ETFDH), which is encoded by *ETFDH*, the electrons are donated to coenzyme Q (Q) and feed into the respiratory chain. Other products of each β-oxidation cycle include NADH, acetyl-CoA, and an acyl-CoA shortened by two carbon atoms. NADH is reoxidized via oxidative phosphorylation (OXPHOS). Acetyl-CoA feeds into the TCA cycle or may be used for ketogenesis. The chain-shortened acyl-CoA reenters the fatty acid β-oxidation cycle. The carbon chain is numbered, with β being the third carbon atom and ω referring to the last carbon atom.

The Acyl-CoA Dehydrogenases

The acyl-CoA dehydrogenase family consists of at least 11 enzymes, most of which play a role in FAO or amino acid catabolism (10). In humans, three different acyl-CoA dehydrogenases have well-established roles in FAO; these are very long chain, medium-chain, and short-chain acyl-CoA dehydrogenases (VLCAD, MCAD, and SCAD, respectively). Together these three enzymes cover the metabolism of acyl-CoAs from long- to medium- and finally to short-chain acyl-CoAs. Whereas VLCAD is associated with the inner mitochondrial membrane, MCAD and SCAD are soluble, matrix-localized enzymes. Two other acyl-CoA dehydrogenases, long-chain acyl-CoA dehydrogenase (LCAD) and acyl-CoA dehydrogenase 9 (ACAD9), may function in FAO. LCAD has a broad substrate specificity covering long- and medium-chain acyl-CoAs as well as specific substrates such as unsaturated and branched-chain acyl-CoAs (11, 12). Whereas this enzyme is crucial in rodents (13–15), its expression level and therefore its contribution to FAO in major fatty

acid–degrading organs in humans are low (13, 17, 18). In humans and in mice, LCAD is expressed in type 2 alveolar cells and may play a role in surfactant production (19, 20). ACAD9 has a substrate specificity that largely overlaps with that of VLCAD (21, 22), but its exact contribution to long-chain FAO remains unclear and may be small (23) or tissue specific (24). The main function of ACAD9 is in the assembly of complex I of the respiratory chain (25–27).

Acyl-CoA dehydrogenases use enzyme-bound FAD as the electron acceptor. The resulting $FADH_2$ needs to be oxidized on the enzyme, and such oxidation is performed by the electron transfer flavoprotein (ETF). Via another enzyme, ETF dehydrogenase, the electrons are subsequently donated to coenzyme Q and ultimately feed into the respiratory chain (**Figure 2**). Defects in this system lead to a disease termed multiple acyl-CoA dehydrogenase deficiency, also known as glutaric acidemia type II (28). This disease affects amino acid and choline metabolism in addition to FAO and is therefore not discussed in this review.

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The Enoyl-CoA Hydratases, (*S*)-3-Hydroxyacyl-CoA Dehydrogenases, and 3-Ketothiolases

Mitochondrial trifunctional protein (MTP) harbors enoyl-CoA hydratase, (*S*)-3-hydroxyacyl-CoA dehydrogenase, and 3-ketothiolase activities that are specific for long-chain intermediates. MTP is a hetero-octamer composed of four α and four β subunits, which are encoded by *HADHA* and *HADHB*, respectively. Long-chain enoyl-CoA hydratase (LCEH) and long-chain (*S*)-3-hydroxyacyl-CoA dehydrogenase (LCHAD) are located on the α-subunit, whereas the long-chain 3-ketoacyl-CoA thiolase (LCKAT) is located on the β-subunit. Similar to VLCAD, MTP is attached to the mitochondrial inner membrane. In addition to MTP, mitochondria have single enzymes in the matrix that can catalyze the same reactions, albeit with the highest specificity for short-chain intermediates. These enzymes are crotonase (short-chain enoyl-CoA hydratase), short-chain (*S*)-3-hydroxyacyl-CoA dehydrogenase (SCHAD), and medium-chain 3-ketoacyl-CoA thiolase (MCKAT). Although acetoacetyl-CoA thiolase (ACAT1) is involved primarily in ketone and isoleucine metabolism, it may also contribute to the last step of FAO.

