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Using Isotopic Tools to Dissect and Quantitate Parallel Metabolic Pathways

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Abstract

4-hydroxyacids are ubiquitous in human physiology. They are derived from the drugs of abuse - hydroxybutyrate (GHB), -hydroxypentanoate(GHP), in addition to the omnipresent lipid peroxidation product 4-Hydroxy-2-(E)-nonenal (4-HNE). Previously we reported that 4-hydroxyacids are catabolized through two parallel pathways. In this report we detail two isotopic tools that have allowed the dissection of this catabolic process, and illustrate how these tools can be used to quantify the relative flux down each pathway. We found that 4-hydroxynonanoate (a 4-hydroxyacid derived from 4-HNE) is primarily catabolized through a pathway that phosphorylates the C-4 hydroxyl and isomerizes it to a C-3 hydroxy compound, which is catabolized through - oxidation.

The existence of parallel metabolic pathways is typically indicative of fundamental pathways that are critical for normal physiology. For example, conversion of glucose to pyruvate is dogmatic across biology, but the eventual fate of pyruvate can proceed down different parallel pathways dictated by physiologic conditions. In aerobic organisms under normal conditions, pyruvate enters the citric acid cycle to be eventually catabolized to carbon dioxide. However, if that organism is transferred to anaerobic conditions, a parallel metabolic step is utilized whereby pyruvate is converted to lactate.¹ This example is by no means unique, with other prominent examples including bile acid biosynthesis occurring via either neutral (classic) or acidic (alternative) pathways² and -oxidation occurring in either the peroxisome or mitochondria. These examples further illustrate that this parallelism may occur in the same compartment, or be separately compartmentalized within the cell.³

4-Hydroxyacids are omnipresent *in vivo*, derived from either exogenous or endogenous sources. Exogenously these molecules are typically introduced through drugs of abuse including -hydroxybutyrate (GHB, the date rape drug)⁴ or its emerging alternative, -hydroxypentanoate.⁵ Endogenously, 4-hydroxyacids are derived from the oxidation and saturation of the ubiquitous lipid peroxidation product, 4-hydroxy-2-(E)nonenal (4-HNE).^{6,7} Since its discovery in 1964,⁸ 4-HNE has been generally accepted as a modulator of numerous cellular systems and implicated in the pathogenesis of a number of degenerative diseases including Alzheimer's disease, atherosclerosis, cataracts, and cancer.^{9,10} The

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Supporting Information Available: Experimental details and compound characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

generally accepted pathogenesis of 4-HNE is linked to the α -hydroxy, α,β -unsaturated aldehyde acting as a strong electrophile, which can form adducts with a variety of cellular nucleophiles via Michael addition or Schiff base formation. This interesting pathogenesis is linked to the general abundance of this molecule. Under physiological conditions typical concentrations of 4-HNE range from 0.1-0.3 μM in all tissues, but under severe oxidative stress this concentration can rise substantially in a localized manner to between 10 μM and 5 mM.¹¹⁻¹³ Consequently, understanding the mechanisms for elimination of 4-HNE is critical, as it may give key insights into the pathogenesis of the molecule.

Despite its physiologic importance, little has been known about the catabolic fate of 4-HNE. Previous reports consisted of a pathway for elimination of 4-HNE via glutathionylation by glutathione *S*-transferase (GST), and subsequent excretion through the kidneys,¹⁴ as well as a report of hepatocyte metabolism through the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH),¹⁵ though little was known about the latter process.

Recently, we reported the catabolic fates of 4-hydroxyacids in perfused rat livers using a combination of metabolomics and mass isotopomer analysis. A key finding of this work is that 4-hydroxyacid catabolism can proceed via two parallel pathways (shown in Figures 1 and supplementary Figure S1) that involve either a phosphorylation and isomerization of the C-4 hydroxyl (denoted Pathway A) or a β -oxidation/ α -oxidation sequence (Pathway B).⁶ This represented the first report on the catabolism of this dogmatic class of biological molecules.

Essential to this finding was the use of isotopically labeled 4-hydroxyacids to define the catabolic pathway for 4-HNE (via 4-hydroxyacids). Here we report the synthesis and evaluation of a series of tools that were critical for this work and demonstrate how these tools can be used to quantify the differential catabolic flux of 4-hydroxyacids down either Pathway A or Pathway B.

The logic behind our labeling strategy can be seen in Figure 1. The ultimate fate of 4-hydroxyacids lies in an initial catabolic step that can proceed via either a phosphorylation and subsequent isomerization to a 3-hydroxyacid (Pathway A) or via an initial round of β -oxidation followed by the loss of formate via α -oxidation (Pathway B).⁶ In order to define the two pathways, we devised a labeling strategy that could incorporate either a single ^{13}C label at position C-3, or a doubly labeled molecule with ^{13}C at positions C-3 and C-4. The label at C-3 was used primarily to confirm that the α -oxidation step in Pathway B leads to [^{13}C]formate. The C-3,4 molecule allowed us to confirm both pathways' existence and as we report here, has allowed us to quantify the relative flux down each pathway.

