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Metabolic biology of 3-methylglutaconic acid-uria: a new perspective

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Summary

Over the past twenty-five years a growing number of distinct syndromes / mutations associated with compromised mitochondrial function have been identified that share a common feature: urinary excretion of 3-methylglutaconic acid (3MGA). In the leucine degradation pathway, carboxylation of 3-methylcrotonyl CoA leads to formation of 3-methylglutaconyl CoA while 3-methylglutaconyl CoA hydratase converts this metabolite to 3-hydroxy-3-methylglutaryl CoA (HMG CoA). In "primary" 3MGA-uria, mutations in the hydratase are directly responsible for the accumulation of 3MGA. On the other hand, in all "secondary" 3MGA-urias, no defect in leucine catabolism exists and the metabolic origin of 3MGA is unknown. Herein, a path to 3MGA from mitochondrial acetyl CoA is proposed. The pathway is initiated when syndrome-associated mutations / DNA deletions result in decreased Krebs cycle flux. When this occurs, acetoacetyl CoA thiolase condenses two acetyl CoA into acetoacetyl CoA plus CoASH. Subsequently, HMG CoA synthase 2 converts acetoacetyl CoA and acetyl CoA to HMG CoA. Under syndrome-specific metabolic conditions, 3-methylglutaconyl CoA hydratase converts HMG CoA into 3-methylglutaconyl CoA in a reverse reaction of the leucine degradation pathway. This metabolite fails to proceed further up the leucine degradation pathway owing to the kinetic properties of 3-methylcrotonyl CoA carboxylase. Instead, hydrolysis of the CoA moiety of 3-methylglutaconyl CoA generates 3MGA, which appears in urine. If experimentally confirmed, this pathway provides an explanation for the occurrence of 3MGA in multiple disorders associated with compromised mitochondrial function.

Keywords

mitochondria; 3-methylglutaconic acid; Krebs's cycle; energy metabolism; electron transport chain

3-methylglutaconic acid (3MGA) uria is a hallmark feature of a heterogeneous collection of syndromes / mutations characterized by defects in mitochondrial function (Gibson et al

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1993; Wortmann et al 2009; Wortmann et al 2012a; Wortmann et al 2013b). The source of elevated 3MGA is unequivocally known only for “primary” 3MGA-uria (OMIM 250950). In this autosomal recessive disorder the leucine degradation pathway is disrupted by mutations in *AUH*, encoding 3-methylglutaconyl CoA hydratase. As a result of this metabolic block, 3-methylglutaconyl CoA accumulates (IJlst et al 2002). Subsequent hydrolysis of the CoA moiety generates the organic acid, 3MGA, which escapes from mitochondria and is excreted in urine. In a functioning leucine catabolic pathway (Figure 1) 3-methylglutaconyl CoA is converted to 3-hydroxy-3-methylglutaryl (HMG) CoA followed by HMG CoA lyase dependent production of acetyl CoA and acetoacetate.

Intriguingly, in numerous other syndromes / mutations that are characterized by 3MGA-uria (Table 1), no defects in leucine metabolism have been identified. These “secondary” 3MGA-urias (Wortmann et al 2013a) include Barth syndrome (BTHS; Clarke et al 2013), Costeff optic atrophy syndrome (Anikster et al 2001), “MEGDEL” syndrome (Wortmann et al 2006; Wortmann et al 2012b), dilated cardiomyopathy with ataxia (DCMA) syndrome (Ojala et al 2012) and mutations in *TMEM70* (Cizkova et al 2008). In addition, 3MGA-uria has been reported to occur in individuals with mutations in *POLG1* (Wortmann et al 2009) as well as the m.3243A>G tRNA^{Leu} variant (De Kremer et al 2001) and DNA depletion syndromes (Figarella-Branger et al 1992; Scaglia et al 2001). Slight elevations in urinary 3MGA have also been reported for mutations in the β subunit of succinyl CoA synthetase (Morava et al 2009). Furthermore, increased urinary excretion of 3MGA has also been reported in cases of statin-induced myopathy (Phillips et al 2002). An apparent connection between the secondary 3MGA-urias is compromised mitochondrial function, although distinctive phenotypic features (e.g. encephalopathy, cardiomyopathy, optic atrophy etc.) distinguish the various syndromes / mutations.

