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On the origin of 3-methylglutaconic acid in disorders of mitochondrial energy metabolism

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Summary

3-methylglutaconic acid (3MGA)-uria occurs in numerous inborn errors of metabolism (IEM) associated with compromised mitochondrial energy metabolism. This organic acid arises from thioester cleavage of 3-methylglutaconyl CoA (3MG CoA), an intermediate in leucine catabolism. In individuals harboring mutations in 3MG CoA hydratase (i.e. primary 3MGA-uria), dietary leucine is the source of 3MGA. In secondary 3MGA-uria, however, no leucine metabolism defects have been reported. While others have suggested 3MGA arises from aberrant isoprenoid shunting from cytosol to mitochondria, an alternative route posits that 3MG CoA arises in three steps from mitochondrial acetyl CoA. Support for this biosynthetic route in IEMs is seen by its regulated occurrence in microorganisms. The fungus, *Ustilago maydis*, the myxobacterium, *Myxococcus xanthus* and the marine cyanobacterium, *Lyngbya majuscula*, generate 3MG CoA (or acyl carrier protein derivative) in the biosynthesis of iron chelating siderophores, iso-odd chain fatty acids and polyketide/nonribosomal peptide products, respectively. The existence of this biosynthetic machinery in these organisms supports a model wherein, under conditions of mitochondrial dysfunction, accumulation of acetyl CoA in the inner mitochondrial space as a result of inefficient fuel utilization drives *de novo* synthesis of 3MG CoA. Since humans lack the downstream biosynthetic capability of the organisms mentioned above, as 3MG CoA levels rise, thioester hydrolysis yields 3MGA, which is excreted in urine as unspent fuel. Understanding the metabolic origins of 3MGA may increase its utility as a biomarker.

Keywords

3-methylglutaconyl CoA; organic aciduria; Barth syndrome; Costeff optic atrophy; leucine; Ferrichrome A; iso-odd fatty acid; curacin A

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Nikita Ikon and Robert O. Ryan declare that they have no conflict of interest.

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Author Contributions

Nikita Ikon played an integral role in this work by conducting literature evaluation, manuscript preparation, manuscript editing, formatting and the selection of references for inclusion. Prepared all figures for the manuscript.

Robert Ryan conceived the concept, probed the literature for evidence, outlined the manuscript design, wrote the first draft, edited subsequent drafts versions and worked closely with the co-author to design effective figures.

Introduction

3-methylglutaconyl CoA (3MG CoA) is an intermediate formed during metabolic breakdown of the branched chain amino acid, leucine (Figure 1). Humans normally ingest abundant leucine, which, along with the two other branched chain amino acids (valine and isoleucine), provide a source of metabolic fuel in muscle. Complete breakdown of leucine in muscle mitochondria generates acetyl CoA that is oxidized via the TCA cycle for ATP production. Interest in 3MG CoA has increased in recent years owing to its apparent connection to an ever-increasing number of inborn errors of metabolism (IEM) associated with compromised mitochondrial energy metabolism (Su and Ryan 2014; Wortmann et al 2013). Despite having distinct origins these disorders share a common feature: increased urinary excretion of 3-methylglutaconic acid (3MGA). In both primary and secondary 3MGA-uria, it is anticipated that build up of 3MG CoA precedes CoA thioester bond hydrolytic cleavage to yield the product, 3MGA, which appears in urine. Since 3MGA is a dead end metabolite in which only two of its six carbons are fully oxidized, its excretion constitutes a waste of potential metabolic energy. Whereas mutations in 3MG CoA hydratase (i.e. primary 3MGA-uria) lead to a buildup of 3MGA in a dietary leucine-dependent manner (Wortmann et al 2014), a noteworthy feature of secondary 3MGA-urias is the absence of defects in leucine metabolism.