On the basis of the different substrate specificities of the individual FAO enzymes, it is assumed that the long-chain acyl-CoAs first undergo two to three β-oxidation cycles by the membrane-bound enzymes VLCAD and MTP. The resulting medium-chain acyl-CoAs are then handled by the matrix-localized enzymes MCAD, crotonase, SCHAD, and MCKAT. Finally, the short-chain acyl-CoAs are metabolized by SCAD, crotonase, SCHAD, and MCKAT. A kinetic mathematic model of FAO, however, suggests that medium-chain acyl-CoAs prefer MTP over crotonase/SCHAD/MCKAT (29). This is a remarkable observation, given that MCAD, which performs the obligatory first step in medium-chain acyl-CoA metabolism, is not colocalized with MTP at the mitochondrial inner membrane.

The Auxiliary Enzymes

In addition to the set of enzymes involved in the β-oxidation cycle, the degradation of mono- and polyunsaturated fatty acids requires the obligatory participation of a set of three different auxiliary enzymes: the 2,4-dienoyl-CoA reductase (DECR), the Δ3,5-Δ2,4-dienoyl-CoA isomerase, and the Δ3,Δ2-enoyl-CoA isomerase (ECI) (30). Through reduction and isomerization, this combined set of auxiliary enzymes can position different combinations of double bonds to the *trans*-2 configuration, allowing reentrance of the enoyl-CoA into the β-oxidation cycle (31).

There are two different ECIs present in mitochondria: ECI1 and ECI2. Whereas ECI1 is localized exclusively in the mitochondria, ECI2 is targeted to mitochondria and peroxisomes (32).

Although both enzymes are reactive with a wide range of substrates, ECI2 shows higher catalytic efficiency with long-chain enoyl-CoAs than does ECI1 (32), likely due to the presence of an acyl-CoA-binding protein domain (33). Despite this difference in specificity, studies have revealed functional redundancy, as Eci2 was able to compensate for Eci1 deficiency in mice (34).

PHYSIOLOGICAL ROLE OF MITOCHONDRIAL FATTY ACID β-OXIDATION

During fasting, the nutritional glucose supply becomes progressively limiting, and glucose production is maintained through glycogen breakdown (glycogenolysis) and de novo glucose synthesis (gluconeogenesis). Glycogen stores are limited, and ultimately gluconeogenic precursors, which include lactate, pyruvate, glycerol, and specific amino acids, are the sole sources of glucose. Because proteolysis is mainly responsible for the net generation of gluconeogenic precursors, oxidation of fatty acids is essential as an alternative to prevent rapid erosion of protein mass (35, 36). This adaptive response is mediated by the neuroendocrine system, which among other mechanisms increases adipose lipolysis. Such lipolysis drives increased FAO and hepatic ketogenesis and thereby leads to decreased glucose uptake and oxidation. This competition between the oxidation of fatty acids and glucose is also known as the glucose–fatty acid, or Randle, cycle.

Tissue-Specific Roles for Fatty Acid β-Oxidation in Common Diseases

The heart has a continuous high energy demand to sustain contractile function. To secure continuous ATP production, the heart is a metabolic "omnivore" that can use many different substrates, depending on their availability (37). FAO, however, is the preferred pathway for energy provision in the heart, with more than half of the ATP production being derived from fatty acid sources (37). In contrast, the prenatal heart relies on glucose and lactate, but in the immediate postnatal period, when dietary fat is abundant, the heart switches to FAO as a source of energy (38). During this period, the myocardial energy demand also increases due to changes in the circulation. This increased energy demand is met by an induction of mitochondrial biogenesis. In general, increased dependence on metabolism of one specific substrate impairs the heart's capacity to adapt to changes in circulating available nutrients and, as such, decreases its metabolic flexibility. Cardiovascular diseases such as heart failure and ischemic heart disease are characterized by significant changes in cardiac metabolism, and as such FAO plays an important role in their pathophysiology (37). Diabetes and obesity also have significant consequences for cardiac metabolism, with increased fatty acid uptake and oxidation possibly contributing to the increased risk of cardiovascular disease (37).

FAO is also crucial for ATP production in muscle, in particular during exercise (39). FAO increases from rest to low-intensity exercise but does not increase further with exercise intensity (40). This pattern is in contrast to that seen for glucose and glycogen utilization, which always increases with exercise intensity (40). The sources of fatty acids also differ depending on the exercise intensity, with the contribution of plasma free fatty acids increasing with exercise intensity (40). Other important sources of fatty acids are circulating triglycerides in very low density lipoprotein particles and intramyocellular lipid droplets. High levels of circulating lipids in obesity and type 2 diabetes overload cellular lipid metabolic processes and cause the accumulation of lipid signaling intermediates such as ceramides, gangliosides, and diacylglycerol. Cellular lipid overload also leads to mitochondrial overload and incomplete FAO. These different mechanisms may play a role in impaired insulin signaling (41).

