Catabolism of 4-Hydroxyacids and 4-Hydroxynonenal via 4-Hydroxy-4-phosphoacyl-CoAs

Guo-Fang Zhang, Rajan S. Kombu, Takhar Kasumov, Yong Han, Sushabhan Sadhukhan, Jianye Zhang, Lawrence M. Sayre, Dale Ray, K. Michael Gibson, Vernon A. Anderson, Gregory P. Tochtrop, and Henri Brunengraber

From the Departments of Nutrition, Chemistry, and Biochemistry and the Cleveland Center for Structural Biology, Case Western Reserve University, Cleveland, Ohio 44106 and the Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931

4-Hydroxyacids are products of ubiquitously occurring lipid peroxidation (C3, C6) or drugs of abuse (C4, C9). We investigated the catabolism of these compounds using a combination of metabolomics and mass isotomer analysis. Livers were perfused with various concentrations of unlabeled and labeled saturated 4-hydroxyacids (C4 to C11) or 4-hydroxynonenal. All the compounds tested form a new class of acyl-CoA esters, 4-hydroxy-4-phosphoacyl-CoAs, characterized by liquid chromatography-tandem mass spectrometry, accurate mass spectrometry, and 31P-NMR. All 4-hydroxyacids with five or more carbons are metabolized by two new pathways. The first and major pathway, which involves 4-hydroxy-4-phosphoacyl-CoAs, leads in six steps to the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoA. The latter are intermediates of physiological β-oxidation. The second and minor pathway involves a sequence of β-oxidation, α-oxidation, and β-oxidation steps. In mice deficient in succinic semialdehyde dehydrogenase, high plasma concentrations of 4-hydroxybutyrate result in high concentrations of 4-hydroxy-4-phosphoacyl-CoAs, which are degradation products of the carbon skeleton. The four-carbon 4-hydroxybutyrate is a physiological neurotransmitter derived from γ-aminobutyrate. Humans with inborn disorder of succinic semialdehyde dehydrogenase have high 4-hydroxybutyrate concentrations in body fluids, mental retardation, and seizures (12). 4-Hydroxybutyrate is also a drug of abuse that impairs the capacity to exercise judgment for unknown reasons. 4-Hydroxybutyrate is used for the treatment of narcolepsy (13). Its known metabolism (14, 15) proceeds via oxidation to succinic semialdehyde and then to succinate, an intermediate of the citric acid cycle. The five-carbon 4-hydroxypentanoate is also a drug of abuse (16). The calcium salt of a compound closely related to 4-hydroxypentanoate, levulinate (4-ketopentanoate, 4-ketovalerate), is used as an oral or intravenous source of calcium in humans.

We conducted a study on the catabolism of C4 to C11 4-hydroxyacids inperfused rat livers using a combination of metabolomics (17, 18) and mass isotomer analysis (19). Our metabolomic strategy concentrated on the identification of carboxylic acids and acyl-CoA esters derived from 4-hydroxyacids. The precursor-to-product relationship between 4-hydroxyacids and metabolites was demonstrated by conducting experiments with unlabeled substrates and with substrates labeled with multiple 13C or 18O atoms. We demonstrated that the 4-hydroxyacids are degraded by two parallel pathways. The first pathway, which involves 4-hydroxy-4-phosphoacyl-CoAs (4-P-acyl-CoAs), leads to the formation of 3-hydroxyacyl-CoAs, which are physiological β-oxidation intermediates. The second pathway is a sequence of β-oxidation, α-oxidation (20, 21), and β-oxidation steps. Via the two pathways, 4-hydroxyacids with five or more carbons are degraded to acetyl-CoA, propionyl-CoA, and formate.

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This work is dedicated to the memory of Dr. Lawrence M. Sayre.

* This article was selected as a paper of the week.

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† Deceased May 8, 2009.

To whom correspondence should be addressed: Dept of Nutrition, Case Western Reserve University, School of Medicine, WG 48, 10900 Euclid Ave., Cleveland, OH 44106-4954. Tel.: 216-368-6548; Fax: 216-368-6560; E-mail: hxb8@case.edu.

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2 Mass isotopomers are designated as M, M1, M2, ..., Mn, where n is the number of heavy atoms in the molecule. The designation m1, m2, ..., mn refers to the isotopic enrichment of the corresponding isotopomer, expressed as a mole percent.

3 The abbreviations used are: 4-P, 4-phospho-; 4-P-acyl-CoA, 4-hydroxy-4-phosphoacyl-CoA; BHB, β-hydroxybutyrate; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry.
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EXPERIMENTAL PROCEDURES

Materials—Sigma-Aldrich supplied most chemicals and the following isotopically labeled compounds: D2O (99%), 13CO2 gas, 4-hydroxy[13C4]butyrolactone, 4-hydroxy-[13CH3]butyrolactone, [2H3]propionic acid, [13C6]glucose, 15NH4Cl, sodium [13C4]acetate, and sodium [13C]formate. [3,3,4,5,5,5-2H5]Levulinate was prepared by isotopic exchange between unlabeled levulinate, D2O, and Na2B4H7 (22). 4-Hydroxy-[3,4-13C2]nonanoate, 4-hydroxy-[3-13C]hexanoate were prepared as in Ref. 23. 4-Hydroxy- [3,4-13C2]nonanoate, and 4-hydroxy-[3-13C]hexanoate were prepared by methods to be reported elsewhere. The purity of synthesized compounds was verified by gas chromatography-mass spectrometry of trimethylsilyl or tert-butyldimethylsilyl derivatives, using analog unlabeled or labeled compounds as internal standards. For 4-Phosphoacyl-CoAs in 4-Hydroxyacid Metabolism

Liver Perfusions—Livers from male Sprague-Dawley rats were perfused (25) with bicarbonate buffer containing 4 mM glucose and either 4% dialyzed, fatty acid-free, bovine serum albumin (recirculating perfusions) or no albumin (non-recirculating perfusions). After equilibration, 0–2 mM of various unlabeled or labeled compounds as internal standards. For 4-Phosphoacyl-CoAs in 4-Hydroxyacid Metabolism