To explain the appearance of 3MGA independent of leucine catabolic pathway defects, an early model invoked a “mevalonate shunt”, wherein intermediates in isoprenoid biosynthesis are dephosphorylated, oxidized and activated with CoA prior to entering the leucine degradation pathway as 3-methylcrotonyl CoA (Edmond and Popjak 1974; Kelley et al 1991). However, this model does not explain why this shunt would lead to 3MGA excretion instead of complete oxidation via the leucine degradation pathway, nor does it explain why 3MGA excretion would specifically occur in IEMs characterized by mitochondrial dysfunction. If this shunt were operative, it may be anticipated that 3-MGA-uria would occur in cases of Smith-Lemli-Opitz disease. However, two recent articles show this is not the case (Roullet et al 2012; Wortmann et al 2013). On the other hand, it was proposed recently that, in secondary 3MGA-urias, 3MG CoA arises wholly within mitochondria via a direct, three step biosynthetic route that is distinct from the classical leucine degradation pathway and the mevalonate shunt (Su and Ryan 2014). According to this model, impaired mitochondrial energy metabolism (resulting from specific gene mutations, DNA deletions, or pharmacological intervention) impairs electron transport chain function such that NADH oxidation is unable to keep pace with its production. Accumulation of NADH in the inner mitochondrial space inhibits key TCA cycle enzymes, thereby impeding metabolite flux through this pathway. When this occurs, acetyl CoA accumulates, driving formation of acetoacetyl CoA via T2 thiolase. Once formed, acetoacetyl CoA reacts with another molecule of acetyl CoA to form (*S*) 3-hydroxy, 3-methylglutaryl CoA (HMG CoA) via mitochondrial HMG CoA synthase 2 (Figure 1, right side). Whereas HMG CoA in mitochondria is normally a substrate for HMG CoA lyase-mediated cleavage to acetoacetate and acetyl CoA, accumulation of acetyl CoA likely inhibits this reaction. Instead, HMG CoA is dehydrated to 3MG CoA by 3MG CoA hydratase. Under normal metabolic conditions, this member of the enoyl CoA hydratase family catalyzes the conversion of 3MG

CoA to HMG CoA in the leucine degradation pathway. However, because this reaction is reversible (Mack et al, 2006), accumulation of HMG CoA drives the reaction in the reverse direction, resulting in formation of 3MG CoA. Once formed, 3MG CoA cannot continue up the leucine pathway because the next step, catalyzed by 3-methylcrotonyl CoA carboxylase, is essentially irreversible. Thus, 3MG CoA accumulates and, ultimately, serves as a substrate for a yet to be identified member of the acyl CoA thioesterase family (Kirkby et al 2010), producing 3MGA that is excreted in urine.

This route to 3MGA predicts 3MG CoA can be synthesized from acetyl CoA in three steps catalyzed by T2 thiolase, HMG CoA synthase 2 and 3MG CoA hydratase, respectively. Given the relative simplicity of this pathway, we investigated whether precedent exists in other organisms that may require a source of 3MG CoA for unrelated biosynthetic purposes. Our investigation yielded specific examples where 3MG CoA (or an acyl carrier protein [ACP] derivative) is synthesized from acetyl CoA (or acetyl ACP) for the purpose of generating unique lipids and secondary metabolites.

