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Metabolic annotation of 2-ethylhydracrylic acid

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Summary

Increased levels of the organic acid, 2-ethylhydracrylic acid (2-EHA) occur in urine of subjects with impaired L(+)-isoleucine metabolism. Chiral intermediates formed during isoleucine degradation are (S) enantiomers. Blockage of (S) pathway flux drives racemization of (2S, 3S) L(+)-isoleucine and its (2S, 3R) stereoisomer, L(+)-alloisoleucine. This non-protein amino acid is metabolized to (R)-2-methylbutyryl CoA via enzymes common to branched chain amino acid degradation. Subsequently, (R) intermediates serve as alternate substrates for three valine metabolic enzymes, generating 2-EHA. Once formed, 2-EHA accumulates because it is poorly recognized by distal valine pathway enzymes. Thus, urinary 2-EHA represents a biomarker of isoleucine pathway defects. 2-EHA levels are also increased in rats exposed to the industrial solvent, ethylene glycol monomethyl ether or the neurotoxin precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. In these cases, a block in (S) pathway isoleucine catabolism occurs at the level of (S)-2-methylbutyryl CoA conversion to tiglyl CoA via inhibition of electron transferring flavoprotein / ubiquinone oxidoreductase dependent reactions. Elevated urinary 2-EHA in propionyl CoA carboxylase deficiency and methylmalonic aciduria results from a buildup of distal intermediates in the (S) pathway of isoleucine degradation. In Barth syndrome and dilated cardiomyopathy with ataxia syndrome, 2-EHA is a byproduct of impeded propionyl CoA entry into the Krebs cycle.

Keywords

isoleucine; alloisoleucine; valine; 2-ethylhydracrylic acid; short / branched chain acyl CoA dehydrogenase; T2 thiolase; propionyl CoA carboxylase deficiency; Barth syndrome; dilated cardiomyopathy with ataxia syndrome

As the field of metabolomics continues to advance in terms of methodology and sensitivity [1], increasingly large amounts of information can be derived from biological samples. In general, metabolomics involves the systematic study of the chemical products or metabolites that cells and organisms generate. One example where significant strides have been made is the human urine metabolome. Comprised of myriad components, it is recognized that

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specific inborn errors of metabolism or disease processes manifest characteristic alterations in this metabolome. To facilitate knowledge transfer, a Urine Metabolome Database (<http://www.urinemetabolome.ca>) has been created that contains a comprehensive, web-accessible resource containing 2651 confirmed urine metabolites or metabolite species (corresponding to 3079 defined structures), their corresponding concentrations and their disease associations. Whereas the potential utility of this information is enormous, it is equally important to comprehend the metabolic biology underlying the presence of a given compound at increased or decreased levels in a sample. Herein, the connection between a unique chemical signature and human physiology and metabolism is illustrated for the organic acid, 2-ethylhydracrylic acid (2-EHA). Normally a minor component of urine, its levels increase in individuals harboring deficiencies in enzymes of isoleucine metabolism and beyond. Based on this understanding it is evident that urinary levels of 2-EHA represent a valuable, readily accessible, biomarker.