Analytical Procedures—The concentrations and mass isotopomer distributions of the various acids and 4-hydroxyacids were assayed by gas chromatography-mass spectrometry of trimethylsilyl or tert-butyldimethylsilyl derivatives, using analog unlabeled or labeled compounds as internal standards. For the concentration and labeling pattern of acyl-CoA esters, powdered frozen liver (∼200 mg), spiked with 10 nmol of [2H3]propionyl-CoA internal standard, was extracted for 1 min with 4 ml of methanol/water 1:1 containing 5% acetic acid using a Polytron homogenizer. The supernatant was run on a 3-ml ion exchange cartridge packed with 300 mg of 2-(pyridyl)ethyl silica gel (Sigma). The cartridge had been preactivated with 3 ml of methanol and then with 3 ml of extraction buffer. The acyl-CoAs trapped on the silica gel cartridge were released with (i) 3 ml of a 1:1 mixture of 50 mM ammonium formate, pH 6.3, and methanol (to release the short- and medium-chain acyl-CoAs) and then (ii) 3 ml of a 1:3 mixture of 50 mM ammonium formate, pH 6.3 and (iii) 3 ml of methanol (to release the medium- and long-chain acyl-CoAs (26)). The combined effluent was dried with nitrogen gas and stored at −80 °C until LC-MS analysis.

After dissolving the acyl-CoAs in 100 µl of buffer A (5% acetonitrile in 100 mM ammonium formate, pH 5.0), 40 µl were injected on a Thermo Scientific Hypersil GOLD C18 column (150×2.1 mm), protected by a guard column (Hypersil GOLD C18 5 µm, 10×2.1 mm), in an Agilent 1100 liquid chromatograph. The chromatogram was developed at 0.2 ml/min (i) for 3 min with 98% buffer A and 2% buffer B (95% acetonitrile in 5 mM ammonium formate, pH 6.3), (ii) from 3 to 25 min with a 2–60% gradient of buffer B in buffer A, (iii) from 26 to 31 min with 10% buffer A, 90% buffer B, (iv) from 32 to 41 min with a 90% to 2% gradient buffer B in buffer A, and (v) for 10 min of stabilization with 98% buffer A before the next injection.

The liquid chromatograph was coupled to a 4000 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) operated under positive ionization mode with the following source settings: turbo-ion-spray source at 600 °C under N2 nebulization at 65 p.s.i., N2 heater gas at 55 p.s.i., curtain gas at 30 p.s.i., collision-activated dissociation gas pressure held at high, turbo ion-spray voltage at 5,500 V, declustering potential at 90 V, entrance potential at 10 V, collision energy at 50 V, and collision cell exit potential at 10 V. The Analyst software (version 1.4.2; Applied Biosystems) was used for data registration.

The concentration of 4-hydroxybutyrate in mouse blood (27) and the labeling of the acetyl moiety of citrate (28), a proxy, i.e. an indicator of mitochondrial acetyl-CoA, β-hydroxybutyrate (BHB) (22), free acetate (29), and formate (29), were assayed as described previously. Exact mass analysis of 4-P-butyryl-CoA and 4-P-pentanoyl-CoA was run on a Thermo Finnigan Fourier transform LTQ mass spectrometer. 31P-NMR spectra of 4-P-butyryl-CoA and of malonyl-CoA standard were run on a 600-MHz Varian Inova equipped with a broad band probe. Spectra were acquired for 26 min with a 45° pulse width and acquisition time of 1.5 s with proton decoupling.

Calculations—Correction of measured mass isotopomer distributions for natural enrichment was performed using the CORMAT software (30). The labeling of the C-1+2 acetyl of BHB, a proxy of mitochondrial acetyl-CoA, was calculated using the mass isotopomer distributions of the whole BHB molecule and of the C-3+4 fragment of BHB. When mitochondrial acetyl-CoA was only M1-labeled, as from 4-hydroxy-[3-13C]nonanoate, the m1 enrichment of the C-1+2 acetyl of BHB was calculated as

\[
m1 \text{of } C-1+2 = ((2m2 + m1) \text{of C-1} \rightarrow 4) - (m1 \text{of C-3} + 4) \tag{1}
\]

When mitochondrial acetyl-CoA was M1- and M2-labeled, as from 4-hydroxy-[3,4-13C2]nonanoate, the m1 and m2 enrichments of the C-1+2 acetyl of BHB were calculated assuming that the m2 and m1 labeling ratios of the two acetylcs of BHB are identical (Equation 2)

\[
R = (m2 \text{of C-3} + 4)/(m1 \text{of C-1} + 2) \tag{3}
\]

The total m2 labeling of BHB is expressed as a function of the labeling of its C-3+4 moiety

\[
(m2 \text{of C-1} \rightarrow 4) = (m2 \text{of C-3} + 4)(1 - (m2 \text{of C-3} + 4)/R) + ((m2 \text{of C-3} + 4)/R)(1 - (m2 \text{of C-3} + 4)) + (m1 \text{of C-3} + 4)^2/R \tag{4}
\]

Equation 3 is solved for R. R is introduced in Equation 2 to solve for the m1 and m2 labeling of C-1+2 of BHB.

4. S. Sandukhan, Y. Han, G. F. Zhang, H. Brunengraber, and G. P. Tochtrop, manuscript in preparation.
Data Presentation and Statistics—We present data from 123 liver perfusion experiments. For a number of conditions, we ran six perfusions in the presence of selected unlabeled or $^{13}$C-labeled substrate(s) with the concentration parameters being allowed to vary. The data points shown in Figs. 3, 5, and 7–10 represent means of duplicate gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry injections, which differed by $<2\%$. The statistical differences between some profiles was tested using a paired $t$ test (GraphPad Prism Software, version 3).