The kidneys are also organs with continuous high energy consumption, and again a significant portion of the ATP is generated through FAO. Mitochondrial FAO has the highest activity in the

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proximal and distal convoluted tubules (42). Transcriptome analysis has confirmed that tubular epithelial cells rely primarily on FAO as their energy source. In humans and mouse models, decreased FAO contributes to the development of kidney fibrosis (43). Disruption of mitochondrial metabolism causes Fanconi's syndrome, further indicating the central role of mitochondrial FAO in proximal tubular function (44).

REGULATION OF MITOCHONDRIAL FATTY ACID β-OXIDATION

Transcriptional Regulation

FAO is regulated by both transcriptional and posttranscriptional mechanisms. Peroxisome proliferator–activated receptor α (PPARα), PPARβ/δ, and PPARγ are a class of ligand-activated nuclear receptors that form heterodimers with retinoid X receptor and are well known for their transcriptional regulation of fatty acid metabolism. PPARs are activated by fatty acids and play specific roles in physiology that are often tissue specific. PPARα controls the hepatic expression of many genes involved in FAO (16, 45) and plays a crucial role in the adaptation of the liver to starvation by also enabling the induction of microsomal ω-oxidation, peroxisomal dicarboxylic acid metabolism, and ketogenesis (46–49). Similarly, PPARα, along with PPARβ/δ, plays a role in the expression of FAO enzymes in skeletal muscle and the heart (47, 50–53).

PPARγ coactivator-1α (PGC-1α) is a transcriptional coactivator with a role in various metabolic processes such as thermogenesis in brown adipose tissue, mitochondrial biogenesis, FAO, and gluconeogenesis. PGC-1α interacts not only with PPARs, but also with estrogen-related receptors (ERRs), another class of nuclear receptors involved in the control of FAO (54). Induction of the expression of PPARα, ERRα, and PGC-1α coincides with increased FAO and increased mitochondrial biogenesis in the postnatal heart (54).

Posttranscriptional Regulation

An important posttranscriptional regulatory step in FAO is the inhibition of CPT1 by malonyl-CoA (55). There are two CPT1 isoforms important for FAO: the ubiquitously expressed CPT1A, also known as liver CPT1, and the muscle- and heart-specific CPT1B. Malonyl-CoA inhibits both CPT1 isoforms. This allosteric regulation of CPT1 is physiologically relevant because Cpt1a activity strongly determines FAO and ketogenic flux in isolated rat hepatocytes (56). Although this point is still under debate, CPT1B also likely controls FAO in heart and skeletal muscle (57). Cellular levels of malonyl-CoA are regulated via production by acetyl-CoA carboxylase and via degradation by malonyl-CoA decarboxylase. Whereas PPAR activation induces transcription of malonyl-CoA decarboxylase, phosphorylation by AMP-activated protein kinase (AMPK) inactivates acetyl-CoA carboxylase. AMPK functions as a metabolic fuel gauge. It is activated by AMP, whose levels rise when ATP levels fall. Therefore, activated AMPK initiates a signaling cascade aimed to restore cellular ATP levels. Thus, the PPAR-mediated increase in malonyl-CoA decarboxylase activity and the AMPK-mediated inactivation of acetyl-CoA carboxylase stimulate FAO by decreasing malonyl-CoA levels. Several other mechanisms related to posttranscriptional regulation of FAO have been reported. The activity of PGC-1α is also regulated by several posttranscriptional mechanisms such as reversible acetylation. AMPK mediates the activation of PGC-1α via SIRT1-mediated deacetylation (58). Hyperacetylation of LCAD in Sirt3 knockout (KO) mice reduced enzymatic activity (59, 60). Interestingly, mitochondrial protein acetylation during fasting is driven by acetyl-CoA from FAO (61). Thus, Sirt3 is important for preserving the activity of enzymes in FAO such as LCAD. Nitric oxide was recently implicated in the regulation of FAO via *S*-nitrosylation of VLCAD (62, 63).