It has been proposed that 3MGA arises from aberrant shunting of isoprenoids generated in the cytosol via the mevalonate pathway to mitochondria (Kelley and Kratz 1995; Walsh et al 1999; Pei et al 2010; Wortmann et al 2012a; Wortmann et al 2013b). Contrary to this, we posit that all secondary 3MGA-urias result from mutation-induced effects on electron transport chain (ETC)-related energy production, causing an increase in the intra-mitochondrial NADH / NAD⁺ ratio such that inhibition of the Krebs cycle enzymes isocitrate dehydrogenase (Gabriel and Plaut 1984) and -ketoglutarate dehydrogenase (Chinopoulos 2013) occurs. In non hepatic tissues including skeletal muscle, heart and brain, this metabolic impediment results in redirection of mitochondrial acetyl CoA toward production of 3MGA.

Overview of tissue specific mitochondrial acetyl CoA metabolism

In mitochondria, four major sources of acetyl CoA exist. These are 1) fatty acid β -oxidation; 2) glucose / amino acid metabolism to pyruvate with subsequent oxidation to acetyl CoA by pyruvate dehydrogenase; 3) ketone body degradation and 4) catabolism of Phe, Leu, Trp, Lys, Tyr, Ile and Thr. Complementing these means of generating acetyl CoA, there are several ways in which intra-mitochondrial acetyl CoA can be utilized. A major route involves condensation with oxaloacetate to form citrate and entry to the Krebs cycle. In muscle, when acetyl CoA levels exceed demand for energy (e.g. immediately following

cessation of vigorous exercise) it is converted to acetyl-carnitine and transported out of mitochondria (Foster and Harris 1987; Longnus et al 2001). Alternatively, in liver, but not muscle or brain (Sluse et al 1971; Gnoni et al 2009), excess acetyl CoA can be transported to the cytosol via the citrate shuttle. Acetyl CoA in liver mitochondria is also efficiently used for ketone body biosynthesis. In skeletal muscle, heart and brain mitochondria, despite the presence of the required enzymes (i.e. acetoacetyl CoA thiolase, HMG CoA synthase 2 and HMG CoA lyase), the presence of succinyl CoA: 3-oxoacid CoA transferase (SCOT) ensures ketone bodies are degraded for use as an energy source (Fukao et al 1997). Thus, in mitochondria of skeletal muscle, heart and brain, the primary fate of acetyl CoA is oxidation via the Krebs cycle, with fine-tuning occurring via the acetyl-carnitine transporter. Thus, under conditions where Krebs cycle flux is impeded, we propose that some portion of acetyl CoA is diverted towards an alternate non energy yielding metabolic fate that, in 4 steps, generates 3MGA.

Metabolic path from acetyl CoA to 3MGA

The pathway to 3MGA from acetyl CoA is presented in Figure 2 and involves four enzyme reactions, as described below:

1. Thiolase-mediated generation of acetoacetyl CoA. Whereas mitochondria possess more than one thiolase activity, that most likely to participate in 3MGA biosynthesis is acetoacetyl CoA thiolase (T2; EC 2.3.1.9), an enzyme that is specific for short chain fatty acyl CoA substrates (Haapalainen et al 2007). T2 catalyzes a reversible Claisen condensation of 2 acetyl CoA, generating acetoacetyl CoA and CoASH. By contrast, another thiolase activity, 3-ketoacyl CoA thiolase (T1; EC 2.3.1.16) is a component of the fatty acid β -oxidation pathway and displays broad-specificity towards short, medium and long chain fatty acyl CoAs (Kunau et al 1995). Whereas T1 is essential for fatty acid β -oxidation, T2 functions in ketone body metabolism. This conclusion is supported by studies demonstrating that brain mitochondria contain T2 and can efficiently oxidize ketone bodies while they possess very little T1 and oxidize fatty acids poorly, if at all (Yang et al 1987).
2. Production of HMG CoA. Mitochondrial HMG CoA synthase 2 is entirely distinct from its cytosolic counterpart that participates in the mevalonate pathway (Hegardt 1999). In liver mitochondria, HMG CoA synthase 2 functions in the biosynthesis of ketone bodies. Non ketogenic tissues, including heart and skeletal muscle also express HMG CoA synthase 2, albeit at far lower levels (Mascaro et al 1995; Puisac et al 2012). In all tissues, HMG CoA is generated in mitochondria as a metabolic intermediate in the degradation of leucine, lysine and tryptophan. Whereas leucine catabolism does not require HMG CoA synthase 2, it does participate in the degradation of lysine and tryptophan (Wu 2013).
3. Conversion of HMG CoA to 3-methylglutaconyl CoA. 3-methylglutaconyl CoA hydratase forms part of the leucine degradation pathway and is missing or defective in primary 3MGA-uria (Figure 1). Whereas this enzyme was originally isolated on the basis of its ability to bind AUUU repeats in RNA (Kurimoto et al 2009), the physiological significance of this observation remains unclear. Insofar as reactions