RESULTS

Identification of 4-P-acyl-CoAs—In extracts from rat livers perfused with C$_4$ to C$_{11}$ 4-hydroxyacids, LC-MS/MS analysis identified the expected CoA esters of the substrates. These, and all subsequently identified CoA esters, showed the typical transitions (31) from the mass of the parent molecular ion to the product ions at m/z = 428 and 261. These correspond to the nucleoside and pantetheine (minus OH) fragments of CoA (supplemental Fig. 1, ions B and E). In addition, we found unexpected CoA esters that migrate faster than the expected esters on the C18 column. This suggested the presence of additional polar group(s) when compared with usual CoA esters. In perfusions with 4-hydroxybutyrate, the new CoA ester had a parent mass of 934 versus 854 for 4-hydroxybutyryl-CoA. When unlabeled 4-hydroxybutyrate was replaced by the $[^{13}$C$_6$]substrate, the m/z of the new CoA ester increased by 6 (not shown). This demonstrated (i) that the two new CoA esters had all the carbon and hydrogen atoms of the corresponding 4-hydroxyacids and (ii) that their masses were 80 Da greater than the corresponding 4-hydroxyacyl-CoAs. The mass of the new CoA ester of 4-hydroxybutyrate was not affected when (i) unlabeled glucose in the perfusate was replaced by $[^{13}$C$_6$]glucose or (ii) when 10 mM $[^{13}$C$_2$]acetate or 5 mM $^{15}$NH$_4$Cl was added to the perfusate. This suggested that the additional polar group did not contain carbon or nitrogen atoms derived from intermediary metabolism.

When we compared the fragmentation patterns of 4-hydroxybutyryl-CoA and of the corresponding new CoA ester, we hypothesized that the unknown ester was 4-hydroxy-4-phospho-pentanoyl-CoA (4-P-pentanoyl-CoA) because the singly charged ion transitions A → C and D → F (supplemental Fig. 1) were accompanied by the loss of m/z 98, which is equivalent to phosphoric acid. Corresponding transitions were observed for the unknown CoA ester derived from 4-hydroxybutyrate, identified as 4-P-butyryl-CoA. Also, in experiments with 4-hydroxy-$[^{13}$C$_6$]butyrate, 4-hydroxy-$[^{1}$H$_6$]butyrate, and

![4-P-pentanoyl-CoA](image-url)
4-hydroxy-[2H6]pentanoate, the masses of fragments A, C, D, and F increased by the same \( m/z \) as the substrates.

To confirm the identity of 4-P-butyryl-CoA and 4-P-pentanoyl-CoA, we isolated these compounds from 10 rat livers perfused with 10 mM 4-hydroxybutyrate or 4-hydroxypentanoate, using semipreparative high pressure liquid chromatography. The isolated esters were analyzed by accurate mass spectrometry, which yielded very good matches between the theoretical and measured masses (supplemental Table 1). In addition, the presumed 4-P-pentanoyl-CoA was analyzed by \(^{31}\)P-NMR (Fig. 1). Comparison between the \(^{31}\)P-NMR spectra of the presumed 4-P-pentanoyl-CoA and of a standard of malonyl-CoA showed that it has one extra phosphorous atom. This confirmed the identity of 4-P-pentanoyl-CoA.

All the saturated C\(_4\) to C\(_{11}\) 4-hydroxyacids tested formed 4-P-acyl-CoA esters (Fig. 2), which accumulated to very

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**TABLE 1**

| Metabolite                  | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 | M10 | M11 |
|-----------------------------|----|----|----|----|----|----|----|----|----|-----|-----|
| 4-P-Nonanoyl-CoA            | 14.6 | 0.5 | 0.2 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.7 | 9.4 | 55.5 |
| Heptanoyl-CoA               | 6.9  | 0.1 | 0.9 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 3.1 | 13.7 | 75.8 |
| Hexanoyl-CoA                | 26.1 | 0.0 | 1.4 | 0.3 | 1.0 | 1.9 | 5.1 | 3.1 | 6.9 | 17.3 | 19.7 |
| Pentanoyl-CoA               | 72.9 | 0.0 | 0.4 | 0.0 | 0.1 | 0.2 | 2.4 | 8.5 | 15.4 |     |     |
| Propionyl-CoA               | 62.1 | 3.8 | 2.3 | 10.5 | 0.0 | 15.5 | 2.7 |     |     |     |     |
| 4-Hydroxynonanoate          | 8.5  | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.4 | 13.3 | 76.8 |
| 4-Hydroxynonenoate          | 1.3  | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 10.6 | 87.7 |
| 2-Hydroxyheptanoate         | 0.0  | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.8 | 13.9 | 84.2 |
different concentrations in the liver. Also, in livers perfused with unlabeled 4-hydroxynonenal or 4-hydroxy-[\textsuperscript{2}H\textsubscript{11}]nonenal (labeled on the terminal five carbons), we identified the corresponding saturated 4-P-acyl-CoAs (Table 1, to be discussed below).

To test whether phospho-acyl-CoAs are generated from other hydroxyacids, we perfused livers, each with 2 mM 2-hydroxyacid (C\textsubscript{3}), 3-hydroxyacids (C\textsubscript{3} to C\textsubscript{5}), or 5-hydroxyacid (C\textsubscript{5}). None of these compounds formed a detectable phospho-acyl-CoA. To gather data that might lead to a hypothesis on the role of 4-P-acyl-CoAs in the metabolism of 4-hydroxyacids, we conducted (i) non-targeted metabolomics analyses on the various liver perfusates and (ii) detailed assays of acyl-CoA profiles in livers perfused with unlabeled and labeled 4-hydroxyacids.

Experiments with 4-Hydroxynonenal—We perfused one liver with recirculating perfusate to which 2 mM [\textsuperscript{2}H\textsubscript{11}]4-hydroxynonenal (labeled on the last five carbons) was added at zero time. In the liver tissue and perfusate collected after 2 h, we identified (Table 1) eight labeled compounds: 4-P-nonanoyl-CoA, four acyl-CoA esters (C\textsubscript{7}, C\textsubscript{9}, C\textsubscript{10}, C\textsubscript{11} C\textsubscript{9}, C\textsubscript{11}, C\textsubscript{11}, C\textsubscript{3}), 4-hydroxynonanoate, 4-hydroxynonenoate, and 2-hydroxyheptanoate. Table 1 shows the mass isotopomer distributions of these compounds. The same but unlabeled compounds were identified in one liver perfused without 4-hydroxynonenal, we could not detect these compounds. It is likely that the metabolism of 4-hydroxy-[\textsuperscript{2}H\textsubscript{11}]nonenal induced the peroxidation of endogenous lipids, resulting in the formation of unlabeled 4-hydroxynonenal. A similar finding has been reported by Cadenas et al. (32), who observed the stimulation of the production of ethane and \textit{n}-pentane by hepatocytes incubated with 2 mM 4-hydroxynonenal.