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Table 2 Symptoms of mitochondrial fatty acid β-oxidation disorders and associated genes

Common clinical symptoms	Causal gene(s)
Hypoketotic hypoglycemia	*CPT1A*, *SLC25A20*, *CPT2*, *SLC22A5*, *ACADVL*, *ACADM*, *HADHA*, *HADHB*
Cardiomyopathy	*SLC25A20*, *CPT2*, *SLC22A5*, *ACADVL*, *HADHA*, *HADHB*
Arrhythmias and conduction defects	*SLC25A20*, *CPT2*, *ACADVL*, *HADHA*, *HADHB*
Skeletal myopathy and rhabdomyolysis	*SLC25A20*, *CPT2*, *SLC22A5*, *ACADVL*, *HADHA*, *HADHB*
Atypical and specific symptoms	**Causal gene(s)**
Polyneuropathy	*HADHA*, *HADHB*
Retinopathy	*HADHA*, *HADHB*
Hyperinsulinemic hypoglycemia	*HADH*
Respiratory chain defects, Leigh syndrome	*ACAD9*, *ECHS1*

DISORDERS OF MITOCHONDRIAL FATTY ACID β-OXIDATION

Human inherited defects have been described for almost all enzymes and transporters involved in FAO. All these defects have autosomal recessive inheritance and may be caused by mutations in *CPT1A*, *SLC25A20*, *CPT2*, *SLC22A5*, *ACADVL*, *ACAD9*, *ACADM*, *ACADS*, *HADHA*, *HADHB*, *ECHS1*, *HADH*, and *ACAT1* (**Table 1**). **Table 2** shows the clinical symptoms commonly observed in FAO disorders and the associated genes. **Table 2** also shows atypical and specific symptoms caused by mutations in FAO genes.

Different types of enzyme deficiency can occur in MTP (64). Mutations in *HADHA* can lead to a deficiency of all three enzymatic functions of MTP or to an isolated defect in LCHAD; the latter is more frequent. Similarly, mutations in *HADHB* may cause complete MTP deficiency or an isolated LCKAT defect; the latter is extremely rare. Mutations in *SLC22A5* lead to primary carnitine deficiency, mainly due to renal carnitine wasting. As a result, tissue carnitine levels are low, which impairs carnitine cycle function and thus FAO (9). Although SCAD deficiency leads to perturbations in specific metabolites consistent with a FAO defect, it is currently regarded as a biochemical phenotype of questionable clinical significance (65).

Metabolic disorders have not been reported for some enzymes in FAO, which are CPT1B, LCAD, and any of the auxiliary enzymes. For two other FAO disorders, the underlying genetic basis has not been established, or the underlying molecular defect causes a secondary defect in FAO. MCKAT deficiency was described in a patient with symptoms typical of a FAO defect after a systematic study of the catalytic activities of nine enzymes of the β-oxidation cycle in cultured fibroblasts (66). So far, a causal molecular defect in *ACAA2* has not been reported in this patient. A deficiency described in the DECR1 enzyme (67) was shown to be secondary to a defect in mitochondrial nicotinamide adenine dinucleotide phosphate (NADP) biosynthesis due to a mutation in *NADK2*, the gene encoding the mitochondrial NAD kinase (68).

Common Clinical Symptoms Associated with Fatty Acid β-Oxidation Disorders

The clinical symptoms that are commonly associated with FAO defects illustrate the importance of this pathway for human physiology, in particular during fasting and in organs with high energy needs. It has been well documented, for example, in MCAD deficiency (69), that patients with FAO disorders are at risk for developing hypoketotic hypoglycemia during catabolic conditions such as illness, fever, fasting, and exercise. Hypoketotic hypoglycemia may progress into life-threatening Reye-like syndrome, leading to coma or death. Survivors of these events can

have sustained neurological damage. Interventions aimed at avoiding catabolism prevent further episodes, which was a main reason for including FAO disorders in neonatal screening programs. For MCAD deficiency, the most frequent FAO disorder in people of Western European descent, neonatal screening has been unequivocally shown to provide benefit (70, 71).

Besides hypoglycemia, hypertrophic or dilated cardiomyopathy can be a presenting symptom in VLCAD, MTP, LCHAD, CACT, CPT2, and OCTN2 deficiencies (72–79). Generally, patients with CPT1A and MCAD deficiencies have no cardiomyopathy (73, 80), although exceptions to his rule, notably for MCAD deficiency, have been published (75). Arrhythmias and conduction defects can also be the presenting symptom in patients with FAO disorders but often without cardiomyopathy (73, 81). There is also some controversy about the occurrence of these symptoms in OCTN2, CPT1A, and MCAD deficiencies (75), and in some of these cases, arrhythmia may be better explained by other factors. For example, in one MCAD-deficient patient, an acquired prolonged QTc interval was speculated to result from hypocalcemia that accompanied an acute metabolic crisis (82).