catalyzed by members of enoyl CoA hydratase enzyme family are generally reversible (Loupatty et al 2004), it may be anticipated that a buildup of HMG CoA will lead to formation of 3-methylglutaconyl CoA. Once formed, 3-methylglutaconyl CoA can conceivably serve as substrate for 3-methylcrotonyl CoA carboxylase. In the forward reaction of leucine degradation, this biotin dependent enzyme utilizes ATP and HCO_3^- to carboxylate 3-methylcrotonyl CoA, forming 3-methylglutaconyl CoA. 3-methylcrotonyl CoA carboxylase is a member of the biotin-dependent carboxylase enzyme family for which a wealth of information is available (Tong 2013). Similar to other prominent members of this family, Schiele and Lynen (1981) reported that 3-methylcrotonyl CoA carboxylase displays a marked kinetic disequilibrium, with the forward reaction proceeding 10 times faster than the reverse reaction. Thus, although the reaction catalyzed by 3-methylcrotonyl CoA carboxylase is formally reversible, the sluggish nature of the reverse reaction would be expected to result in accumulation of 3-methylglutaconyl CoA.

4. Thioester hydrolysis to yield 3MGA. Accumulation of 3-methylglutaconyl CoA promotes hydrolytic cleavage of its CoA moiety, generating 3MGA and CoASH. Cells possess at least 13 distinct acyl CoA thioesterase enzymes (ACOTs; Kirkby et al 2010). These are distributed throughout the cell and show distinct tissue expression patterns and substrate specificities. Of interest to the present discussion is the observation that ACOT4 and ACOT8 are known to hydrolyze dicarboxylic CoA esters (Westin et al 2005). The specific ACOT that hydrolyzes 3-methylglutaconyl CoA to 3MGA must meet certain criteria including tissue / organelle expression and substrate specificity. 3-methylglutaconyl CoA likely represents an alternate substrate for the ACOT activity involved in production of 3MGA.

Metabolic rationale for the formation of 3MGA

In muscle tissue, under normal physiological circumstances, acetyl CoA generated by fatty acid β -oxidation rapidly enters the Krebs cycle. When entry to the cycle is impeded (as a result of syndrome-specific mutations described above, for example), acetyl CoA may be used as a substrate for T2 thiolase, generating acetoacetyl CoA. In the presence of NADH, acetoacetyl CoA can be converted to β -hydroxy butyryl CoA, an intermediate in the fatty acid β -oxidation pathway. β -hydroxy butyryl CoA can be further metabolized in the reverse direction of β -oxidation but, ultimately, reaches a dead end that stifles further metabolism of acetoacetyl CoA via this route. Alternatively, acetoacetyl CoA can condense with acetyl CoA to form HMG CoA. Mitochondrial HMG CoA has fewer options than that generated in the cytosol such that, under normal physiological conditions, this metabolite is converted to acetoacetate plus acetyl CoA by HMG CoA lyase (see Figure 1). However, in keeping with the fact that skeletal muscle, heart and brain do not generate ketone bodies for export, HMG CoA lyase activity is low in these tissues (Puisac et al 2010). Whereas hepatic tissue exports acetoacetate and β -hydroxy butyrate as ketone bodies, this process does not occur in muscle, heart or brain because SCOT converts acetoacetate to acetoacetyl CoA. When acetyl CoA metabolism via the Krebs cycle is impeded by syndrome-specific mutations, T2 thiolase