Ferrichrome A biosynthesis in *Ustilago maydis*

The pathogenic fungus, *Ustilago maydis*, is the causative agent of maize smut (Brefort et al 2009). One of the key challenges for growth of *U. maydis* is iron availability. Although iron is abundant in nature, its bioavailability is extremely low due to the poor solubility of ferric oxyhydroxides. To facilitate iron recruitment from the environment, microorganisms synthesize and secrete potent iron chelators, termed siderophores (Miethke and Marahiel 2007). *U. maydis* produces two siderophores, ferrichrome and ferrichrome A, that exist as cyclic hexapeptides. Significantly, ferrichrome A contains three N⁵-hydroxyornithine residues that are N⁵-acylated with 3MG moieties (Figure 2). Ferrichrome A biosynthesis begins with the conversion of acetyl CoA plus acetoacetyl CoA to HMG CoA, via HMG CoA synthase 1. Although this enzyme is required for ferrichrome A biosynthesis, the *hcs1* gene is not part of the co-regulated gene cluster described below, as it is also required for sterol biosynthesis (i.e. the mevalonate pathway) in this fungal species. The remaining enzymes in the pathway, however, are found in the *fer* gene cluster that encodes proteins specifically required for ferrichrome A biosynthesis (Winterberg et al 2010). Of interest to the present discussion is the *fer4* gene product, an enoyl CoA hydratase (i.e. 3MG CoA hydratase) that converts HMG CoA to 3MG CoA. Once formed, 3MG CoA serves as a substrate for an acylase encoded by *fer5*. 3MG CoA reacts with N⁵-hydroxyornithine to generate 3MG-N⁵-hydroxyornithine and CoASH. The next step in the pathway is catalyzed by the *fer3* gene product, a nonribosomal peptide synthase, which catalyzes cyclization of three 3MG-N⁵-hydroxyornithines, one glycine and two serine via peptide bonds to complete formation of ferrichrome A. The 3MG moieties are indispensable in the product molecule as they participate directly in iron chelation (van der Helm and Winkelmann 1994). Thus, when iron is limiting, *U. maydis* induces coordinated transcription of a gene cluster that encodes proteins required for biosynthesis of 3MG CoA that is used in biosynthesis of ferrichrome A.

Iso-odd chain fatty acid and secondary metabolite biosynthesis in myxobacteria

CoA thioesters of the short branched-chain carboxylic acids, isovaleryl-CoA, isobutyryl-CoA and 2-methylbutyryl-CoA are formed during the degradation of leucine, valine, and isoleucine, respectively. In certain organisms these metabolites serve as biosynthetic precursors of secondary metabolites. In *Streptomyces avermitilis*, for example, isobutyryl CoA and 2-methylbutyryl CoA participate in biosynthesis of the macrocyclic lactone, avermectin (Yoon et al 2003). In myxobacteria, isovaleryl CoA is used in biosynthesis of the secondary metabolites geosmin (Dickschat et al 2005a), myxothiazol (Silakowski et al 1999), and aurafuron (Frank et al 2007). Likewise, isovaleryl CoA serves as a primer in the biosynthesis of “iso-odd chain” fatty acids (Bode et al 2006). These unusual branched chain fatty acids effectively substitute for unsaturated fatty acids as a means to control membrane fluidity at different temperatures. In all myxobacteria species investigated to date, iso-odd chain fatty acids are the dominant molecular species, contributing up to 75% of the fatty acids present. Interestingly, in mutant strains of *Myxococcus xanthus* and *Stigmatella aurantiaca* in which branched chain amino acid catabolism is blocked, synthesis of iso-odd chain fatty acids and other isovaleryl CoA-derived metabolites continues (Bode et al 2006; Dickschat et al 2005b). This finding indicates a biosynthetic route to isovaleryl CoA exists that does not arise from leucine degradation (Figure 3). In studies of this process, Muller and coworkers described an operon (*aib*) that controls expression of genes that participate in *de novo* biosynthesis of isovaleryl CoA (Li et al 2013). In *M. xanthus*, HMG CoA synthase, encoded by the *mvaS* gene, catalyzes formation of HMG CoA from acetoacetyl CoA and acetyl CoA. Subsequently, LiuC, a 3MG CoA hydratase, converts HMG CoA into 3MG CoA (Bode et al 2009). At this point, a decarboxylase, composed of a heterodimer of AibA and AibB, converts 3MG CoA to 3-methylcrotonyl CoA (also referred to as 3,3 dimethylacrylyl CoA). Finally, isovaleryl CoA is generated by reduction of 3-methylcrotonyl CoA by the oxidoreductase AibC (Li et al 2013). Isovaleryl CoA generated by this pathway is directly incorporated into iso-odd chain fatty acids. Thus, in the absence of leucine-derived isovaleryl CoA, myxobacteria can synthesize this intermediate through the *de novo* synthesis of 3MG CoA from acetyl CoA, using enzymes found in humans (HMG CoA synthase and 3MG CoA hydratase), and subsequent processing of 3MG CoA to isovaleryl CoA using enzymes not found in humans (decarboxylase and oxidoreductase).