Patients with a FAO defect may also present with or develop skeletal myopathy characterized by rhabdomyolysis, myalgia, and muscle weakness (73, 74). These symptoms are the main manifestations in patients presenting at later age. Rhabdomyolysis may be triggered by prolonged exercise, infections, fasting, cold, or emotional stress. Muscle biopsies may show moderate lipid storage, mainly in type 1 fibers (83–86). A recent cross-sectional study of lower body muscle using MRI revealed specific abnormalities between FAO disorders (87). Whereas VLCAD-deficient patients had proximal abnormalities likely reflecting differences in lipid accumulation, similar findings appeared more distal in patients with LCHAD deficiency. MRI changes consistent with inflammation were observed in patients with elevated plasma creatine kinase (CK) levels, which point to rhabdomyolytic episodes (87). Although patients with MCAD deficiency may have fatigue, muscle pain, and/or reduced exercise tolerance (80), the occurrence of rhabdomyolysis is rare and may develop only in exceptional circumstances (88, 89).

Hepatomegaly is another common symptom, which is very frequent among all FAO disorders and likely reflects fatty liver (74). Polyneuropathy (peripheral neuropathy) (64, 76–77) and retinopathy (90–92) are specific symptoms for patients with LCHAD and MTP deficiencies. The primary focus of all referenced studies was on presenting symptoms in FAO disorders. Follow-up or cross-sectional studies have not yet been published.

Atypical Symptoms Associated with Fatty Acid β-Oxidation Genes

Although SCHAD deficiency due to mutations in *HADH* has a biochemical phenotype consistent with a FAO defect, for example, an aberrant acylcarnitine profile, its clinical presentation is generally atypical. Patients present with protein- or leucine-induced hyperinsulinemic hypoglycemia (93). Detailed studies using an Hadh KO mouse model have illustrated a crucial role for this protein in the pancreatic β cell, which regulates insulin secretion via interaction with glutamate dehydrogenase (94).

Mutations in *ECHS1*, which encodes crotonase, cause Leigh syndrome (95, 96). Crotonase functions not only in FAO, but also in the oxidation of specific amino acids. In crotonase deficiency, highly reactive metabolites, including methacrylyl-CoA derived from valine, accumulate and damage other proteins such as pyruvate dehydrogenase and respiratory chain enzymes, likely explaining the brain pathology (95). These findings do not rule out that crotonase also plays a role in FAO.

Mutations in *ACAD9* cause a deficiency of complex I with symptoms characteristic of respiratory chain defects (25–27). These symptoms include encephalopathy, exercise intolerance, hypertrophic cardiomyopathy, and lactic acidemia.

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Diagnosis of Patients Affected by a Fatty Acid β-Oxidation Disorder

Introduction of tandem mass spectrometry has revolutionized the diagnosis of patients suspected to suffer from an inborn error of metabolism, including patients with FAO disorders (97). Indeed, diagnosis of patients with a FAO disorder used to be extremely problematic and often required in vivo loading tests with sunflower oil or phenylbutyrate and/or fasting tests. Not only is plasma acylcarnitine analysis using tandem mass spectrometry the first test of choice when a patient is suspected to suffer from a FAO deficiency, it is also the method by which neonatal screening for these disorders is performed.

It is generally assumed that an abnormal acylcarnitine profile reflects the intramitochondrial accumulation of acyl-CoAs and as such the substrate of the deficient enzyme in vivo. The accumulating acyl-CoAs are exported out of the mitochondria as acylcarnitines mediated by CPT2, carnitine acetyltransferase (CRAT), and CACT (98–101). For instance, a patient with elevated C8:0 acylcarnitine levels most likely has MCAD deficiency, whereas elevated C14:1 acylcarnitine levels point to VLCAD deficiency. The molecular mechanism of export of acylcarnitines across the cell membrane is unresolved (**Figure 1**).

