Metabolism of propionic acid to a novel acyl-coenzyme A thioester by mammalian cell lines and platelets

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Abstract  Metabolism of propionate involves the activated acyl-thioester propionyl-CoA intermediate. We employed LC-MS/MS, LC-selected reaction monitoring/MS, and LC-high-resolution MS to investigate metabolism of propionate to acyl-CoA intermediates. We discovered that propionyl-CoA can serve as a precursor to the direct formation of a new six-carbon mono-unsaturated acyl-CoA. Time course and dose-response studies in human hepatocellular carcinoma HepG2 cells demonstrated that the six-carbon mono-unsaturated acyl-CoA was propionate-dependent and underwent further metabolism over time. Studies utilizing [13C3]propionate and [2H2]propionate suggested a mechanism of fatty acid synthesis, which maintained all six-carbon atoms from two propionate molecules. Metabolism of 2,2-[2H2]propionate to the new six-carbon mono-unsaturated acyl-CoA resulted in the complete loss of two deuterium atoms, indicating modification at C2 of the propionyl moiety. Coelution experiments and isotopic tracer studies confirmed that the new acyl-CoA was trans-2-methyl-2-pentenoyl-CoA. Acyl-CoA profiles following treatment of HepG2 cells with mono-unsaturated six-carbon fatty acids also supported this conclusion. Similar results were obtained with human platelets, mouse hepatocellular carcinoma Hepa1c1c7 cells, human bronchoalveolar carcinoma H358 cells, and human colon adenocarcinoma LoVo cells. Interestingly, trans-2-methyl-2-pentenoyl-CoA corresponds to a previously described acylcarnitine tentatively described in patients with propionic and methylmalonic acidemia. We have proposed a mechanism for this metabolic route consistent with all of the above findings.—Snyder, N. W., S. S. Basu, A. J. Worth, C. Mesaros, and I. A. Blair. Metabolism of propionic acid to a novel acyl-coenzyme A thioester by mammalian cell lines and platelets. J. Lipid Res. 2015. 56: 142–150.

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CoA thioesters, which are members of the fatty acyl class of lipids (1), are critical for adequate control of cellular bioenergetics (2), as well as the regulation of important cellular functions (3). Furthermore, anabolic and catabolic carbon processing is extensively dependent on a pool of acyl-CoA intermediates, which are available as activated and localized substrates (4). Specifically, synthesis of fatty acids in mammalian cells is initiated through activation of acetyl- and malonyl-CoA, with elongation of the nascent chain via addition of the acetyl moiety from acetyl-CoA (3). This system is in contrast to the ability of certain bacteria, which condense propionate, branched chain amino acids, and odd chain fatty acids is accomplished through propionyl-CoA (6). Thus, a fundamental understanding of the acyl-CoA chemical space is relevant to a wide spectrum of cellular biochemical transformations.

Perturbations of acyl-CoA biosynthesis are associated with diseases that have high morbidity and mortality, including a spectrum of inborn errors of metabolism. For example, deregulated propionyl-CoA metabolism caused by deficiency or aberrant function of propionyl-CoA carboxylase results in metabolic acidosis, coma, mental development delays, and cardiac complications (7). Modulation of levels of other acyl-CoAs can result in cellular responses such as histone acetylation, which modulates transcriptional activation (8). In addition, exogenous exposures such as the pesticide and complex I inhibitor, rotenone, disrupt specific acyl-CoA metabolism and cause Parkinson’s disease-like symptoms (9).

Increasing use of LC-MS/MS, LC-selected reaction monitoring (SRM)/MS, and LC-high-resolution MS (HRMS)
provides orthogonal separations and structural information that are ideal for analysis of complex analytes such as acyl-CoAs that are present in biological matrices (10). Development of new model systems, such as isolated human platelets, may provide a future platform integrating acyl-CoA analysis into quantitative metabolic studies (11). Furthermore, coupling of stable isotopic labels, including [13C1]- and [2H]labeled precursors, to metabolic analysis can provide new insight into cellular metabolism in humans, as well as in animal models (12).