Curacin A biosynthesis

Curacin A is a major lipid component of the cyanobacterium, *Lyngbya majuscula*. This mixed polyketide/nonribosomal peptide displays potent antimitotic activity by inhibiting microtubule assembly (Blokchin et al 1995). In studies of curacin A biosynthesis, Chang et al (2004) reported the existence of a biosynthetic gene cluster, *cur*, that includes genes encoding a β -keto acyl ACP synthase (*curC*), HMG ACP synthase (*curD*) and 3MG ACP hydratase (enoyl ACP hydratase; *curE*; Geders et al 2007; Figure 4). In this system, *curB* encodes the ACP onto which intermediates in the pathway are bound. Once formed, 3MG ACP is decarboxylated by the *curF* gene product, generating 3-methylcrotonyl ACP, the metabolite that ultimately gives rise to the cyclopropyl ring of curacin A. Interestingly, it has

been observed that tandemly arranged genes involved in this part of the curacin A biosynthetic pathway (*curB-curF*) form an HMG CoA synthase (HCS)-like cassette that is utilized in the biosynthesis of numerous metabolites across different species (Gu et al 2006). For example, Jamaicamide A synthesis in *L. majuscula* employs an HCS-like cassette (Edwards et al 2004) as does *Pseudomonas fluorescens* in the biosynthesis of mupirocins (El-Sayed et al 2003). It has also been reported that HCS-like cassettes are involved in synthesis of apratoxin by *Lyngbya bouillonii* (Grindberg et al 2011), pksX synthesis by *Bacillus subtilis* (Calderone et al 2006) as well as myxovirescin A synthesis by *M. xanthus* (Simunovic et al 2007). Thus, coordinated expression of genes that function in *de novo* biosynthesis of 3MG ACP is a highly conserved process.

Conclusions

Taken together, these examples illustrate that biosynthesis of 3MG CoA (or ACP derivatives) from acetyl CoA and acetoacetyl CoA occurs via a common route that is distinct from the classical leucine degradation pathway and is not reliant on leucine availability. 3MG CoA moieties generated via this pathway serve varied functions including a) structural component of the iron chelating siderophore, ferrichrome A; b) precursor of isovaleryl CoA used in iso-odd chain fatty acid biosynthesis and c) precursor of 3-methylcrotonyl ACP used in curacin A biosynthesis. Several species possess a conserved HCS-like gene cassette that permits coordinated production of enzymes involved in this pathway. The existence of essentially identical pathways in fungi, myxobacteria and cyanobacteria provides precedent for the existence of this biosynthetic route to 3MG CoA in humans, albeit under different circumstances. While the aforementioned organisms express the required enzymes in the cytosol, as part of coordinately regulated gene clusters, in humans the enzymes are present in mitochondria. Although the biosynthetic route described does not normally operate in humans, IEMs and other factors that compromise mitochondrial energy metabolism can initiate this process via a buildup of acetyl CoA. When this occurs the bidirectional enzymes T2 thiolase and 3MG CoA hydratase reverse direction and, together with HMG CoA synthase 2, generate 3MG CoA. Because humans lack the biosynthetic machinery to employ 3MG CoA for other biosynthetic purposes, as this metabolite accumulates, a yet to be identified acyl CoA thioesterase hydrolyzes 3MG CoA to 3MGA and CoA, sealing its fate for excretion in urine. Understanding this pathway not only provides insight into IEMs but other forms of mitochondrial impairment as well. For example, a major effect of statin-mediated HMG CoA reductase inhibition is reduced ubiquinone (coenzyme Q) synthesis (Davidson et al 1997). Owing to coenzyme Q's integral role in the electron transport chain and mitochondrial energy production, it is not unexpected that pharmacological inhibition of coenzyme Q biosynthesis by statins leads to 3MGA-uria (Phillips et al 2002; Pei et al, 2010). Recent studies have also revealed that defects in a range of mitochondrial enzymes can inhibit activation of mitochondrial repair programs, thereby decreasing mitochondrial fitness (Liu et al 2014). While such "suboptimal" mitochondria may show no impairment under basal conditions, increased energy demand could precipitate the type of metabolic overload that is known to result in 3MGA production in IEMs.