Rapid confirmation of a particular suspected enzyme deficiency can be performed in lymphocytes, as these cells express all enzymes involved in FAO (102). If an enzyme is deficient, subsequent genomic analysis of the encoding gene should be done to identify the underlying molecular defect. Cultured fibroblasts can also be used for diagnostics via enzyme analysis and via acylcarnitine profiling after fatty acid loading (an in vitro probe assay) (102, 103). FAO flux analysis in cultured fibroblasts using tritium-labeled fatty acids can be used for diagnosis (104) but appears better suited to determining the extent of FAO deficiency, as it may correlate with clinical severity in patients with VLCAD deficiency (105).

Mouse Models

Several mouse models have been developed to study the pathophysiology of FAO defects. Most commonly used are the VLCAD KO (14, 106) and LCAD KO (15) mouse models. Using these models, we and others have shown that LCAD and VLCAD have overlapping and distinct roles in murine FAO (13–15). In fibroblasts, the absence of VLCAD in mice appears largely compensated for, with no obvious changes in FAO flux and acylcarnitine production. Although LCAD plays an essential role in the oxidation of unsaturated fatty acids such as oleic acid, it seems redundant in the oxidation of saturated fatty acids (13). These in vitro findings reflect the biochemical derangements observed in vivo (14, 15). These observations also explain the relatively subtle phenotype of the VLCAD KO mouse, which comprises mild hepatic steatosis, mild microvesicular lipid accumulation in the heart, facilitated induction of polymorphic ventricular tachycardia, and reduced exercise capacity (14, 106–108). In contrast, the LCAD KO mouse has a more pronounced phenotype, resembling more aspects of human VLCAD deficiency than does the VLCAD KO mouse, including fasting-induced hypoketotic hypoglycemia and marked fat accumulation in the liver. LCAD KO hearts are hypertrophic, display triglyceride and ceramide accumulation, and develop fasting-induced cardiac dysfunction (13–15, 110–113).

Several other mouse models are available to investigate different aspects of FAO. Mutations in *Acads* (BALB/cByJ) and *Slc22a5* (juvenile visceral steatosis) have occurred spontaneously and represent good models for SCAD deficiency (114, 115) and primary carnitine deficiency (116–118), respectively. All other existing mouse models have been generated through homologous recombination. *Cpt1a* and *Cpt1b* KO mice are embryonic lethal (119, 120), whereas *Hadha* KO mice die in the early neonatal period (121). *Hadh* KO mice are a good model for hyperinsulinemic hypoglycemia (94, 122). *Acadm*, *Decr1*, and *Eci1* KO mice have mild phenotypic presentations, making

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them less attractive for pathophysiological studies (34, 123–125). Most recently, a cardiac-specific VLCAD-deficient mouse model of dilated cardiomyopathy and cold intolerance was reported (126).

PATHOPHYSIOLOGY OF DISORDERS OF FATTY ACID β-OXIDATION

Hypoglycemia

Several human and mouse studies have addressed the pathogenetic mechanisms underlying fasting-induced hypoglycemia. Patients with a FAO disorder are expected to have an increased glucose demand because they have a diminished capacity to generate ATP from fatty acids and ketones. Indeed, patients with CPT2 or VLCAD deficiency compensate for impaired FAO during prolonged low-intensity exercise by increasing glucose metabolism, which is in this case driven by enhanced muscle glycogenolysis (127, 128). Similarly, fasting-induced hypoglycemia in LCAD KO mice is initiated by an increased glucose requirement in peripheral tissues, leading to rapid hepatic glycogen depletion (113). Gluconeogenesis cannot compensate for the increased glucose demand due to a shortage in the supply of glucogenic precursors, which is explained by a suppressed glucose-alanine cycle, by decreased branched-chain amino acid metabolism, and ultimately by impaired protein mobilization (113). Similar results have been described in a model of acute FAO inhibition (129).

Whereas overnight fasted LCAD KO mice have relatively low, but stable, plasma glucose levels, human patients with a FAO disorder have progressive hypoglycemia, with the lowest recorded values <1 mmol/L. Moreover, during hypoglycemia, patients frequently have lactic acidemia. Both observations are consistent with the inhibition of gluconeogenesis. The mechanism underlying this inhibition is currently unclear. It is known that pharmacological inhibition of FAO suppresses gluconeogenesis in perfused rat livers (130); such suppression may be caused by low levels of acetyl-CoA that limit the activity of the rate-controlling enzyme pyruvate carboxylase (131). Similarly, low acetyl-CoA levels may also explain ammonia elevation, which may be observed in patients with Reye-like symptoms. The first reaction in the urea cycle is catalyzed by carbamoyl phosphate synthase, which requires *N*-acetylglutamate as a natural activator. *N*-Acetylglutamate is synthesized from acetyl-CoA and glutamate by *N*-acetylglutamate synthase. Thus, the origin of hypoglycemia in FAO disorders likely involves multiple mechanisms that include increased peripheral glucose need, limited availability of gluconeogenic precursors, and ultimately failure of gluconeogenesis.