Metabolomics of Metabolites of Saturated 4-Hydroxyacids Released by the Perfused Rat Liver—To expand on the data of perfusions with 4-hydroxynonenal, we did a metabolomic study of carboxylic acids released by livers perfused with saturated 4-hydroxyacids of various chain length. We identified a pattern of metabolite production, which is exemplified in Fig. 3, with the products of the metabolism of 4-hydroxyacids with 6, 9, and 11 carbons. With each of these 4-hydroxyacids, we observed the progressive accumulation of (i) a 3-hydroxyacid with the same number of carbons, (ii) a 2-hydroxyacid with two carbons less than the starting substrate, and (iii) acids with two and three carbons less than the starting substrate (not shown on Fig. 3). This suggested the existence of two mechanisms of degradation of 4-hydroxyacids with at least five carbons. These mechanisms are outlined in Fig. 4 using 4-hydroxynonanoate (compound 1) as the starting substrate. The first mechanism (Fig. 4, pathway A) is the isomerization of 4-hydroxyacyl-CoA (compound 3) to 3-hydroxyacyl-CoA (compound 10), the normal intermediate of \textbeta-oxidation, via 4-P-acyl-CoA and other CoA esters to be described below.

This is followed by regular \textbeta-oxidation cycles producing (i) acetyl-CoA and propionyl-CoA (in the case of odd-chain 4-hydroxyacids) or (ii) acetyl-CoA (in the case of even-chain 4-hydroxyacids). The second mechanism (Fig. 4, pathway B), starting at 4-hydroxyacyl-CoA (compound 3), involves one cycle of \textbeta-oxidation, followed by one \alpha-oxidation step, and cycles of \textbeta-oxidation. Pathway B leads to formate (compound 13, via \textalpha-oxidation of 2-hydroxyacyl-CoA and formyl-CoA hydrolysis (21, 33)), and either (i) acetyl-CoA + propionyl-CoA (in the case of even-chain 4-\textit{OH}-fatty acids) or (ii) acetyl-CoA (in the case of odd-chain 4-hydroxyacids with at least five carbons). This double mechanism was confirmed by the identification and mass isotopomer distribution of acyl-CoAs and of carboxylic acids formed during the degradation of 4-hydroxyacids labeled with \textsuperscript{13}C or \textsuperscript{2}H. The evidence is described in the following paragraphs.
Experiments with 4-Hydroxy-[3-13C]Nonanoate and 4-Hydroxy-[3,4-13C2]Nonanoate—We synthesized these labeled compounds because both would lead to the formation of [13C]formate via pathway B (Fig. 4, follow the fate of C-3 of 4-hydroxynonanoate, shown in red). 4-Hydroxy-[3-13C]nonanoate would lead to the formation of M1 acetyl-CoA via pathway B. 4-Hydroxy-[3,4-13C2]nonanoate would lead to the formation of M1 acetyl-CoA via pathway B and M2 acetyl-CoA via pathway A (Fig. 4, follow the fates of C-3 and C-4 of 4-OH-nonanoate, shown in red and green, respectively). In livers perfused with recirculating buffer containing 2 mM 4-hydroxynonanoate that was unlabeled, 3-13C-labeled or 3,4-13C2-labeled, we observed the M0, M1, or M2 compounds listed in supplemental Table 2 and the time-dependent accumulation of [13C]formate in perfusions with labeled substrates (Fig. 5). The mass isotopomer distribution of these compounds is compatible with the scheme presented in Fig. 4. We also observed the formation of the unlabeled and labeled 4-ketoacids corresponding to the 4-hydroxyacids (supplemental Table 2 and Fig. 4, compound 2). Evidence for the reversible interconversion of 4-hydroxyacids and 4-ketoacids, as well as their CoA esters, is presented below.

We also perfused livers with non-recirculating buffer containing increasing concentrations of 4-hydroxy-[3-13C]nonanoate or 4-hydroxy-[3,4-13C2]nonanoate. Careful examination of the LC-
MS/MS spectra of the CoA esters eluting in the vicinity of the 4-phospho-acyl-CoAs identified a number of additional CoA esters of interest. Fig. 6 shows, for a perfusion with 4-hydroxy-[3,4-\textsuperscript{13}C\textsubscript{2}]nonanoate, 10 acyl-CoA esters identified by (i) the \(m/z\) of the molecular ion, (ii) the corresponding ion \(A\) at \(m/z = 428\) (supplemental Fig. 1), and (iii) the retention time. The first nine strips of Fig. 6 correspond each to one multiple reaction monitoring transition; the 10th strip (acetyl-CoA) corresponds to two transitions. The acyl-CoAs listed in Fig. 6 are presented in the order that corresponds to the metabolic scheme we had.
hypothesized (Fig. 4). The labeling of these acyl-CoAs, i.e. M2 of all C9 and C7, M1 of C6, and M1 and M2 of C2, confirms the hypothesis. The acyl-CoA profiles assayed in perfusions with unlabeled or 4-hydroxy-[3-13C]nonanoate (not shown) further confirm the hypothesis.