When employing LC-MS/MS to examine the utilization of isotopically labeled precursors including [15C1]propionate and [13C3]propionate for acyl-CoA biosynthesis, we discovered a high abundance unknown chromatographic spectral feature which was inconsistent with existing mammalian metabolic models. The present study was designed to test the hypothesis that the unknown feature resulted from propionate metabolism to a previously undescribed acyl-CoA.

MATERIALS AND METHODS

Chemicals and reagents

5-Sulfosalicylic acid, ammonium formate solution, glacial acetic acid, formic acid, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). [13C1]propionate, [13C3]propionate, and 2,2-[2H2]propionate were purchased from Cambridge Isotopes (Woburn, MA). Optima LC-MS grade methanol, acetonitrile (ACN), 2-propanol, and water were purchased from Fisher Scientific (Pittsburgh, PA). DMEM:F12 medium, FBS, streptomycin, and penicillin were purchased from Invitrogen (Carlsbad, CA). RPMI1640 medium, FBS, streptomycin, and penicillin were purchased from Fisher Scientific (Pittsburgh, PA). Calcium pantothenate was purchased from Isosciences (King of Prussia, PA).

Cell line studies

HepG2 cells were grown and maintained in DMEM:F12 with 2% FBS and 2 mM glutamine with 100,000 units/ml penicillin and 100 mg/ml streptomycin. For all experiments, cells were grown to 80% confluence prior to treatment. For dose response studies, solutions of sodium propionate in maintenance medium were prepared by serial dilution from the most concentrated stock after filtration through a 0.2 μm sterile filter (Corning Inc., Corning, NY). Spent medium was aspirated and fresh medium was added at varying concentrations.

Extraction of acyl-CoAs from cell lines and platelets

The procedures for cell line extraction have been described in detail previously (11, 14–17). Briefly, cells or platelets were gently lifted and transferred to 15 ml conical tubes, centrifuged at 500 g for 5 min and resuspended in 1 ml of ice-cold 10% TCA. They were then pulse-sonicated for 30 s on ice using a sonic dismembrator (Fisher), followed by a 5 min centrifugation at 15,000 g at 4°C. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by solid-phase extraction as follows: Oasis HLB 1 cm3 (30 mg) solid-phase extraction columns (Waters) were conditioned with 1 ml of methanol followed by 1 ml of water. The collected supernatant was applied, washed with 1 ml of water, and finally eluted using three subsequent injections of 0.5 ml of methanol containing 25 mM ammonium acetate. Eluted compounds were dried down under nitrogen and resuspended in 50 μl of 5% 5-sulfosalicylic acid. Injections of 10 μl were made for LC-ESI/MS analysis.

LC-MS/MS and LC-SRM/MS analysis

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Acyl-CoAs were separated using a reversed-phase HPLC Phenomenex Luna C18 column (2.0 × 150 mm, particle size 5 μm) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in 95/5 ACN/water (v/v) as solvent B, and 80/20/0.1 ACN/water/formic acid (v/v/v) as solvent C. Gradient conditions were as follows: 2% B for 1.5 min, increased to 25% over 3.5 min, increased to 100% B in 5 min and held for 8.5 min, washed with 100% C for 5 min before equilibration to 2% B for 5 min. The flow rate was 200 μl/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive ESI mode. Samples (10 μl) were injected using a Leap CTC autosampler (CTC Analytics, Switzerland) where they were maintained at 4°C, and data were analyzed with Analyst 1.4.1 software. The column effluent was diverted to the mass spectrometer from 8 to 23 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), gas 2 (15 units), and collision-induced dissociation gas (5 units). The ESI probe temperature was 450°C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. A constant neutral loss of 507 Da was monitored for each acyl-CoA. LC-SRM/MS analysis was conducted using similar instrument parameters and specific selected ions as noted in the text. LC-HRMS was conducted on a LTQ-Orbitrap XL.
operated in positive ion mode at a resolution of 100,000 as previously described (17), except that an ESI source was used and the mass spectrometer was coupled to the LC system described above.