Cardiomyopathy and Heartbeat Disorders

Cardiomyopathy associated with FAO disorders is likely due to the FAO defect in the heart (intrinsic origin) and not caused by pathophysiological processes in other tissues that can affect the heart (extrinsic origin). Given the importance of FAO for the heart, changes in cardiac substrate use resulting in impaired metabolic flexibility or ATP production may be the primary events leading to disease. Alternatively, lipotoxicity due to the accumulation of metabolites may cause tissue damage, which is suggested by the potential toxic effects of fatty acids, acyl-CoAs, and acylcarnitines that have been revealed in vitro and in cultured cells (132–135). These hypotheses have now been addressed in mouse models using in vivo magnetic resonance spectroscopy measurements as well as complementary approaches.

The LCAD KO mouse has cardiac hypertrophy (15), which does not affect cardiac performance and does not appear to progress to cardiomyopathy (111, 136). In response to fasting,

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left ventricular ejection fraction and diastolic filling rate decreased (111). Myocardial triglyceride content was higher in LCAD KO mice in the fed state and further increased upon fasting, which coincided with higher myocardial ceramide content (111). Whereas fasting led to a pronounced decrease in myocardial glucose uptake in wild-type (WT) animals, myocardial glucose uptake did not change in LCAD KO mice (110, 113). Indeed, pyruvate dehydrogenase activity followed a similar pattern, with a much more pronounced fasting-induced decrease in WT animals (110). This finding provides in vivo evidence for decreased metabolic flexibility in LCAD KO hearts, in which the defect in FAO leads to sustained glucose use upon fasting. This decreased metabolic flexibility impacts cardiac energy status, as fasting decreases the phosphocreatine (PCr)/ATP ratio (110). Thus, a combination of toxicity of accumulating lipid metabolites and impaired metabolic flexibility may be responsible for the fasting-induced impairment of cardiac function observed in the LCAD KO mouse.

VLCAD KO mice have cardiac hypertrophy, which is much less pronounced than in LCAD KO mice (112) and may be only transient (137). Cardiac function is unremarkable at a young age (2–3 months old) (106, 137), but a progressive development of systolic dysfunction was observed in older mice (6 and 12 months old) (137). Similar to the LCAD KO hearts, VLCAD KO hearts display increased lipid accumulation (14, 106), higher myocardial glucose uptake, and a decreased PCr/ATP ratio (137). In ex vivo perfusion experiments, working VLCAD-null mouse hearts maintained values similar to those of the controls for functional parameters and for the contribution of exogenous palmitate to FAO (138).

Evidence for a heartbeat disorder has been found in the VLCAD KO mouse. VLCAD KO mice can develop polymorphic ventricular tachycardia (106) and display a prolonged rate-corrected QT (QTc) interval under all conditions examined (138). These phenotypes may be explained by impaired intracellular ion homeostasis (109). Alternatively, hearts of VLCAD KO (138) and LCAD KO (111, 136) mice contained lower levels of docosahexaenoic acid (DHA), which is an antiarrhythmogenic fatty acid. There is currently no good biochemical explanation for the decreased DHA levels.

Skeletal Myopathy and Rhabdomyolysis

The pathophysiology of myopathy and rhabdomyolysis may involve impaired sodium/calcium homeostasis due to reduced metabolic flexibility or lipotoxicity. However, in contrast to the hypoglycemia and cardiac phenotypes in the available mouse models, the skeletal muscle appears unaffected in these animals, with no reports of rhabdomyolysis or elevated plasma CK levels. VLCAD KO mice display reduced exercise capacity, but the underlying mechanism has not been revealed (108). A recently generated new model in which one VLCAD allele was deleted on the background of the LCAD KO mouse did show fasting-induced inactivity, which was not explained by rhabdomyolysis but rather reflected the overall reduced capacity of these mice to generate heat (139). Thus, more insight into the cause of skeletal myopathy and rhabdomyolysis is urgently needed.