functions in the direction of acetoacetyl CoA synthesis, creating a “futile cycle” that consumes succinyl CoA (Figure 3). Under conditions where succinyl CoA is depleted or HMG CoA lyase activity is limiting (e.g. product inhibition by acetoacetate and/or acetyl CoA), HMG CoA levels will rise. At this point the metabolic options for HMG CoA are reduced to reversal of the reaction catalyzed by 3-methylglutaconyl CoA hydratase. Thus, when entry to the Krebs cycle is impeded and acetyl CoA buildup leads to production of acetoacetyl CoA, HMG CoA is generated. When HMG CoA lyase or SCOT activity fail to keep up, HMG CoA is diverted toward 3-methylglutaconyl CoA in a reaction catalyzed by 3-methylglutaconyl CoA hydratase, which functions in the reverse direction of leucine catabolism.

The relationship between cardiolipin defects and 3MGA-uria

BTHS is an X-linked recessive disorder characterized by cardiomyopathy, skeletal muscle weakness, neutropenia and 3MGA-uria (Clarke et al 2013). BTHS is caused by mutations in the *TAZ* gene locus (Whited et al 2013) encoding the phospholipid transacylase, tafazzin (Xu et al 2006b). Loss of tafazzin activity leads to alterations in the composition of cardiolipin molecular species and the appearance of monolysocardiolipin (Valianpour et al 2005). BTHS patients manifest mitochondrial ultrastructure changes (Bissler et al 2002) and defective energy metabolism (Ferri et al 2013). Numerous studies in non primate models of BTHS, including yeast (Gu et al 2004), *Drosophila* (Xu et al 2006a), zebrafish (Khuchua et al 2006) and mice (Acehan et al 2011) provide support for these general concepts.

Cardiolipin is an important structural component of the protein-rich inner mitochondrial membrane. In heart and muscle tissue mitochondria, this membrane is highly enriched in tetra-linoleoyl cardiolipin (Houtkooper and Vaz 2008). ETC proteins interact directly with cardiolipin (Zhang et al 2005; Bazan et al 2013) such that their function is compromised by the defective cardiolipin content present in BTHS mitochondria. NADH and FADH₂ generated as products of acetyl CoA combustion are normally oxidized by ETC complexes in reactions that lead to formation of the proton gradient that drives ATP synthesis. Indeed, optimal muscle contraction during exercise relies on efficient coupling of fuel utilization, NADH and FADH₂ oxidation and ATP synthesis. Accumulation of NADH will inhibit key Krebs cycle enzymes and, when this occurs, intra-mitochondrial acetyl CoA levels rise. Cardiac and skeletal muscle cells respond by forming acetyl-carnitine with subsequent transport of acetyl units out of mitochondria via the carnitine translocase system. In the cytosol of muscle and heart, acetyl CoA released from acetyl-carnitine serves as a substrate for acetyl CoA carboxylase 2, generating malonyl CoA, a potent inhibitor of carnitine palmitoyl transferase I (Lopaschuk et al 2010). As a result, long chain fatty acyl-carnitines are unable to enter mitochondria and β -oxidation stalls. When this happens, intra-mitochondrial carnitine levels decline, thereby preventing continued formation of acetyl-carnitine. Ultimately, the cell turns to glycolysis in an effort to maintain ATP production. In BTHS, a reliance on glucose utilization by working muscle can be seen by their respiratory use coefficient >1.0 (Spencer et al 2011). Increased glycolytic activity, together with impaired Krebs cycle flux, leads to a buildup of pyruvate. Extra-mitochondrial pyruvate is converted to lactic acid by lactate dehydrogenase. Lactate migrates to the liver where it can be used as a substrate for gluconeogenesis. A side effect of this process is lactic acidosis, a

known feature of BTHS. Under the metabolic conditions prevailing in cardiac and muscle mitochondria, pyruvate dehydrogenase complex will be inhibited by acetyl CoA (Lopaschuk et al 2010). In the face of continuing energy demand, muscle proteins degradation will occur, yielding amino acids that are metabolized to acetyl CoA.