The (R) pathway of isoleucine metabolism

2-EHA, also known as 2-ethyl-3-hydroxypropionic acid or 2-(hydroxymethyl)butyric acid (Figure 1), is an organic acid normally present in urine at low levels (2.1 μM /mM creatinine; range 1.3 – 2.9) [2]. Routinely measured by gas chromatography-mass spectrometry, urinary 2-EHA levels can be elevated up to ten fold in individuals with specific inborn errors of metabolism or following ingestion of certain toxic substances. 2-EHA is considered to be an intermediate / product of the alternative “(R) pathway” of isoleucine metabolism [3,4]. In general, the (R) pathway operates when there is an impediment in the classical (S) pathway of L(+)-isoleucine metabolism (Figure 2). Since the second and third carbons of isoleucine are chiral centers, this branched chain amino acid has four unique enantiomeric forms. The predominant enantiomer is L(+)-isoleucine (2S-amino-3S-methylpentanoic acid). The first step in L(+)-isoleucine metabolism is transamination to (S)-2-keto-3-methylvalerate, with loss of one chiral center. Under normal circumstances, metabolism via the (S) pathway generates propionyl CoA and acetyl CoA. When an impediment in this pathway exists, such as in individuals with mutations in *ACADSB*, encoding short/branched chain acyl CoA dehydrogenase (SBCAD; EC 1.3.99.3), increased amounts of 2-EHA appear in urine [5]. Other common symptoms include hypotonia, developmental delay, hypoglycemia, and ketoacidosis. SBCAD is a member of the acyl CoA dehydrogenase family [6], catalyzing the FAD dependent dehydrogenation of (S)-2-methylbutyryl CoA to tiglyl CoA. When SBCAD activity is inhibited, the substrate (S)-2-methylbutyryl CoA, as well as proximal intermediates of the (S) pathway, accumulate. In this case, some portion of (S)-2-methylbutyryl CoA forms a glycine conjugate (i.e. 2-methylbutyryl glycine) that is excreted in urine. In addition, loading studies have demonstrated racemization of isoleucine stereoisomers [4]. Racemization is the result of incomplete transamination of L(+)-isoleucine by branched chain amino acid aminotransferase via enzyme-bound ketimine-enamine tautomerization [7,8,9] (Figure 3). The prevailing concept is that an impediment in (S) pathway flux leads to redirection of the enzyme bound enamine intermediate toward the (R) pathway metabolites, L(+)-alloisoleucine and (R)-2-keto-3-methylvalerate.

The (R) pathway utilizes valine catabolic enzymes

Once formed, L(+)-alloisoleucine and (R)-2-keto-3-methylvalerate can be further metabolized to (R)-2-methylbutyryl CoA by the combined action of branched chain amino acid aminotransferase and branched chain α -keto acid dehydrogenase (in the case of L(+)-alloisoleucine) or the latter enzyme alone (in the case of (R)-2-keto-3-methylvalerate). Both of these enzymes are common to metabolism of the three branched chain amino acids and correspond to reactions (1) and (2) in Figure 2. Henceforth, the (R) pathway of isoleucine metabolism employs three successive enzymes specific to valine metabolism [10] wherein (R) series metabolites serve as alternate substrates (Figure 4). Insofar as the structure of (R)-2-methylbutyryl CoA is reminiscent of the valine metabolite, isobutyryl CoA, it serves as an alternate substrate for the FAD dependent enzyme, isobutyryl CoA dehydrogenase (ACAD8), leading to formation of 2-ethylacrylyl CoA and FADH₂. 2-ethylacrylyl CoA is converted to 2-ethylhydracrylyl CoA via the action of a second valine pathway enzyme, methacrylyl CoA hydratase. Subsequently, 2-ethylhydracrylyl CoA is converted to 2-EHA by a third valine catabolic enzyme, 3-hydroxyisobutyryl CoA hydrolase.

2-EHA: dead end or metabolic intermediate?

It is currently a point of contention whether 2-EHA is a “dead end” metabolite destined solely for excretion in urine or a pathway intermediate. If the latter does occur, two additional reactions in the valine catabolic pathway, catalyzed by 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase, would be expected to generate butyryl CoA, a β -oxidation pathway intermediate.