The six panels of Fig. 7 show the concentrations of unlabeled or labeled acyl-CoA esters assayed in livers perfused with increasing concentrations of 4-hydroxy-[3-13C]nonanoate (left side) or 4-hydroxy-[3,4-13C2]nonanoate (right side). For each labeled substrate, the acyl-CoA metabolites

FIGURE 7. Accumulation of acyl-CoAs in livers perfused with increasing concentrations of 4-hydroxy-[3-13C]nonanoate (A–C) and 4-hydroxy-[3,4-13C2]nonanoate (D–F). Unlabeled, singly labeled, and doubly labeled compounds are designated as M, M1, and M2. The three levels of panels group acyl-CoAs formed (i) before the bifurcation of the pathways outlined in Fig. 4 (A and D), (ii) in pathway A (B and E), and (iii) in pathway B (C and F).
are presented in three groups: (i) CoA esters formed before the bifurcation of 4-hydroxynonanoate metabolism (4-hydroxynonanoyl-CoA and 4-ketononanoyl-CoA, upper level), (ii) CoA esters in pathway A (4-P-nonanoyl-CoA, 3-hydroxy-4-P-nonanoyl-CoA, heptanoyl-CoA, and pentanoyl-CoA, middle level), and (iii) CoA esters in pathway B (dihydroxynonanoyl-CoA, 2-hydroxyheptanoyl-CoA, and hexanoyl-CoA, lower level). The concentration profiles of these unlabeled and labeled CoA esters are also compatible with the scheme presented in Fig. 4. In the perfusates of these livers, we also observed the accumulation of all the free acids corresponding to the acyl-CoAs, except for 4-P-nonanoyl-CoA (not shown).

Fig. 8, A and B, show the labeling of acetyl-CoA and of three proxies of acetyl-CoA (acetyl moiety of citrate (28, 34), C-1/H110012 acetyl of BHB (35), and free acetate (35, 36)) in livers perfused with 4-hydroxy-[3-13C]nonanoate (Fig. 8A) or 4-hydroxy-[3,4-13C2]nonanoate (Fig. 8B). In the presence of 4-hydroxy-[3-13C]nonanoate, which forms M1 acetyl-CoA via pathway A (Fig. 4), the M1 labeling of acetyl-CoA and its three proxies was similar. In the presence of 4-hydroxy-[3,4-13C2]nonanoate, which forms M2 acetyl-CoA via pathway A and M1 acetyl-CoA via pathway B (Fig. 4), the M2 labeling of acetyl-CoA and its three proxies was 5–6 times higher than the M1 labeling.

The mass isotopomer distribution of acetyl-CoA labeled from 4-hydroxy-[3,4-13C2]nonanoate allows us to calculate the contributions of pathways A and B to the production of acetyl-CoA from this substrate. Pathways A and B yield three and four acetyl-CoA, respectively, with one acetyl-CoA being labeled in each pathway. Thus, the contribution of pathway A to acetyl-CoA production is three times the m2 enrichment of acetyl-CoA (Fig. 8C, middle curve). Likewise, the contribution of pathway B to acetyl-CoA is four times the m1 enrichment of acetyl-CoA (Fig. 8C, lower curve). The total contribution of 4-hydroxy-[3,4-13C2]nonanoate to acetyl-CoA plateaued at 55–60% (Fig. 8C, upper curve). Thus, pathway A is the predominant pathway of 4-hydroxy-[3,4-13C2]nonanoate me-
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The presence in the perfusate of [2H4]lactate resulting, presumably, nyl-CoA in the liver (Fig. 9 of 3-hydroxypentanoate (Fig. 9 not be identified in the liver. Note that the main mass isotope-derived from M5 [3,3,5,5,5-2H5]levulinate, shown in supplemental Table 2).

We also conducted one recirculating liver perfusion experiment with 2 mM 4-hydroxy-[3-13C]hexanoate. This compound also led to the accumulation in the perfusate of M1 formate (Fig. 5), 4-ketohexanoate, 3-hydroxyhexanoate, dihydroxyhexanoate (presumably 3,4), and 2-hydroxybutyrate. In the liver, we identified M1 4-P-hexanoyl-CoA, 3-hydroxy-4-P-hexanoyl-CoA, and butyryl-CoA. This spectrum of labeled compounds is compatible with the scheme shown in Fig. 4 if one starts with 4-hydroxyhexanoate as compound 1.

Experiments with 4-Hydroxy-[3,3,4,5,5,5-2H6]Pentanoate or [3,3,5,5,5-2H5]Levulinate—In orientation perfusion experiments, we found that unlabeled 4-hydroxypentanoate is partly converted to the 4-keto analog levulinate (4-ketopentanoate). Likewise, in perfusions with unlabeled levulinate, we observed the formation of 4-hydroxypentanoate. Such keto-hydroxy interconversion should impact on the labeling pattern of 4-hydroxypentanoate deuterated in positions 3, 4, and 5, as well as on the labeling pattern of its metabolites. This is why we conducted liver perfusion experiments with 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate or [3,3,5,5,5-2H5]levulinate. Fig. 9 shows the mass isotopomer distribution of the 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate substrate and of three metabolites (3-hydroxypentanoate, propionyl-CoA, and lactate). Note that 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate was prepared by NaBH₄ reduction of [3,3,5,5,5-2H5]levulinate, which itself was prepared by equilibrating unlabeled levulinate with a solution of NaO₂H in 100% H₂O. Because the isotopic exchange was not complete, the 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate and its metabolites assayed in liver perfusate (3-hydroxypentanoate, lactate) and in liver tissue (propionyl-CoA).  B, scheme of the metabolism of M6 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate in liver. ²H atoms are designated as D. The compounds shown are: 4-hydroxypentanoate M6 (compound 1), 4-hydroxypentanoyl-CoA M6 (compound 2), 3-hydroxypentanoyl-CoA M5 (compound 3), released as the free acid, 3-keto-pentanoyl-CoA M4 (compound 4), propionyl-CoA M4 (compound 5), lactyl-CoA M4 (compound 6), and lactate M4 (compound 7).