Thus, alterations in cardiolipin content and composition caused by mutations in tafazzin lead to membrane defects that impair ETC function. This impairment leads to inefficient oxidative phosphorylation and accumulation of NADH and FADH₂. NADH-mediated inhibition of key Krebs cycle enzymes leads to increased intra-mitochondrial acetyl CoA. When faced with demand for ATP under conditions where acetyl CoA oxidation via the Krebs cycle is suboptimal, excess acetyl CoA, which serves as a direct inhibitor of pyruvate dehydrogenase and an indirect inhibitor of fatty acid β -oxidation, is diverted toward the non energy yielding metabolite, 3MGA, that is excreted in urine. Utilization of this pathway has the net effect of decreasing intra-mitochondrial acetyl CoA levels, potentially relieving its inhibitory effects on fatty acid oxidation and pyruvate dehydrogenase activity.

Defective mitochondrial protein import

Davey et al. (2006) described an autosomal recessive condition, termed DCMA syndrome, in the Canadian Dariusleut Hutterite population. This syndrome is characterized by early onset dilated cardiomyopathy with conduction defects, non-progressive cerebellar ataxia, testicular dysgenesis, growth failure and 3MGA-uria. A disease-associated mutation has been identified in *DNAJC19*, encoding a DNAJ domain containing protein. The DNAJC19 protein was previously localized to mitochondria in cardiac myocytes, and is similar to the yeast mitochondrial inner membrane proteins, Mdj2p and Tim14. Insofar as Tim14 is a component of the yeast inner mitochondrial membrane presequence translocase, it is likely that the phenotype associated with DCMA syndrome is the result of defective mitochondrial protein import. Since nearly all mitochondrial proteins in humans are nuclear encoded, successful transport of cytoplasm-derived precursor proteins into mitochondria is critical for optimal function of this organelle (Rehling et al 2003). Curiously, although they arise from distinct mutations, DCMA syndrome and BTHS share a number of phenotypic features in common.

Costeff Syndrome

Individuals with Costeff optic atrophy syndrome harbor mutations in *OPA3*. Whereas the *OPA3* gene product localizes to mitochondria (Powell et al 2011), the precise function of this protein remains a mystery. Major phenotypic features of Costeff syndrome include optic atrophy and dystonia. In an engineered mouse model expressing a mutant opa3 protein, Wells et al. (2012) provided evidence of impaired non-shivering thermogenesis. The authors observed a reduction in lipid utilization by brown adipose tissue although there was no defect in cellular lipid uptake. Based on this, a yet to be determined relationship between opa3 and lipid metabolism exists. In the context of the present discussion, however, the findings of lipid accumulation in brown fat and 3MGA-uria are entirely consistent with a potential function of opa3 in some aspect of ETC function. In this scenario, mutations in opa3 would compromise mitochondrial energy metabolism, leading to accumulation of

NADH and decreased Krebs cycle flux. Subsequent accumulation of intra-mitochondrial acetyl CoA will inhibit fatty acid β -oxidation, explaining both the presence of lipid droplets and the production of 3MGA. The underlying mechanism responsible for opa3 mutation-dependent effects, however, must await a more complete understanding of its biological function in mitochondria.

Phosphatidylglycerol metabolism defect

“MEGDEL” syndrome is caused by mutations in *SERAC1*, which encodes a protein harboring a serine-lipase domain (see Wortmann et al 2012b). The function of this protein was unknown prior to the identification of disease-causing mutations. Studies using fibroblasts from patients showed that *serac1* localizes to contact sites between mitochondria and the endoplasmic reticulum where it plays a role in phosphatidylglycerol remodeling. Biochemically, patients carrying mutations in *SERAC1* display alterations in the composition of phosphatidylglycerol molecular species, low levels of bis (monoacylglycerol) phosphate, and accumulation of free cholesterol. Given the fact that mutations that affect cardiolipin content and composition are associated with 3MGA-uria, it is reasonable to consider that defects in phosphatidylglycerol molecular species composition, trafficking or remodeling that result from mutations in *SERAC1* could lead to inner membrane defects that compromise ETC function such that acetyl CoA is diverted toward synthesis of 3MGA.