Early studies of organic acids in a subject with β -keto thiolase (i.e. T2 thiolase; ACAT1, EC 2.3.1.9) deficiency revealed that 2-EHA was present together with ethylmalonic acid [3]. This observation led to the suggestion that a precursor – product relationship exists between these compounds. The presence of 2-EHA in subjects with T2 thiolase deficiency may be explained by the role this enzyme plays in (S) pathway isoleucine metabolism. An inability to metabolize the (S) pathway intermediate, 2-methyl-3-ketobutyryl CoA, to acetyl CoA and propionyl CoA (see Figure 2 reaction 6) leads to decreased flux through the (S) pathway of isoleucine metabolism. Subsequently, racemization of (2S, 3S) L(+)-isoleucine (see Figure 3) generates metabolites that are converted to 2-EHA via the valine catabolic path. On the other hand, the appearance of ethylmalonic acid in urine of subjects with T2 thiolase deficiency almost certainly results from the fact that the deficient enzyme also functions in the β -oxidation pathway, converting acetoacetyl CoA into 2 acetyl CoA. When this thiolytic cleavage reaction is blocked, the upstream pathway metabolite, butyryl CoA, accumulates. As its concentration increases, butyryl CoA serves as an alternate substrate for propionyl CoA carboxylase [11], leading to formation of ethylmalonyl CoA. Whereas some portion the ethylmalonyl CoA generated by this reaction can be metabolized by methylmalonyl CoA mutase to methylsuccinyl CoA, and beyond, the bulk of ethylmalonyl CoA is subject to thioester hydrolysis and excretion in urine as ethylmalonate. Thus, the appearance of 2-EHA in a subject with T2 thiolase deficiency is the result of a block in isoleucine metabolism while ethylmalonic aciduria arises from aberrant metabolism of butyryl CoA. Consistent

with this interpretation, Mamer and Tjoa [3] reported that, following an isoleucine load, control subjects excreted large amounts of 2-EHA but no ethylmalonic acid.

In loading experiments with a racemic mixture, comprised of (*R*)- and (*S*)-2-methylbutyrate, a rapid buildup of 2-EHA was observed [4,12] suggesting it is poorly metabolized. It is conceivable that the next enzyme in the valine catabolic path, 3-hydroxyisobutyrate dehydrogenase, is unable to efficiently recognize 2-EHA as an alternate substrate. Labeling studies by Mamer et al [4], however, raise the possibility that 2-EHA is converted to ethylmalonate semialdehyde with subsequent reversion to the alcohol. In this case, it must be considered that inefficient use of ethylmalonate semialdehyde by methylmalonate semialdehyde dehydrogenase may be responsible for 2-EHA accumulation / excretion in urine. Whether some portion of 2-EHA generated from isoleucine metabolism via the (*R*) pathway actually continues down the valine catabolic pathway to butyryl CoA is difficult to ascertain because this “product” is readily metabolized via β -oxidation and the TCA cycle. Notwithstanding this caveat, Mamer et al [4] was able to recover deuterated ethylmalonate in urine of rats fed a labeled racemic mixture of (*R*)- and (*S*)-2-methylbutyrate. Despite the appearance of label in ethylmalonate, levels of this organic acid did not rise above normal while 2-EHA levels increased dramatically. Distinguishing between these possibilities is important because, if 2-EHA can be metabolized to butyryl CoA, then the (*R*) pathway of isoleucine catabolism constitutes a bona fide alternate metabolic route from L(+)-isoleucine to energy yielding metabolites. At present, however, convincing evidence that significant amounts of 2-EHA are actually converted to butyryl CoA, is lacking.

Isoleucine pathway enzyme deficiencies

Given the discussion above, it may be anticipated that a deficiency in any enzyme of (*S*) pathway isoleucine metabolism will lead to increased excretion of 2-EHA. It should be noted that a deficiency in the proximal enzyme, branched chain α -keto acid dehydrogenase (giving rise to “maple syrup urine” disease; [13]), does not result in an accumulation of 2-EHA because the same enzyme is required for metabolism of the corresponding (*R*) pathway intermediate. The effect of a deficiency in SBCAD has been discussed above and elsewhere [5]. Proceeding down the (*S*) pathway (see Figure 2), no deficiencies in tiglyl CoA hydratase have been reported. Beyond this step, deficiencies in 2-methyl-3-hydroxybutyryl CoA dehydrogenase [14,15] or T2 thiolase [3,16] are associated with elevated levels of 2-EHA in urine. The latter deficiency is accompanied by ketoacidosis and hyperglycemia. In these cases, it is logical to consider that blockage of isoleucine catabolism via the (*S*) pathway leads to a build up of proximal metabolites with racemization of L(+)-isoleucine as described above.