![FIGURE 9. Identification of the metabolites of 4-hydroxy pentanoate by mass isotopomer analysis. A, mass isotopomer distribution of synthetic 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate and its metabolites assayed in liver perfusate (3-hydroxy pentanoate, lactate) and in liver tissue (propionyl-CoA). B, scheme of the metabolism of M6 4-hydroxy-[3,3,4,5,5,5-2H6]pentanoate in liver. ²H atoms are designated as D. The compounds shown are: 4-hydroxy pentanoate M6 (compound 1), 4-hydroxy pentanoyl-CoA M6 (compound 2), 3-hydroxy pentanoyl-CoA M5 (compound 3), released as the free acid, 3-keto pentanoyl-CoA M4 (compound 4), propionyl-CoA M4 (compound 5), lactyl-CoA M4 (compound 6), and lactate M4 (compound 7).](image)

4-hydroxy-[3,4-13C]nonanoate can provide a substantial fraction of the energy generated via the citric acid cycle. This fraction is similar to what would be generated by [13C]-oleate.² Note that 4-hydroxynonanoate is rapidly taken up by the perfused rat liver; 0.32 ± 0.04 μmol·min⁻¹·g⁻¹ in three recirculating perfusions with an initial substrate concentration of 2 mM. This rate is similar to the uptake of oleate by the perfused rat liver (37). Note that part of the carbon derived from the metabolism of 4-hydroxy-[3,4-13C]nonanoate is not converted to acetyl-CoA but is released as shorter carboxylic acids into the perfusate (supplemental Table 2).

We conducted orientation perfusion experiments with NaB₂H₄ reduction of [3,3,5,5,5-2H₅]levulinate, which itself was prepared by equilibrating unlabeled levulinate with a solution of NaO₂H in 100% H₂O. Because the isotopic exchange was not complete, the 4-hydroxy-[3,3,4,5,5,5-2H₆]pentanoate and its metabolites assayed in liver perfusate (3-hydroxypentanoate, lactate) and in liver tissue (propionyl-CoA).  B, scheme of the metabolism of M6 4-hydroxy-[3,3,4,5,5,5-2H₆]pentanoate in liver. ²H atoms are designated as D. The compounds shown are: 4-hydroxypentanoate M6 (compound 1), 4-hydroxypentanoyl-CoA M6 (compound 2), 3-hydroxypentanoyl-CoA M5 (compound 3), released as the free acid, 3-keto pentanoyl-CoA M4 (compound 4), propionyl-CoA M4 (compound 5), lactyl-CoA M4 (compound 6), and lactate M4 (compound 7).

Experiments with 4-Hydroxybutyrate—In livers perfused with 4-hydroxybutyrate, we detected 4-P-butryl-CoA, which was present at much lower concentrations than in perfusions with other 4-hydroxyacids (Fig. 2, expanded inset). We did not detect any additional CoA ester, which would be part of pathway A or B (Fig. 4). In perfusions with 4-hydroxy-[13C₄]butyrate, we did not identify 3-hydroxy-[13C₄]butyrate, which would be formed via pathway A. Also, we could not identify M2 glycolate, which would be formed by β-oxidation of the substrate, as was hypothesized by Vamecq et al. (38). Lastly, we

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² F. Bian, and H. Brunengraber, unpublished data.
could not detect label in acetyl-CoA. In contrast, we identified M4 succinate and other multilabeled citric acid cycle intermediates. These are formed by the oxidation of 4-hydroxybutyrate to succinate via succinate semialdehyde, as shown by the Kaufman group (14, 39, 40). This is the only known pathway of 4-hydroxybutyrate catabolism. We will present separately a study of the hepatic metabolism of 4-hydroxybutyrate via the citric acid cycle.

Perfusions of 4-Hydroxyacids in 100% 2H2O Buffer—To gain additional information on the metabolism of 4-hydroxyacids, we perfused two livers for 2 h with recirculating buffer made with 100% 2H2O and containing 2 mM unlabeled 4-hydroxyacid. This is the only known pathway of 4-hydroxybutyrate catabolism. To gain more insight on this question, we assayed the concentrations of all newly identified CoA esters in livers perfused with increasing concentrations of 4-hydroxyacids. Supplemental Fig. 3 shows the concentrations of a number of CoA esters in livers perfused with increasing concentrations of 4-hydroxyacids with 4–9 carbons. For substrates with 5–9 carbons, we observed (i) large accumulations of CoA esters formed in the initial metabolism of the substrates (supplemental Fig. 3), (ii) large decreases in free CoA (Fig. 10A), and (iii) large decreases in acetyl-CoA and malonyl-CoA (Fig. 10, B and C). Thus, the metabolism of high concentrations of 4-hydroxyacids can lead to substantial CoA trapping, which, in some cases, has been linked to metabolic perturbations (41).

Experiments with Mice Deficient in Succinic Semialdehyde Dehydrogenase—Mice deficient in succinic semialdehyde dehydrogenase (SSADH−/−) were developed (42) as a model to study the biochemical perturbations found in humans with 4-hydroxybutyric aciduria (12). We assayed the concentrations of 4-P-butyryl-CoA in quick-frozen livers and brains from control, heterozygote, and homozygote mice. The concentration of 4-P-butyryl-CoA was 9 and 40 times greater in livers and brains of SSADH-deficient mice than in livers and brains of control and heterozygote mice (Fig. 11). The high concentration of 4-hydroxybutyrate in their plasma of SSADH−/− mice is clearly related to the high concentration of 4-hydroxybutyrate in their plasma (935 ± 97 μM, S.E., n = 20; range 264–1683 μM). The concentration of 4-hydroxybutyrate in the plasma of control and heterozygote mice was undetectable.

DISCUSSION

Our findings demonstrate the existence of the previously unknown phospho-acetyl-CoAs, which appear to be derived only from 4-hydroxyacids. These 4-P-acetyl-CoAs are intermediates in the catabolism of 4-hydroxyacids with at least five carbons. The combination of metabolomics and mass isotopomer analysis reveals that the 4-hydroxyacids are metabolized by two pathways. The main pathway (Fig. 4, pathway A) involves the isomerization of 4-hydroxyacyl-CoAs to 3-hydroxyacyl-CoA. We propose that this isomerization proceeds via the scheme shown in Fig. 4. According to this scheme, the phosphorylation
of 4-hydroxyacyl-CoA is followed by dehydrogenation and hydration, forming a 3-hydroxy-4-P-acyl-CoA. The latter would be dephosphorylated to the enol form of 3-ketoacyl-CoA, which undergoes thiolic cleavage to a β-cleaved acyl-CoA. Pathway A (isomerization followed by β-oxidation) is supported by the finding that in perfusions with 4-hydroxy-[3,4-13C2]nonanoate (Fig. 6), the following intermediates are doubly labeled: 4-P-nonanoyl-CoA, 3-hydroxy-4-P-nonanoyl-CoA, 3-hydroxynonanoate, heptanoyl-CoA, heptanoate, the C-1+2 acetyl unit of BHB, and acetyl-CoA.