RESULTS

Metabolism of propionate in HepG2 cells

Treatment of HepG2 cells with 10 mM propionate revealed an upregulation of certain acyl-CoA species after analysis by the constant neutral loss 507 Da scans to survey short chain acyl-CoA content (Fig. 1). The expected increase of propionyl-CoA with a protonated molecule (MH⁺) at m/z 824 was coupled to an unexpected increase in an intense ion at m/z 864 corresponding to MH⁺ of a new C6 mono-unsaturated acyl-CoA (Fig. 1B). Treatment under the same conditions with [13C₁]propionate revealed a similar acyl-CoA profile except that MH⁺ for the new acyl-CoA appeared at m/z 866 (Fig. 1C) corresponding to a C6 acyl-CoA isotopologue containing both [13C]labeled carbon atoms. Furthermore, [13C₃]propionate treatment revealed a highly abundant ion at m/z 870, which would correspond to MH⁺ of a C6 mono-unsaturated acyl-CoA containing all six [13C]atoms (Fig. 1D). Finally, treatment of the cells with trans-2-methyl-2-pentenoic acid resulted in the formation of a C6 acyl-CoA with an MH⁺ at m/z 864 and similar constant neutral loss mass spectrum to that observed with propionate (Fig. 1E).

Treatment of HepG2 cells with other mono-unsaturated six-carbon fatty acids for 1 h resulted in the substrate-specific acyl-CoA profiles as demonstrated by constant neutral loss scans of 507 Da (supplementary Fig. 1). However, the LC-MS chromatograms observed after treatment of cells

Fig. 1. Propionate treatment in HepG2 cells induced distinct acyl-CoA species. MH⁺ observed from constant neutral loss (507 Da) scans from HepG2 cells extracted with no treatment (A), 10 mM propionate (B), 10 mM [13C₁]propionate (C), 10 mM [13C₃]propionate (D), or 10 mM 2-methyl-2-pentenoic acid (E). A new acyl-CoA with MH⁺ at m/z 864 was strongly induced by propionate treatment with a corresponding species at m/z 866.4 (+2 Da) and m/z 870.4 (+6 Da) for the 10 mM [13C₁]propionate and [13C₃]propionate treatments, respectively. The pattern of acyl-CoA formation following 10 mM propionate treatment was identical to that observed from treatment with 10 mM trans-2-methyl-2-pentenoic acid.
with propionate, \[^{13}\text{C}1\text{]}\text{propionate}, \text{or } \[^{13}\text{C}3\text{]}\text{propionate} could only be reproduced by the addition of trans-2-methyl-2-pentenoic acid. Importantly, the ion at \(m/z\) 838 corresponding to MH\(^+\) of butyryl-CoA was only observed after treatment with unbranched hexanoyl fatty acids. This confirmed the differential metabolism of a 2-methyl-substituted pentanoate when compared with an unbranched fatty acid β-oxidation. 

Correspondingly, propionate did not increase the formation of butyryl-CoA, confirming that neither propionyl-CoA nor its metabolites were catabolized via unbranched fatty acid β-oxidation. Treatment of other cell lines with propionate, including mouse hepatocellular carcinoma Hepa1c1c7 cells, human bronchoalveolar carcinoma H358 cells, and human colon adenocarcinoma LoVo cells, also resulted in generation of the new acyl-CoA with MH\(^+\) at \(m/z\) 864 (supplementary Fig. 2).

**Dose-response and time-course experiments with propionate**

Formation of the new acyl-CoA increased in a dose-dependent manner with increasing propionate treatment over the range of 0–1 mM. Its formation reduced with 10 mM and 100 mM propionate suggesting that this new metabolic pathway could be inhibited at high propionate concentrations or that the new acyl-CoA underwent further propionate-dependent metabolism (Fig. 2A). An increase of almost an order of magnitude in concentration of the new acyl-CoA was observed between 1 and 100 μM propionate with almost another order of magnitude increase between 100 μM and 1 mM. Following treatment with 1 mM propionate, the MH\(^+\) at \(m/z\) 864 increased rapidly to a maximum at 1 h, with a stable plateau of levels from 1 h to 12 h, followed by a decrease until 24 h (Fig. 2B).