Impaired ATP synthase assembly

The *TMEM70* gene encodes an undefined “factor” involved in biogenesis of ATP synthase (Cizkova et al 2008; Honzik et al 2010). A defect in this gene was recently identified as the cause of autosomal recessive ATP synthase deficiency. In addition to 3MGA-uria, patients harboring mutations in *TMEM70* display a distinctive phenotype characterized by neonatal onset of severe muscular hypotonia, hypertrophic cardiomyopathy, facial dysmorphism and profound lactic acidosis (Spiegel et al 2011). It is plausible to consider that an inability to generate ATP via oxidative phosphorylation negatively impacts ETC function, causing a buildup of NADH and acetyl CoA, with subsequent production of 3MGA.

Mutant Krebs cycle enzyme

The *SUCLA2* gene encodes the β -subunit of the ADP-forming succinyl-CoA synthetase (SCS), a heterodimeric enzyme that exists in two isoforms (Morava et al 2009). SCS is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA. Whereas the reverse reaction occurs in the Krebs cycle, the forward reaction produces succinyl-CoA for activation of ketone bodies (see Figure 3) and heme biosynthesis. As such, SCS plays a key role in mitochondrial metabolism. Mutations in this enzyme have been reported to be associated with modest increases in 3MGA (Wortmann et al 2009), possibly the result of increased acetoacetyl CoA formation from ketone bodies owing to elevated levels of succinyl CoA. Since levels of 3MGA observed in cases of mutant *SUCLA2* are only slightly above background, additional studies are required confirm this association.

Statin-mediated inhibition of isoprenoid biosynthesis

The “statins” are a family of structurally related compounds that inhibit HMG CoA reductase, the rate-limiting enzyme of isoprenoid biosynthesis. Statins are widely used to treat hypercholesterolemia, a major risk factor for development of coronary heart disease and stroke. Despite documented clinical efficacy, up to 10 % of the ~13 million Americans who take statins experience undesirable side effects, including myopathy (Larsen et al 2013). Phillips et al. (2002) reported that statin-induced myopathy is accompanied by elevated urinary excretion of 3MGA. Whereas no mechanism was ascribed at the time, it is now recognized that inhibition of HMG CoA reductase not only decreases cholesterol biosynthesis but coenzyme Q₁₀ (CoQ₁₀) as well. CoQ₁₀ is a long chain modified isoprenoid that is abundant in the inner mitochondrial membrane, where it shuttles electrons between ETC complexes. A deficiency in CoQ₁₀ caused by statin treatment would be anticipated to impair ETC function and, ultimately, lead to production of 3MGA. Consistent with this interpretation, Muraki et al. (2012) showed that, in mice, CoQ₁₀ supplementation reverses mitochondrial dysfunction associated with statin treatment.

Metabolite shunting from the cytosol as the source of 3MGA

In contrast to the pathway to 3MGA described in Figure 2, several authors have proposed alternative schemes to explain the metabolic origin of 3MGA-uria (see Wortmann et al 2012a for a review). In every case it has been proposed that 3MGA-uria arises from an imbalance in cytosolic isoprenoid homeostasis that induces shunting of isoprene units into mitochondria where they enter the leucine catabolic pathway and, ultimately, accumulate as 3MGA. Left unexplained is why 3-methylglutaconyl CoA would not be metabolized via the leucine catabolism pathway, since secondary 3MGA-urias are distinguished by the absence of a blockage in leucine metabolism. Edmond and Popjack (1974) first proposed a “mevalonate shunt” to explain the results of studies in which radiolabel from ¹⁴C-mevalonate was recovered in fatty acids. On close inspection, however, the mevalonate shunt offers no physiological rationale why trafficking of isoprenoid metabolites generated in the cytosol to the mitochondria would occur as a result of the various mutations described above.