Congenital multiple acyl CoA dehydrogenase deficiency (MADD)

Members of the acyl CoA dehydrogenase enzyme family catalyze analogous chemical reactions yet are distinguished by their unique substrate specificities. For example, SBCAD catalyzes the dehydrogenation of (*S*)-2-methylbutyryl CoA to tiglyl CoA in the isoleucine pathway while butyryl CoA dehydrogenase converts butyryl CoA to crotonyl CoA as part of the fatty acid β -oxidation pathway. Both enzymes have an FAD prosthetic group that

extracts 2 protons to yield the corresponding unsaturated product (i.e. tiglyl CoA and crotonyl CoA, respectively) along with enzyme-associated FADH₂. In order for members of this enzyme family to function in another catalytic event, FADH₂ must be re-oxidized to FAD. This is achieved by the activity of electron transferring flavoprotein (ETF) which transfers an electron pair from FADH₂ to the iron sulfur protein, ETF:ubiquinone oxidoreductase. In turn, the oxidoreductase transfers an electron pair to the electron transport chain by reducing coenzyme Q (Figure 5). As such, mutations in ETF or ETF:ubiquinone oxidoreductase lead to inhibition of reactions catalyzed by members of the acyl CoA dehydrogenase family. The term multiple acyl CoA dehydrogenase deficiency (MADD) is used to describe deficiencies in these enzymes since reactions catalyzed by all acyl CoA dehydrogenase family members are adversely affected. Because of its broad impact on metabolism, MADD has devastating physiological consequences. Although it may be anticipated that MADD would lead to redirection of L(+)-isoleucine to the alternative (*R*) pathway, 2-EHA is not generated because the key valine pathway enzyme, isobutyryl CoA dehydrogenase, is also subject to inhibition. Thus, although MADD leads to accumulation of (*S*)-2-methylbutyrate and ethylmalonic acid, 2-EHA is not produced.

Acquired MADD

The compound hypoglycin A, found in unripe ackee fruit [17] and box elder seeds [18], is the cause of Jamaican vomiting disease and equine seasonal pasture myopathy, respectively. Hypoglycin A inhibits acyl CoA dehydrogenase activity, causing severe hypoglycemia in humans. The observation that 2-EHA does not accumulate in Jamaican vomiting disease or seasonal pasture myopathy is consistent with the fact that hypoglycin A is a potent general inhibitor of acyl CoA dehydrogenase enzyme activity such that inhibition of isobutyryl CoA dehydrogenase activity prevents metabolism of (*R*)-2-methylbutyrate to 2-EHA.

At the same time, exposure of rats to the industrial solvent, ethylene glycol monomethyl ether (EGME) results in elevated urinary 2-EHA [19]. Based on the finding that EGME exposure leads to metabolic perturbations in choline oxidation, fatty acid β -oxidation and branched chain amino acid catabolism, it may be surmised that EGME functions as a direct or indirect inhibitor of acyl CoA dehydrogenase enzyme activity. Indeed, similar urinary and serum metabolite signatures are seen in humans suffering from MADD. Unlike congenital MADD, however, exposure to sublethal doses of EGME leads to increased levels of 2-EHA in urine (referred to as 3-hydroxy-2-ethylpropionate in [19]). The authors propose that increased 2-EHA is the result of EGME-dependent inhibition of SBCAD with diversion of some portion of L(+)-isoleucine toward the (*R*) pathway. In this case, isobutyryl CoA dehydrogenase may be less susceptible to inhibition by EGME, permitting (*R*) pathway metabolism to 2-EHA to proceed. Whether rat isobutyryl CoA dehydrogenase activity is resistant to EGME toxicity or the dose administered did not completely inhibit the enzyme, remains to be determined.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin precursor of MPP⁺, (1-methyl-4-phenylpyridinium ion) that causes permanent symptoms of Parkinson's disease by destroying dopaminergic neurons in the substantia nigra of the brain [20]. Treatment of rats with MPTP results in elevated levels of 2-EHA in urine [21]. Interestingly, however,

other metabolites expected to accumulate if a MADD-like inhibition pattern is operative were not present. Thus, it is conceivable that, in the rat, at the dose administered, MPTP serves as a specific inhibitor of SBCAD activity, either by direct inhibition or by preventing FADH₂ oxidation. Based on the selective nature of the effect, 2-EHA may serve as a biomarker of MPP⁺ toxicity.