**Stable isotope labeling by essential nutrients in cell culture**

\[^{13}\text{C}4\text{,}^{15}\text{N}1\text{]}\text{pantothenic acid} was used to label all CoA species in mouse hepatocellular carcinoma Hepa1c1c7 cells, as previously described (14). Coelution experiments conducted after the addition of unlabeled propionate provided further support for the identification of the new metabolite as trans-2-methyl-2-pentenoyl-CoA. LC-SRM/MS analyses of the transition \(m/z\) 864 (MH\(^+\)) to \(m/z\) 357 (MH\(^+\)-507), as well as the corresponding \[^{13}\text{C}3\text{]}^{15}\text{N}1\text{]}\text{acyl-CoA} transition \(m/z\) 868 (MH\(^+\)) to \(m/z\) 361 (MH\(^+\)-507), demonstrated coelution of the propionate-derived acyl-CoA with MH\(^+\) at \(m/z\) 864 with the \[^{13}\text{C}4\text{,}^{15}\text{N}1\text{]}\text{acyl-CoA} obtained from the stable isotope labeling by essential nutrients in cell culture Hepa1c1c7 cells (Fig. 3A). Likewise, the trans-2-methyl-2-pentenoic acid derived analyte with MH\(^+\) at \(m/z\) 864 coeluted with the corresponding \[^{13}\text{C}3\text{,}^{15}\text{N}1\text{]}\text{acyl-CoA} (Fig. 3B).

Finally, LC-SRM/MS analysis of the trans-2-methyl-2-pentenoic acid-derived acyl-CoA (SRM transition \(m/z\) 864 to \(m/z\) 357), the \[^{13}\text{C}3\text{,}^{15}\text{N}1\text{]}\text{acyl-CoA} (SRM transition \(m/z\) 868 to \(m/z\) 361), and the \[^{13}\text{C}4\text{]}\text{propionate-derived }[^{13}\text{C}4\text{]}\text{acyl-CoA} (SRM transition \(m/z\) 870 to \(m/z\) 363) all coeluted (Fig. 3C).

LC-HRMS analysis was employed to confirm that the MH\(^+\) at \(m/z\) 864 arose from a C6 mono-unsaturated acyl-CoA (Table 1). An intense MH\(^+\) was observed at \(m/z\) 864.1776 which corresponded to a molecular formula of \(\text{C}_{27}\text{H}_{45}\text{N}_{7}\text{O}_{17}\text{P}_{3}\text{S}^{+} \) (M0, Δm = -2.77 ppm). The acyl-CoA from \[^{13}\text{C}1\text{]}\text{propionate-treated cells} gave an intense MH\(^+\) at \(m/z\) 866.1846 corresponding to a molecular formula of \(\text{C}_{27}\text{H}_{47}\text{N}_{7}\text{O}_{17}\text{P}_{3}\text{S}^{+} \) (M2, Δm = -2.42 ppm). Importantly, LC-HRMS was able to distinguish MH\(^+\) of the \(^{13}\text{C}\)-isotopologue arising from the natural abundance of \(^{13}\text{C}\) \[^{13}\text{C}4\text{,}^{15}\text{N}1\text{]}\text{acyl-CoA}, which had the same nominal mass but different accurate mass \(\text{C}_{27}\text{H}_{47}\text{N}_{7}\text{O}_{17}\text{P}_{3}\text{S}^{+} \) (\(m/z\) 866.1846) from the MH\(^+\) of hexanoyl-CoA, which had the same nominal mass but different accurate mass \(\text{C}_{25}\text{H}_{47}\text{N}_{7}\text{O}_{17}\text{P}_{3}\text{S}^{+} \) (\(m/z\) 866.1956, Δm 12.70 ppm). Interestingly, the acyl-CoA derived from 2,2-[\(^2\text{H}_{2}\)] propionate revealed intense MH\(^+\) at \(m/z\) 865.1840 (M1, Δm = -2.65 ppm) and \(m/z\) 866.1908 (M2, Δm = -2.07 ppm), indicating the incorporation of either one or two deuterium atoms into the new acyl-CoA.