The accumulation of intermediates in pathway A, especially of 4-P-acyl-CoAs (supplemental Fig. 3), and the extensive 2H-labeling of 4-P-acyl-CoAs in perfusions conducted in 100% 2H2O (Table 2) suggest that (i) one of the distal steps of pathway A (Fig. 4) is limiting and that (ii) the dehydrogenation and hydration steps are reversible. Thus, our data are compatible with the following sequence of pathway A: 4-hydroxyacyl-CoA → 4-P-acyl-CoA ↔ 4-P-2-enoyl-CoA ↔ 3-hydroxy-4-P-acyl-CoA → 3-enol-acyl-CoA ↔ 3-ketoacyl-CoA ↔ 3-hydroxyacyl-CoA. The latter undergoes β-oxidation via 3-ketoacyl-CoA.

Pathway B (β-oxidation followed by α-oxidation and β-oxidation) is supported by the finding that, in perfusions with 4-hydroxy-[3,4-13C2]nonanoate (Fig. 6), (i) the following metabolites are doubly labeled: dihydroxynonanoyl-CoA, dihydroxynonanoate, 2-hydroxyheptanoyl-CoA, and 2-hydroxyheptanoate, whereas (ii) the subsequent metabolites are singly labeled: hexanoyl-CoA, hexanoate, the C-1+2 acetyl unit of BHB, and acetyl-CoA. Through our careful review of the literature, this seems to be the only example of an α-oxidation step...
4-Hydroxynonanoate and related compounds are derived from the lipid peroxidation product 4-hydroxynonenal (1), the concentration of which in microsomal membranes can reach mM levels during bursts of lipid peroxidation (43). Although mM concentrations of 4-hydroxynonenal, as used in our study, can have cytotoxic effects (11), our study concentrated on the degradative pathways of the 4-hydroxynonenal carbon skeleton. 4-Hydroxynonanoate is converted to 4-hydroxyhexanoate, 4-hydroxyoctanoate, and 4-hydroxyhexnone (for reviews, see Refs. 6, 8, and 11). The release of [3H]water after injection of 4-hydroxynonenal-3H-labeled on C-4 or C-2 has been interpreted as demonstrating that part of the substrate undergoes β-oxidation (8). Actually, 3H on C-4 of 4-hydroxyhexanoate must be released to water at the interconversions of (i) 4-hydroxy- and 4-ketohexanoate and (ii) 4-hydroxy- and 4-ketohexanoyl-CoA (Fig. 4). 3H on C-2 of 4-hydroxyhexanoate must be released to water in the first β-oxidation cycle of pathway B and in the reversible dehydrogenation and hydration step of pathway A (4-P-acyl-CoA ↔ 4-P-2-enoyl-CoA ↔ 3-hydroxy-4-P-acyl-CoA). Our data demonstrate the complete oxidation of 4-hydroxyhexanoic acid observed by labeling from 4-hydroxy-[3,4-13C2]nonanoate (Table 1). Also, our experiments with 4-hydroxy-[3,4-13C2]nonanoate (Figs. 4–8) demonstrate the complete oxidation of the carbon skeleton of 4-hydroxyhexanoic acid to acetyl-CoA, propionyl-CoA, and formate.

The trapping of CoA in intermediates of the degradation of 4-hydroxyacids with five or more carbons (supplemental Fig. 3) results in the decrease in the liver concentration of free CoA, acetyl-CoA, and malonyl-CoA (Fig. 10). Trapping of CoA by the metabolism of some drugs has been implicated in the deleterious effects of the drugs (44). Also, in some inborn errors of metabolism, the accumulation of CoA esters has been implicated in the physiopathology of the diseases (41). However, in the case of 4-hydroxyhexanoate, this trapping of CoA does not prevent the substrate from (i) being taken up at a rate similar to that of a long-chain fatty acid like palmitate and (ii) contributing 55–60% of acetyl-CoA produced by the liver. However, in some situations, the trapping of CoA by the metabolism of 4-hydroxyacids could result in metabolic perturbations.

Note that some of the reactions of 4-hydroxyhexanoic acid metabolism probably occur in peroxisomes. This is the site of fatty acid β-oxidation (20), which, in the present case, forms [13C]formate from 4-hydroxyhexanoate labeled on C-3. Given the low labeling of acetyl-CoA and its proxies formed from 4-hydroxy[3,13C2]nonanoate (Fig. 8A), it is likely that pathway B is peroxisomal and that pathway A is mitochondrial.

Our data on the accumulation of 4-P-butyl-CoA in the brain and liver of mice unable to convert 4-hydroxybutyrate to succinate (Fig. 11) suggest that 4-P-butyl-CoA may contribute to the perturbation of brain metabolism in these mice who experience severe epileptic seizures (42). Also, 4-P-butyl-CoA may contribute to the severe mental retardation of patients with 4-hydroxybutyric aciduria (12). Lastly, 4-P-butyl-CoA may be implicated in acute mental dysfunction of subjects who ingested 4-hydroxybutyrate. Similar mental dysfunction is likely to be caused by the accumulation of 4-P-pentanoyl-CoA in subjects ingesting 4-hydroxypentanoic as an alternate drug of abuse to 4-hydroxybutyrate (16).

Unlike 4-hydroxyacids with five or more carbons, 4-hydroxybutyrate is not degraded to acetyl-CoA, as demonstrated by the absence of M2 acetyl-CoA and M2 + M4 BHB in livers perfused with 4-hydroxy-[13C4]butyrate. Other metabolites, not yet identified, may contribute to the brain toxicity of 4-hydroxybutyrate, in addition to 4-P-butyryl-CoA.