Thus, when acetyl CoA production exceeds utilization capacity, the 3MGA pathway functions as an "overflow valve" generating an organic acid waste product that is excreted as

unspent fuel. In addition to accounting for the incidence of 3MGA-uria in a number of disparate IEMs, the present model provides an explanation for the inconsistent appearance of 3MGA-uria among individuals with the same disorder. Just as statin-induced myopathy subjects only experience symptoms under conditions of increased energy demand (e.g. exercise), we posit that patients with IEMs associated with 3MGA-uria will only display consistently elevated levels of 3MGA when placed under metabolic challenge. Testing patients under conditions of increased energy demand, such as strenuous exercise or increased metabolic impairment (e.g. increased statin dose), will likely reveal a higher and more consistent level of 3MGA-uria. Increased knowledge of the metabolic origin of 3MGA, and its facile detection in urine, increases its potential value as a biomarker of mitochondrial function in affected individuals.

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Synopsis

Processes in microorganisms provide support for *de novo* biosynthesis of 3-methylglutaconate from acetyl CoA in human subjects with inherited disorders that compromise mitochondrial energy metabolism.

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Figure 1. Catabolic and anabolic routes to 3-methylglutaconyl CoA

Left side) The classical leucine catabolism pathway is depicted. Right side) Anabolic route to 3MG CoA from acetyl CoA. Note that the reversible enzyme, 3MG CoA hydratase, is shown in both pathways. In the leucine degradation path 3MG CoA hydratase catalyzes hydration of 3MG CoA to HMG CoA while in the *de novo* anabolic route it catalyzes dehydration of HMG CoA to form 3MG CoA. The names of equivalent enzymes involved in 3MG CoA biosynthesis in other organisms are identified in all figures by a box that is rectangular (thiolase), dashed (HMG CoA synthase) or rounded (3MG CoA hydratase).

Figure 2. Involvement of 3MG CoA in biosynthesis of ferrichrome A by *Ustilago maydis*
 3MG CoA synthesized from acetyl CoA and acetoacetyl CoA (left side) condenses with N⁵-hydroxyornithine (right side) to generate the modified amino acid, 3MG-N⁵-hydroxyornithine, that is incorporated into the cyclic peptide, ferrichrome A.

Figure 3. Biosynthetic route to isovaleryl CoA from acetyl CoA in *Myxococcus xanthus*

When the leucine degradation pathway is blocked, a route to isovaleryl CoA is used that involves *de novo* synthesis of 3MG CoA from acetyl CoA (left side). Once formed, 3MG CoA is metabolized to isovaleryl CoA via two enzymatic reactions that do not exist in humans. Isovaleryl CoA subsequently serves as primer for iso-odd chain fatty acid biosynthesis.

Figure 4. De novo synthesis of 3-methylcrotonyl ACP in biosynthesis of curacin A

The coordinated synthesis of 3-methylcrotonyl ACP for incorporation into *Lyngbya majuscula* curacin A involves condensation of acetyl ACP and acetoacetyl ACP to form HMG ACP. Subsequent dehydration to 3MG ACP is followed by decarboxylation of 3MG ACP to form 3-methylcrotonyl ACP that is incorporated into curacin A. This pathway utilizes a reaction scheme for generation of 3MG ACP that is analogous to reactions in other systems that utilize CoA derivatives.