**MID analyses**

HepG2 cells treated with 1 mM \[^{13}\text{C}4\text{]}\text{propionate} revealed the anticipated M3 enrichment into MH\(^+\) of propionyl-CoA (Fig. 4A). Furthermore, propionyl-CoA extracted from HepG2 cells treated with 1 mM 2,2-[\(^2\text{H}_{2}\)] propionate revealed nearly equal enrichment in M1 and M2 of the MH\(^+\), indicating a portion of deuterium was lost through protium exchange. Similar to the observations in the constant neutral loss experiments, \[^{13}\text{C}4\text{]}\text{propionate} treatment resulted in approximately 80% of the new acyl-CoA to be enriched in the M6 isotopologue.

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**Fig. 2.** Formation of the new acyl-CoA was dose- and time-dependent following propionate treatment in HepG2 cells. A: LC-SRM/MS analysis of the transition \(m/z\) 864 (MH\(^+\)) to \(m/z\) 357 (MH\(^+\)-507) for the new acyl-CoA following treatment with the indicated doses of sodium propionate for 1 h. B: Time course of generation of the new acyl-CoA by LC-SRM/MS analysis following treatment of HepG2 cells with 1 mM sodium propionate.
Human platelet studies

Platelets from healthy volunteers were isolated for isotopic tracer studies using a previously described procedure (11). Studies were conducted using platelets because although they are anuclear, they are rich in mitochondria, and produce much higher levels of mitochondrial metabolites than lymphocytes (11) or neutrophils (data not shown). Thus, platelets have a much higher basal oxygen consumption rate when compared with lymphocytes or neutrophils (19). In contrast to platelets, lymphocytes primarily only utilize oxidative phosphorylation under basal conditions and they have a limited capacity to increase glycolytic flux (19). Neutrophils have little or no dependence on oxidative phosphorylation and glycolysis is not increased when mitochondrial ATP synthase is inhibited (19). Furthermore, platelets are amenable to isolation and purification, and do not require culturing, which could otherwise introduce changes in cellular metabolism. Finally, alterations in platelet mitochondrial function have been demonstrated in a variety of diseases so that they have been proposed to serve as potential markers of systemic mitochondrial dysfunction (11, 20).

The M0 through M3 isotopologue of MH+ from propionyl-CoA, and the M0 through M6 isotopologue of MH+ from the new acyl-CoA were monitored by LC-SRM/MS. After adjustment for natural isotopic abundance, treatment with unlabeled propionate gave essentially 100% M0 isotopologue at MH+ for both propionyl-CoA (Fig. 5A) and the new acyl-CoA (Fig. 5B). [13C1]propionate treatment resulted in a predominant M1 isotopologue at MH+ for propionyl-CoA (Fig. 5A). However there was a predominant M2 isotopologue at MH+ for the new acyl-CoA (Fig. 5B). Similarly, [13C3]propionate resulted in a predominant M3 isotopologue at MH+ for propionyl-CoA (Fig. 5A), whereas there was a predominant M6 isotopologue at MH+ for the new acyl-CoA (Fig. 5B). In contrast, 2,2-[2H2]propionate treatment resulted in a similar mixture of M0, M1 and M2 isotopes in MH+ for both propionyl-CoA (Fig. 5A) and the new acyl-CoA (Fig. 5B).

Characterization of the new acyl-CoA as 2-methyl-2-pentenoyl-CoA and mechanism of formation

The LC-MS/MS properties of the new six carbon mono-unsaturated acyl-CoA were identical with the acyl-CoA that was formed by the addition of trans-2-methyl-2-pentenoic acid to human cell lines and platelets. This finding together with the isotope labeling data provided compelling evidence for the structural assignment of the new acyl-CoA as trans-2-methyl-2-pentenoyl-CoA (Fig. 6). Its mechanism of formation must account for the results of three isotope-labeling experiments: 1) conservation of the [13C4]atom from each precursor propionate; 2) conservation of all [13C1]atoms from precursor propionate; and 3) exchangeable deuterium labeling with a maximum of two total conserved [2H]atoms from precursor propionate (Fig. 6). The proposed mechanism involves initial formation of propionyl-CoA through the action of a short chain CoA synthetase (Fig. 6). The limited protium/deuterium exchange observed in the formation of propionyl-CoA from 2-methyl-2-pentenoic acid-derived acyl-CoA with MH+ at trans-2-methyl-2-pentenoic acid.