Smith-Lemli-Opitz syndrome

Support for a connection between the mevalonate pathway and 3MGA-uria comes from a study of individuals with Smith-Lemli-Opitz syndrome (SLOS). SLOS is caused by missing or defective sterol 7-reductase activity, resulting in accumulation of 7-dehydrocholesterol / 8-dehydrocholesterol and defective steroid metabolism. Kelley and Kratz (1995) reported that 7 of 35 SLOS subjects studied had elevated blood levels of 3MGA. Based on this, they reasoned that defective cholesterol production in SLOS leads to increased flux through the mevalonate pathway, resulting in “overflow” of intermediates into mitochondria via the Popjack shunt. Subsequent attempts to confirm an association between SLOS and 3MGA-uria, however, have failed. Rouillet et al. (2012) found no evidence for mevalonate shunting in a study of moderately affected SLOS patients. Likewise, Wortmann, Kluijtmans et al. (2013b) did not detect 3MGA in eight SLOS patients under treatment with cholesterol and

simvastatin. These authors suggested that, insofar as the 3MGA positive SLOS subjects identified by Kelley and Kratz were untreated and had very low plasma cholesterol levels, 3MGA detected in these individuals may be a secondary effect related to membrane dysfunction owing to a lack of cholesterol.

Studies with mutant *opa3* zebrafish

In studies investigating the origin of 3MGA in Costeff syndrome (see above), Pei et al. (2010) employed zebrafish. These authors reasoned that, since the shunt described by Edmond and Popjak (1974) occurs at the level of mevalonate, statin-mediated inhibition of HMG CoA reductase should interfere with 3MGA production. However, elevated 3MGA levels were observed in both statin-treated wild type zebrafish embryos and *opa3* mutant zebrafish embryos. On the basis of these data, and leucine supplementation experiments, the authors proposed that *opa3* functions to facilitate metabolism of cytosolic 3MGA derived from extra-mitochondrial HMG CoA through a novel, mevalonate-independent “HMG salvage pathway”. Insofar as *opa3* localizes to mitochondrial membranes, it seems more likely that this protein functions in some aspect of mitochondrial energy production. Indeed, the association of 3MGA with simvastatin treated zebrafish is entirely consistent with the known effects of this compound on CoQ₁₀ biosynthesis. By the same token, leucine supplementation would be expected to increase catabolism of this amino acid. Whereas wild type zebrafish are fully capable of metabolizing acetyl CoA generated from metabolism of the supplemented leucine, impairment of ETC / oxidative phosphorylation function caused by mutations in *opa3* may be expected to lead to NADH-induced Krebs cycle inhibition, with diversion of acetyl CoA toward 3MGA synthesis.

Limitations of the proposed pathway

Based on evidence presented herein, if the pathway to 3MGA described in Figure 2 is valid, then it may be anticipated that other single gene mutations that disrupt mitochondrial energy metabolism should also lead to 3MGA-uria. For example mutations in Krebs cycle enzymes, such as alpha ketoglutarate dehydrogenase (Bonnefont et al 1992) or fumarase (Deschauer et al 2006) should interfere with Krebs cycle flux and, thereby, lead to 3MGA formation. By the same token, disruptive mutations in genes encoding various subunits units of oxidative phosphorylation complexes (Bird et al 2013) would be anticipated to affect NADH levels and subsequently, inhibit Krebs cycle function, leading to 3MGA production. In another example, the appearance of 3MGA in glycogen storage disease 1b (GSD1b; Law et al 2003) is difficult to fit into the developing model. GSD1b is caused by mutations in the glucose 6 phosphate transporter (*SLC37A4*) and results in defects in glucose processing. Law and coworkers found that elevated urinary 3MGA levels in the propositus correlated with elevated levels of Krebs cycle intermediates and that partial dietary correction of urinary fumarate / malate levels partially corrected 3MGA levels as well. Thus, an as yet undefined mechanistic relationship between GSD1b and 3MGA-uria may exist.

Conclusions and future directions

In conclusion, we propose that secondary 3MGA-urias are the result of direct or indirect effects of specific mutations on Krebs cycle flux, ETC function or oxidative phosphorylation. Clearly, experimental validation of this proposed pathway is required. Considering that the alternative path to 3MGA (i.e. isoprenoid shunting) requires components derived from the cytosol, examination of 3MGA formation by isolated mitochondria could provide evidence that cytosol is not required for 3MGA production *in vitro*. If so, then the “isoprenoid shunt” pathway becomes less tenable. If the pathway described herein is correct, then isolated mitochondria derived from experimental models of disease should be capable of producing 3MGA and, furthermore, wild type mitochondria should generate 3MGA when incubated with acetyl CoA and NADH. A more detailed evaluation (i.e. validation) of the pathway may be obtained through studies with isolated enzymes. With the exception of the thioesterase (step 4 of the proposed pathway; see Figure 2) each of the enzymes is well known. Thus, *in vitro* reconstruction of the pathway should be possible, allowing for characterization of kinetic aspects of metabolite flux.