Enzyme deficiencies in distal steps of L(+)-isoleucine catabolism

The products of the (S) pathway of isoleucine metabolism are acetyl CoA and propionyl CoA, generated by the action of T2 thiolase on 2-methyl-3-ketobutyryl CoA. Whereas acetyl CoA can enter the TCA cycle directly by condensation with oxaloacetate, propionyl CoA entry requires additional enzymatic steps including propionyl CoA carboxylase, methylmalonyl CoA epimerase and methylmalonyl CoA mutase, ultimately giving rise to the TCA cycle intermediate, succinyl CoA. Insofar as propionyl CoA also arises from metabolism of valine, methionine, threonine, thymidine and cholesterol, a deficiency in propionyl CoA carboxylase activity will quickly lead to an accumulation of propionyl CoA. Individuals with propionyl CoA carboxylase deficiency suffer from hypotonia, hyperammonemia, ketoacidosis and excrete of a variety of organic acids, including 2-EHA [22]. Przyrembel et al [23] reported the presence of 2-EHA in urine of a patient with propionyl CoA carboxylase deficiency after a two-day L(+)-isoleucine load. The finding that isoleucine loading also led to increased urinary excretion of 2-methyl-3-ketobutyrate and N-tiglylglycine is consistent with an impediment in (S) pathway isoleucine metabolism. Conceivably, this occurs because propionyl CoA accumulation inhibits T2 thiolase mediated conversion of 2-methyl-3-ketobutyryl CoA to acetyl CoA and propionyl CoA, thereby inhibiting isoleucine metabolic flux via the (S) pathway. If so, it may be anticipated that increased L(+)-isoleucine racemization (see Figure 3) will occur, leading to increased 2-EHA production via the valine catabolic pathway. Essentially the same arguments hold for methylmalonyl CoA mutase deficiency wherein propionyl CoA accumulation also occurs.

Barth syndrome

2-EHA has been reported to occur in urine of subjects with Barth syndrome [24] and, more recently, in individuals suffering from dilated cardiomyopathy with ataxia (DCMA) syndrome [25,26]. Barth Syndrome is an X-linked disorder caused by mutations in the tafazzin (*TAZ*) gene, encoding a transacylase involved in remodeling cardiolipin molecular species while DCMA is caused by mutations in *DNAJC19*, a protein predicted to function in mitochondrial protein import. Both disorders are characterized by cardiomyopathy, growth delay and 3-methylglutaconic aciduria. The occurrence of 2-EHA in these syndromes is curious insofar as no defect in isoleucine pathway enzymes has been reported. However, based on our current understanding of organic aciduria in disorders characterized by compromised mitochondrial energy metabolism [27], it is likely that 2-EHA in these syndromes is a byproduct of impaired oxidative metabolism and the inability of propionyl CoA to enter the Krebs cycle. In Barth syndrome, alterations in cardiolipin content and composition interfere with electron transport chain function, resulting in a buildup up of NADH. As its levels rise, NADH inhibits key TCA cycle enzymes [28]. In heart and muscle tissue mitochondria, acetyl CoA buildup drives T2 thiolase activity in the reverse direction,

from cleavage to condensation, forming acetoacetyl CoA that is ultimately metabolized to 3-methylglutaconic acid, the primary organic acid in urine of Barth Syndrome and DCMA subjects [27]. It must also be considered that, when acetyl CoA is unable to enter the TCA cycle, the same holds true for propionyl CoA. Thus, when TCA cycle flux is impaired and substrate entry is blocked, propionyl CoA levels will rise. When this occurs in the presence of acetyl CoA, T2 thiolase catalyzes a condensation reaction between acetyl CoA and propionyl CoA, generating the (*S*) pathway isoleucine intermediate, 2-methyl-3-ketobutyryl CoA [29]. As this metabolite (possibly in combination with 2-methyl-3-hydroxybutyryl CoA) accumulates, it may be anticipated that forward isoleucine metabolism via the (*S*) pathway will be affected, with redirection of L(+)-isoleucine toward the (*R*) pathway. Consistent with this interpretation, Kelley et al [24] reported that isoleucine feeding to a subject with Barth syndrome resulted in increased urinary excretion of 2-EHA.