Fig. 3. Coelution experiments confirmed that the new acyl-CoA is 2-methyl-2-pentenoyl-CoA. Hepa1c1c7 cells, grown with or without [13C3,15N1]pantothenate, were treated with propionate to generate [13C8,15N1]propionyl-CoA, which was then converted to the new [13C3,15N1]acyl-CoA with propionate. LC-SRM/MS analysis was performed and the chromatogram compared with those obtained for the new-CoAs derived from propionate, [13C5]propionate, or trans-2-methyl-2-pentenoic acid. LC-SRM/MS transitions for m/z 864–357, m/z 868–361, m/z 870–363 were monitored. Coelution was observed with: the propionate-derived new acyl-CoA with MH+ at m/z 864 and the [13C5]labeled analyte (A), the trans-2-methyl-2-pentenoic acid-derived acyl-CoA with MH+ at m/z 864 and the [13C5]labeled analyte (B), and the 2-methyl-2-pentenoic acid-derived acyl-CoA with MH+ at m/z 864 with the [13C5]labeled new acyl-CoA from propionate and the [13C5] propionate-derived new acyl-CoA (C). A peak corresponding to succinyl-CoA in the m/z 868–361 channel was completely resolved at retention time 9.6 min.

(Fig. 4B); indicating direct incorporation of six carbon atoms from two molecules of propionyl-CoA without any carbon loss. Deuterium labeling in the new acyl-CoA was almost identical to the labeling in propionyl-CoA, with nearly equal enrichment of the M1 and M2 of MH+ and virtually no labeling in M3 or M4 (Fig. 4C). M3 labeling, which was present in succinyl-CoA only for the [13C3] propionate treatment, arose from anaplerosis into the Krebs cycle (Fig. 4D).
A novel acyl-CoA from human cell lines and platelets was converted to the final trans-2-methyl-2-pentenoyl-CoA molecule by a transacylase (Fig. 6). All deuterium atoms that were originally on C2 from the first propionate molecule were lost during the dehydration step (Fig. 6). The remaining deuterium atoms at C2 from the second propionate molecule were partially exchanged for protium (Fig. 5B) in a similar manner to that observed in propionyl-CoA (Fig. 5A).

### DISCUSSION

Diseases of propionic acid metabolism, most notably, propionic and methylmalonic acidemias, result in a widely dysregulated metabolome (21–23). This follows from the

| Predicted Formula (MH⁺) | Found Mass | Predicted Mass | Delta (ppm) |
|------------------------|------------|----------------|-------------|
| M0                     | C₂₇H₄₅N₇O₁₇P₃S⁺ | 864.1776 | 864.1800 | -2.77 |
| [1³C₁]propionate       | C₂₇H₄₅N₇O₁₇P₃S⁺ | 866.1846 | 866.1867 | -2.42 |
| M2                     | C₂₇H₄₅N₇O₁₇P₃S⁺ | 864.1784 | 864.1800 | -1.85 |
| 2,2-[²H₂]propionate   | C₂₇H₄₅N₇O₁₇P₃S⁺ | 865.184 | 865.1863 | -2.65 |
| M1                     | C₂₇H₄₅N₇O₁₇P₃S⁺ | 866.1908 | 866.1926 | -2.07 |
| M2                     | C₂₇H₄₅N₇O₁₇P₃S⁺ | 865.184 | 865.1863 | -2.65 |