Finally, given that 3MGA levels are highly variable, and frequently intermittent (Wortmann et al 2009), urinary content of this organic acid provides a unique window into mitochondrial function, especially when combined with knowledge of an underlying causal gene defect. Future studies with animal models or human subjects will reveal the precise nature of the relationship between 3MGA-uria and metabolic dysregulation as it pertains to specific syndromes or mutations. It is conceivable that dietary intervention strategies and/or exercise modulation will be reflected in urinary 3MGA content. Indeed, it is plausible to consider that a medical device capable of routine, rapid detection of 3MGA in urine would improve home management (i.e. personalized outpatient care) of syndromes associated with 3MGA-uria. Such a device could be in the form of a “dip-stick” based enzyme linked immunosorbant assay. If so, urinary 3MGA levels could be monitored in real time at low cost. With knowledge of the correlation between 3MGA-uria and aberrant mitochondrial function, an ability to inform subjects diagnosed with specific inborn errors of metabolism about their metabolic status should permit them to lead a more normal life, akin to a diabetic monitoring their blood glucose.

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Synopsis

A pathway from acetyl CoA to 3-methylglutaconic acid is described that occurs in metabolically compromised mitochondria of heart, muscle and brain tissue.

Figure 1. Leucine degradation pathway in mitochondria

Leucine is initially transaminated to α -ketoisocaproic acid by branched chain amino acid aminotransferase, with α -ketoglutarate as the amino group acceptor. Branched chain α -keto acid dehydrogenase generates isovaleryl CoA and acyl CoA dehydrogenase converts isovaleryl CoA to 3-methylcrotonyl CoA. 3-methylcrotonyl CoA carboxylase, 3-methylglutaconyl CoA hydratase, and HMG CoA lyase complete the metabolic path to acetyl CoA and acetoacetate.

Figure 2. Proposed metabolic path to 3MGA from acetyl CoA in a metabolically compromised mitochondria

The four enzymes reactions are (1) T2 Thiolase; (2) HMG CoA synthase 2; (3) 3-Methylglutaconyl CoA hydratase and (4) Thioester hydrolase. 3MG CoA, 3-methylglutaconyl CoA.

Figure 3. Acetoacetyl CoA metabolism in metabolically compromised muscle/heart mitochondria
Under conditions where acetyl CoA entry to the Krebs cycle is impeded, acetoacetyl CoA is generated by T2 thiolase, which is converted to HMG CoA by HMG CoA synthase 2 (HMGCS2). Together with HMG CoA Lyase and SCOT, a "futile cycle" is created that regenerates acetoacetyl CoA. An alternative metabolic fate of HMG CoA, that escapes this cycle, is conversion to 3-methylglutaconyl CoA (3MG CoA).

Table 1
Syndromes associated with 3MGA-uria

Syndrome ^a	Mutated gene ^a	Gene product function	Location ^b
Barth	TAZ	Cardiolipin remodeling	MIM
Costeff	OPA3	Unknown	MOM/MIM
MEGDEL	SERAC1	Phosphatidylglycerol trafficking	MOM/MAM
DCMA	DNAJC19	Mitochondrial protein import	MOM
-	TMEM70	ATP synthase assembly	MIM
MELAS ^c	m.3243A>G	tRNA ^{Leu}	Matrix
-	POLG1	Mitochondrial DNA polymerase	Matrix
Statin-induced myopathy	HMG CoA reductase ^d	Coenzyme Q ₁₀ biosynthesis	MIM

^a Syndrome / Gene abbreviations are described in the text.

^b MIM, mitochondrial inner membrane; MOM, Mitochondrial outer membrane; MAM, Mitochondrial associated membrane

^c MELAS, Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke

^d Wild type enzyme inhibited by statin treatment.