The present report illustrates the potential of the association of metabolomics and mass isotopomer analysis for pathway discovery. Our data open the way to extensive studies that will identify and characterize the enzymes that catalyze the reactions involved in the isomerization of 4-hydroxyacyl-CoA to 3-hydroxyacyl-CoAs (Fig. 4, pathway A). Our identification of new acyl-CoAs derived from 4-hydroxyacids was based on mass, mass fragmentation, chromatographic properties, and isotopomer distribution. Confirmation of the identity of these acyl-CoAs will require designing synthetic techniques to prepare them. The difficulty of this endeavor is illustrated by the failure to chemically synthesize 4-hydroxybutyryl-CoA because of the propensity of 4-hydroxyacids to form lactones (45). However, once these difficulties are resolved, future studies will provide a clear picture of the catabolism of 4-hydroxyacids, especially those derived from the lipid peroxidation products 4-hydroxynonenal and 4-hydroxyhexenal. The catabolism of these compounds may modulate their cytotoxic effects.

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Note Added in Proof—It has come to our attention that a reaction similar to the conversion of 4-phospho-3-hydroxyacyl-CoA to 3-enol-acyl-CoA (compounds 7 and 8 in Fig. 4) has been proposed by Hecht et al. (46) and Gräwert et al. (47). Such reductive elimination of an unactivated phosphate ester vicinal to an alcohol would occur in the final steps in the deoxy-D-sulfoxydole pathway to dimethyllypropophosphate and isopenlenylpropophosphate, i.e. the initial steps in the nonmevalonic acid pathway to isoprenoids in some bacteria.

REFERENCES

1. Schneider, C., Porter, N. A., and Brash, A. R. (2008) J. Biol. Chem. 283, 15539–15543
2. Ishikawa, T., Esterbauer, H., and Sies, H. (1986) J. Biol. Chem. 261, 1576–1581
3. Bennnaars-Eiden, A., Higgins, L., Hertzel, A. V., Kapphahn, R. J., Ferrington, D. A., and Bernlohr, D. A. (2002) J. Biol. Chem. 277, 50693–50702
4. Benderdour, M., Charron, G., DeBlois, D., Comte, B., and Des Rosiers, C. (2003) J. Biol. Chem. 278, 45154–45159
5. Siems, W. G., Pimenov, A. M., Esterbauer, H., and Grune, T. (1998) J. Biochem. 123, 534–539
6. Siems, W., and Grune, T. (2003) Mol. Aspects Med. 24, 167–175
7. Schaur, R. J. (2003) Mol. Aspects Med. 24, 149–159
8. Poli, G., Schaur, R. J., Siems, W. G., and Leonarduzzi, G. (2008) Med. Res. Rev. 28, 569–631
9. Luckey, S. W., and Petersen, D. R. (2001) Arch. Biochim. Biophys. 389, 77–83
10. Choudhary, S., Srivastava, S., Xiao, T., Andley, U. P., Srivastava, S. K., and Ansari, N. H. (2003) Invest Ophthalmol. Vis. Sci. 44, 2675–2682
11. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Free Radic. Biol. Med.
11. (1998) Neuroupediatrics 29, 14–22.
12. Gibson, K. M., Hoffmann, G. F., Hodson, A. K., Bottiglieri, T., and Jakobs, C. (1998) Neuropediatrics 29, 14–22.
13. Pardi, D., and Black, J. (2006) CNS Drugs 20, 993–1018.
14. Kaufman, E. E., and Nelson, T. (1991) Neurochem. Res. 16, 965–974.
15. Wong, C. G., Chan, K. F., Gibson, K. M., and Snead, O. C. (2004) Toxicol. Rev. 23, 3–20.
16. Anderson, I. B., Kim, S. Y., Dyer, J. E., Burkhardt, C. B., Iknoian, J. C., Walsh, M. J., and Blanc, P. D. (2006) Ann. Emerg. Med. 47, 177–183.
17. Fiehn, O. (2002) Plant Mol. Biol. 48, 155–171.
18. Goodacre, R. (2007) J. Nutr. 137, 259S–266S.
19. Brunengraber, H., Kelleher, J. K., and Des Rosiers, C. (1997) Annu. Rev. Nutr. 17, 559–596.
20. Foulon, V., Sniekers, M., Huysmans, E., Asselberghs, S., Mahieu, V., Manneaerts, G. P., Van Veldhoven, P. P., and Casteels, M. (2005) J. Biol. Chem. 280, 9802–9812;
21. Verhoeven, N. M., Schor, D. S., Previs, S. F., Brunengraber, H., and Jakobs, C. (1997) Eur. J. Pediatr. 156, Suppl. 1, S83–S87.
22. Des Rosiers, C., Montgomery, J. A., Desrochers, S., Garneau, M., David, F., Mamer, O. A., and Brunengraber, H. (1988) Anal. Biochem. 173, 96–105.
23. Gioacchini, A. M., Calonghi, N., Boga, C., Cappadone, C., Masotti, L., Roda, A., and Traldi, P. (1999) Rapid Commun. Mass Spectrom. 13, 1573–1579.
24. Coleman, T. M., Li, N., and Huang, F. Q. (2005) Tetrahedron Lett. 46, 4307–4310.
25. Minkler, P. E., Kerner, J., Ingalls, S. T., and Hoppel, C. L. (2008) Anal. Biochem. 376, 275–276.
26. Silva, M. F., Aires, C. C., Luis, P. B., Ruiter, J. P., Ijlst, L., Duran, M., Wanders, R. J., and de Almeida, I. T. (2008) J. Inherit. Metab. Dis. 31, 205–216.
27. Scherf, U., and Buckel, W. (1991) Appl. Environ. Microbiol. 57, 2699–2702.
28. Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kie K., Bacher, A., Arigoni, D., and Rohdich, F. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 14837–14842.
29. Graewert, T., Rohdich, F., Span, I., Bacher, A., Eisenreich, W., Epping, J., and Groll, M. (2009) Angew. Chem. Int. Ed. Engl. 48, 5756–5759.
30. Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kie K., Bacher, A., Arigoni, D., and Rohdich, F. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 14837–14842.