Fig. 4. MID analysis revealed the stoichiometry and positional specificity of incorporation of propionate into 2-methyl-2-pentenoyl-CoA. Labeling percent of HepG2 derived acetyl-, propionyl-, succinyl-, and the new acyl-CoA. Treatment with [¹³C₃]propionate resulted in enrichment of M₃ propionyl-CoA (A) and M₆ in the new acyl-CoA (B), with virtually no detectable enrichment in acetyl-CoA (C) and minimal M₃ labeling in succinyl-CoA (D). 2,2-[²H₂]propionate treatment yielded nearly equal M₁ and M₂ labeling in propionyl-CoA (A), as well as the new acyl-CoA (B), with very little enrichment in acetyl-CoA (C) and succinyl-CoA (D). Taken together these findings suggest a direct condensation of 2 propionyl-CoA molecules to the new acyl-CoA independently of anaplerosis into the Krebs cycle.
acids, and C5-ketone bodies are precursors in the formation of threonine, methionine, cholesterol, odd-chain fatty acids, isoleucine and valine, via propionyl-CoA. Propionyl-CoA can also be formed directly from activation of amino acids, an intermediate, such as catabolism of the branched chain metabolite used in this study range from below to above physiological relevance. However, previous untargeted metabolomics surveys have most likely observed trans-2-methyl-2-pentenoylcarnitine but lacked sufficient depth of experimental information to characterize the exact structure or propose a mechanism of formation (23). Clearly, further research is needed to examine the biochemistry of the newly identified trans-2-methyl-2-pentenoic acid in propionic acidemia patients in crisis (29). Although we observed no immediate cellular toxicity by light microscopy within the time frame of treatment at 10 mM and 100 mM, we cannot discount the seemingly likely conclusion that the decrease in trans-2-methyl-2-pentenoic acid formation at these doses was due to cell death. The lack of detection of the intermediate at basal conditions may relate to the fact that there is no sodium propionate added to DMEM base media. Thus, the doses of propionate used in this study range from below to above physiological relevance.

Understanding of propionate metabolism has been driven not only by human disease but also by the critical nature of propionate for branched chain hydrocarbons and hormones in insects (6, 30). In fact, 2-methyl-2-pentenoic acid is an aggregation pheromone of the grain borer, and is produced by the grain borer when feeding (31). As sources of propionate are relatively energetically expensive to a developing insect, branched chain products are likely to be biologically important (32). Such branched chain products include 2-methyl branched alkanes, critical components of the cuticular lipids that compose significant portions of the outer shells of insects.

Absolute quantification of intracellular metabolites serves an indispensable role in identifying and characterizing new metabolic pathways. For this purpose, LC-SRM/MS is the gold standard for quantification because it affords high sensitivity and specificity from complex biological matrices, especially when coupled to the isotopically labeled analogs of target analytes as internal standards to adjust for variation in extraction and analysis (33).
However, it is also useful in combination with stable isotope labeling for examining alterations that can occur to cellular metabolite pools (9, 13). Monitoring the uptake and conversion of isotope-labeled nutrients into downstream metabolic pathways can shed light on unknown components of metabolism (12). The utility of stable isotopes for metabolic elucidation is demonstrated by the findings in this report, where \(^{13}\text{C}_1\)-, \(^{13}\text{C}_3\)-, and \(^2\text{H}_2\)-labeled propionate provided complementary metabolic information in conjunction with LC-SRM/MS and LC-HRMS. For quantitative studies, stable isotope analog internal standards utilizing carbon or nitrogen labels are more desirable than deuterium, which causes small LC retention time shifts and can potentially undergo protium/deuterium exchange. However, deuterated analogs may provide insight into unexpected metabolic pathways, as demonstrated in this study and in the recent elucidation of folate-dependent generation of NADPH (34). Constantly improving capabilities of LC-SRM/MS and LC-HRMS may warrant reexamination of previously studied metabolic pathways, and may make identification of previously unrecognized pathways possible.

In summary, we have used absolute quantification in combination with MID analysis to show both the propionate-dependent metabolism and labeling from upstream metabolic sources of \(^\text{trans}\)-2-methyl-2-pentenoyl-CoA in multiple biological systems. Future work could elucidate the enzymology of this pathway completely, as well as assess the contribution of \(^\text{trans}\)-2-methyl-2-pentenoic acid to metabolic crisis in propionic academia patients.

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