Our synthetic approach is illustrated in Figure 2 (details can be found in supporting information), and is based on a convergent route that minimizes the number of reactions needed to be optimized. For the C-3 labeled molecule we began with hexyl bromide and introduced the carbon via a Grignard reaction with labeled carbon dioxide. Esterification and reduction with diisobutylaluminum hydride (DIBAL-H) gave the convergent heptanal (circled in Figure 2). Introduction of the labeled carbon for the C-3,4 molecule began with isotopically labeled ethyl bromoacetate, and built up the non-labeled carbon in the opposite direction (as compared to the C-3 labeled molecule) via a Wittig olefination and catalytic reduction. DIBAL-H reduction gave the convergent heptanal. C-1 and C-2 are introduced via an additional Wittig olefination, and the C-4 hydroxyl group is introduced through the use of selenium dioxide under carefully optimized solvent conditions. Typically we would convert this molecule to the saturated lactone by hydrogenation, and a two step hydrolysis/acidic laconization sequence. For stability, we store these molecules as the lactone and hydrolyze just prior to any animal experiments.

Our experimental system for studying this catabolic pathway is via perfusion of live rat liver, which has been reported on previously.¹⁶ In short, rat livers are perfused with 150 ml recirculating buffer containing 4% bovine serum albumin (fat-free), 4 mM glucose and 0 to 2 mM of 4-hydroxyacids. We subsequently monitor key metabolic intermediates via LCMS and GCMS (a table of these intermediates is provided in supplementary information). A key attribute of this labeling pattern is the ability to quantify the relative fluxes down either Pathway A or Pathway B. This could presumably be done with either the C-3 or C-3,4 labeled molecules. For the C-3 labeled molecule, it would be accomplished by quantitatively measuring the ratio of M-1 isotopically enriched formate to that of M1 enriched acetyl-CoA. This would be difficult as formate is only produced in Pathway B, and there are other sources of formate in the liver. We chose, therefore, to utilize the C-3,4 labeled molecule as we could quantify the relative fluxes by comparing M1 acetyl-CoA to M2 acetyl-CoA (rationalized in Figure 1). This approach has the distinct advantage of only monitoring acetyl-CoA. We therefore do not have to worry about variability in how the molecules will behave on LC/GC, or differences in ionization during the mass spectrometry. As shown in Figure 3 (A), the plateau of M2 acetyl-CoA is 15.9% (contribution from pathway A), and the plateau of M1 acetylCoA is 2.9% (contribution from pathway B). The other 81.2% acetyl-CoA is unlabeled (contribution from endogenous sources and the unlabeled carbons of the 4-hydroxyacids). The M2/M1 ratio of acetyl-CoA is therefore 5.5, which means the contribution of pathway A is 5.5 times that of pathway B. To further confirm that the source of M2 acetyl-CoA is from Pathway A and not from a reprocessing of labeled formate we performed an analogous series of perfusions utilizing the C-3 labeled molecule. As expected, we see a plateau of M1 acetylCoA derived from Pathway A, and no M2 acetyl-CoA.

This catabolic pathway is impressively robust. In our experiments we observed that under high concentrations of 4-hydroxyacids the liver can utilize the liberated acetyl-CoA, propionyl-CoA, and formate as its primary carbon source (data not shown). Given the robust nature and the carefully evolved parallelism, we have concluded that this pathway represents a fundamental process in normal physiology. The implications of these observations and the role that this pathway may play in either normal physiology or disease pathogenesis not yet known. However, the disease relevance of 4-HNE (and consequently 4-hydroxyacids) has been clearly delineated in the literature. For example, there is increasing evidence that 4-HNE is partially responsible for the development of alcoholic liver disease.¹⁷⁻²² A highly plausible hypothesis is that loss of the ability to catabolically process lipid peroxidation products precedes their accumulation and their contribution towards disease progression.

Other questions abound. What is the implication of having two parallel pathways? It is possible that under different physiologic stresses, the predominant pathway may switch from Pathway A to Pathway B. Alternatively, different pathways may predominate in different tissues. Regardless, with these tools we now have the ability to answer these questions about this and other fundamental physiologic processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
 Parallel pathways of the catabolism of 4-hydroxy nonanoic acid

Figure 2.
Convergent synthetic routes for 4-Hydroxy-[3-¹³C]nonanoic acid lactone and 4-hydroxy-[3,4-¹³C₂]nonanoic acid lactone.

Figure 3.

The M1 and M2 acetyl-CoA enrichment from 0 to 1 mM of 4-OH-3,4-¹³C₂-nonanoic acid (A) and 4-OH-3-¹³C-nonanoic acid (B) non-recirculating perfusions. Ratio of M1 and M2 acetyl-CoA (from A) demonstrate the flux of the two pathways.