TREATMENT

There are multiple treatment options, some of which are useful only in specific FAO disorders (140). Carnitine supplementation is crucial in patients with primary carnitine deficiency. Patients with other FAO disorders may display secondary carnitine deficiency, which is also often treated by carnitine supplementation. The association of acylcarnitines with ventricular fibrillation in a cat model of acute ischemia has sparked debate on the use of carnitine (140, 141). Animal studies

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show harmful as well as beneficial effects of carnitine (132, 136). Available human studies have failed to show beneficial effects (140).

Many patients with a defect in one of the long-chain-specific enzymes are on dietary long-chain triglyceride restriction and medium-chain triglyceride (MCT) supplementation, which in theory would limit the supply of long-chain fatty acids with impaired degradation and provide, as an alternative, a medium-chain fatty acid substrate that can bypass the enzymatic defect. Indeed, the ketogenic properties of MCTs are well established and are expected to be beneficial in long-chain FAO disorders. It is, however, unclear whether MCTs should be supplied continuously or only before or during conditions of increased energy demand. Indeed, work in VLCAD KO mice suggests that MCTs are beneficial prior to exercise, whereas long-term MCT treatment induced tissue lipid accumulation (142). Also, in patients with long-chain FAO disorders, MCT supplementation prior to exercise improved substrate oxidation and cardiac performance (143, 144).

One hypothesis is that the cardiac and muscle pathology in FAO disorders is a result of leakage of catalytic intermediates from the TCA cycle (cataplerosis) (5). This hypothesis led to the development of triheptanoin, an odd MCT that, upon oxidation of the heptanoate, generates anaplerotic propionyl-CoA in addition to acetyl-CoA (5). Interestingly, recent work in the LCAD KO mouse confirmed a fasting-induced defect in anaplerosis (110, 113), and clinical improvement was observed upon triheptanoin treatment of patients with a FAO disorder (5, 145). These observations warrant further studies on the use of this oil.

Bezafibrate, a PPAR agonist, increases residual CPT2 and VLCAD activity in cells of patients with relatively mild missense mutations and as such improves FAO flux (6, 146). In addition, bezafibrate improves myopathy and rhabdomyolysis in patients with CPT2 deficiency (147, 148), although a recent randomized clinical trial of CPT2 and VLCAD deficiency suggested that bezafibrate is ineffective (149).

In conclusion, therapeutic options for FAO disorders are scarce. Moreover, the effectiveness of all treatments needs to be confirmed in a larger cohort of patients, preferably in double-blind studies.

FUTURE PROSPECTS

Although the currently available (mouse) models have provided significant understanding of the pathophysiology associated with FAO disorders, they also have limitations. Current technological advances such as CRISPR/Cas9 genome editing and the generation of induced pluripotent stem cells (iPSCs) will provide new research models. With genome editing, the generation of knock-in mouse models has become cheaper and faster. Given the differences between human FAO and mouse FAO, new models are needed. We anticipate that knock-in mouse models for common mutations of *CPT2* (p.S113L) and *HADHA* (p.E510Q) will be extremely valuable. The possibility of generating iPSC lines from patient fibroblasts may result in the ability to study disease pathogenesis in relevant cell types of each individual patient. A recent study modeled the mitochondrial cardiomyopathy of Barth syndrome with iPSC and heart-on-chip technologies (150). We suggest that FAO disorders should be studied in a similar way.

Exome sequencing has significantly improved our capabilities to identify causal genes in rare genetic diseases. Although most FAO disorders are diagnosed by aberrant metabolite profiles, exome (or whole-genome) sequencing may identify unexpected phenotypes caused by a FAO gene defect, which will further improve our understanding of the physiological functions of FAO.

As is the case for most other inborn errors of metabolism, the genotype at the primary defective locus does not explain all observed variability in a patient's clinical symptoms. Other factors such

as environment and variation at modifier loci will likely contribute to disease severity. There is an urgent need for methods that help predict and identify these modifiers in rare diseases.

CONCLUSION

Although much progress has been made in the understanding of the physiological functions of FAO and the pathophysiology of FAO disorders, many questions remain unresolved. Most notably, there is a pronounced lack of understanding of the pathophysiological mechanisms underlying the myopathy and rhabdomyolysis observed in FAO disorders. Continued research efforts are needed to further progress the field and will enable the design of novel, rational therapeutic strategies.

DISCLOSURE STATEMENT

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