Conclusions

The discussion above illustrates the complementary, if not synergistic, relationship that exists between compound identification in a biological sample and understanding of the metabolic biology underlying its production and/or accumulation. When this information is aligned, the true power of metabolomics can be used to reveal pathway flux perturbations that reflect inborn errors of metabolism or exposure to toxic materials. Annotation of signature metabolites, such as 2-EHA, provides a framework to define the root cause of metabolic disorders and develop strategies treat and/or prevent deleterious physiological consequences from occurring.

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Highlights

- 2-ethylhydracrylic acid (2-EHA) is derived from L(+)-isoleucine
- 2-EHA production utilizes valine metabolic enzymes
- Increased 2-EHA in urine is a biomarker of isoleucine pathway defects
- Enzyme deficiencies distal to isoleucine metabolism may also give rise to 2-EHA
- Annotation of signature metabolites provides a framework to define the cause of metabolic disorders

Figure 1. Chemical structure of 2-ethylhydracrylic acid (2-EHA)

Figure 2. Pathway of L(+)-isoleucine catabolism

The six enzymes involved are (1) Branched chain amino acid aminotransferase; (2) Branched chain keto acid dehydrogenase; (3) Short / branched chain acyl CoA dehydrogenase; (4) Tiglyl CoA hydratase; (5) 2-Methyl-3-hydroxybutyryl CoA dehydrogenase and (6) T2 thiolase (β-ketoacyl CoA thiolase).

Figure 3. Interconversion of (S) pathway and (R) pathway isoleucine metabolites

Racemization is thought to occur via an enamine intermediate formed during transamination of L(+)-isoleucine by branched chain amino acid aminotransferase. Formation of an enzyme bound pyridoxal-5'-phosphate (PLP) Schiff base intermediate between the substrate amino group and the transaminase PLP coenzyme precedes rearrangement to a ketimine intermediate that tautomerizes to the corresponding enamine, resulting on loss of chirality at carbon 3. This enzyme bound achiral intermediate can either revert to corresponding amino acid [L(+)-isoleucine or L(+)-alloisoleucine] or undergo hydrolysis to yield the product (R)-

or (*S*)- α -keto acid and a pyridoxamine-enzyme. α -ketoglutarate (α -KG) serves as terminal acceptor of the enzyme bound amino group, yielding glutamate (Glu) and a regenerated enzyme-PLP. * depicts a chiral center.

Figure 4. (R) Pathway isoleucine metabolism via the valine catabolic pathway

Following racemization and formation of (R) 2-keto-3-methylvalerate, branched chain α -keto acid dehydrogenase catalyzes formation of (R) 2-methylbutyryl CoA. Metabolism of this substrate using successive valine pathway enzymes yields 2-EHA, which is excreted in urine (see arrow). While evidence suggests 3-OH isobutyrate dehydrogenase may reversibly form the corresponding semialdehyde, this product is able to revert to 2-EHA. Unknown (see “?”) is whether any fraction of ethylmalonate semialdehyde is converted to butyryl CoA via methylmalonate semialdehyde dehydrogenase. * depicts a chiral center.

Figure 5. Conversion of (S) 2-methylbutyryl CoA to tiglyl CoA

Diagram depicts the flow of electrons derived from (S) 2-methylbutyryl CoA to a short/branched chain acyl CoA dehydrogenase (SBCAD)-bound FAD prosthetic group to the electron transport chain (ETC). ETF, electron transferring flavoprotein; CoQ, Coenzyme Q.