Functional loss of ketogenesis in odontocete cetaceans

Michael J. Wolfgang[1,2,4*] Joseph Choi[1], Susanna Scafidi[3]

[1]Department of Biological Chemistry, [2] Pharmacology and Molecular Sciences, [3]Anesthesiology and Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[4]Lead Contact

*Address correspondence to**: Michael J. Wolfgang, Ph.D,** Department of Biological Chemistry, Johns Hopkins University School of Medicine, 855 N. Wolfe St., 475 Rangos Building, Baltimore, MD 21205, Tel: 443-287-7680, Email: mwolfga1@jhmi.edu

Highlights

- Odontocete cetaceans (e.g. dolphins) have a loss in *Hmgcs2* and β-hydroxybutyrate.
- Liver acylcarnitines suggest a switch from fatty acid to protein catabolism.
- Acetoacetate catabolism is retained for protein catabolism.

ABSTRACT

Odontocete cetaceans exhibit genomic mutations in key ketogenesis genes. In order to validate an inferred lack of ketogenesis made by observations from genome sequencing, we biochemically analyzed tissues from several odontocete cetacean species and demonstrate that they indeed do not exhibit appreciable hepatic β-hydroxybutyrate (βHB) or its carnitine ester. Furthermore, liver tissue exhibited significantly lower long chain acylcarnitines and increased odd chain acylcarnitines indicative of a decreased reliance on hepatic long chain fatty acid oxidation in these carnivorous mammals. Finally, we performed Single Molecule, Real-Time next generation sequencing of liver and brain RNA of *T. truncatus* and demonstrate that the succinyl-CoA transferase required for acetoacetate catabolism is expressed in the nervous system. These data show that odontocete cetaceans have lost the ability to perform ketogenesis

and suggest a hepatocentric Coenzyme A recycling function rather than a predominantly systemic-bioenergetic role for ketogenesis in other ketogenic competent mammals like humans.

INTRODUCTION

The consumption of a ketogenic, carbohydrate-limited diet requires adaptations to processes such as gluconeogenesis and ketogenesis to maintain sufficient fuel for the nervous system, especially for large brained mammals like humans. The ketone bodies (acetone, acetoacetate and β-hydroxybutyrate (βHB)) are produced in the liver from acetyl-CoA derived primarily from fatty acid β-oxidation (Lee et al., 2016) and can change dramatically between the fed and fasted state (Cahill, 2006). Ketogenesis is thought to perform several important functions (Puchalska and Crawford, 2017). Ketone bodies can be used by most cells outside of the liver including the brain as an alternative oxidative substrate. This not only provides the brain with an alternative fuel but also staves off muscle wasting by reducing the need for amino acid carbon skeletons for gluconeogenesis. Additionally, the generation of ketone bodies in the liver is necessary for the liberation of Coenzyme A from acetyl-CoA for the continued oxidation of fatty acids (Arima et al., 2021; Cotter et al., 2014). Without this, fatty acid oxidation would cease and would no longer be able to enable gluconeogenesis (**Fig. 1A**). Finally, ketone bodies, particularly βHB, have been suggested to exhibit signaling properties well beyond their role in organismal metabolism (Newman and Verdin, 2017).

These putative important roles of ketone bodies have been challenged by the identification of multiple distant mammalian lineages that exhibit a genomic loss of Hydroxymethyl-CoA Synthase 2, *hmgcs2*, an enzyme required for ketogenesis. This gene has been lost or made nonfunctional in the genomes of all odontocete cetaceans sequenced to date (dolphins and whales), elephants and Old World fruit bats (Jebb and Hiller, 2018). While it has been shown that fasting in fruit bats can be lethal, dolphins and elephants can fast for relatively long periods of time suggesting an adaptive mechanism to maintain vital macronutrients. In fact, dolphins maintain a relatively high blood glucose concentration following a fast (Houser et al., 2021; Venn-Watson et al., 2011; Venn-Watson and Ridgway, 2007). How then can carnivorous dolphins consume a natural ketogenic diet yet maintain high circulating glucose concentrations during fasting in the absence of the generation of ketone bodies?

The loss of key ketogenesis genes in odontocete cetaceans suggests two mutually exclusive hypotheses. Either they do not perform ketogenesis or they have evolved an alternative ketogenic pathway utilizing for example Hmgcs1. In order to validate the inferred lack of ketogenesis made by observations from cetacean genome sequencing, we biochemically analyzed tissues from several odontocete cetacean species for hallmarks of ketogenesis. We found that in agreement with the genomic sequencing, odontocete cetaceans do not produce βHB and exhibit a liver acylcarnitine profile suggestive of a suppression in fatty acid catabolism and reliance on protein catabolism to fuel liver function. Finally, we utilized Single Molecule, Real-Time (SMRT) next generation sequencing of liver and brain RNA of *T. truncatus* to gain insight into their unique metabolic adaptations. These comparative organismal data suggest an hepatocentric rather than systemic-bioenergetic role for ketogenesis in ketogenic competent mammals.

MATERIELS AND METHODS

Animals

Mice were housed in ventilated racks with a 14-hr/10-hr light/dark cycle and fed a standard chow diet (2018SX, Teklad Global) in a controlled environment with a room temperature of 21C, and 50% humidity. Livers were harvested from 9-week old male C57BL/6J mice that were food deprived for 4 hours (11AM to 3PM). The mice were first anesthetized with isoflurane until the toe pinch reflex was absent, then decapitated and livers collected and frozen in liquid nitrogen (LN_2). All procedures were performed in accordance with the NIH's Guide for the Care and Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee. Tissue samples from odontocete cetaceans were provided by Craig Harms (North Carolina State University) with permission from the National Marine Fisheries Services (**Table S1**).

Metabolite measurements

Tissue βHB was measured by liquid chromatography-mass spectroscopy (Selen and Wolfgang, 2021). Liver samples were homogenized in an 80% methanol-water mixture, vortexed for 30 seconds and centrifuged at 13,000g for 10 mins at 4 degrees Celsius. The supernatant was then transferred to a new tube and placed into a speed-vac over-night until dried. The pellet was resuspended in 0.5M sodium hydroxide and used to quantify the protein concentration for data normalization by BCA assay (Thermo Scientific). Dried samples were reconstituted in 200ul water just before LCMSMS analysis. A kinetex C18 column (2.6um, 50mm, 2.1mm, Phenomenex, Torrance, CA) was used for liquid chromatography. Mobile phases were A: water+0.2% formic acid and B: ACN+0.2% formic acid. The data was collected via a Shimadzu Nexera UHPLC (Shimadzu, Columbia, MD), coupled to a 4500 triple quadruple mass spectrometer (AbSciex, Redwood, CA). The total run time was 5 minutes, with a flow rate of 0.2ml/min. The gradient was applied as follows; 0%B at 0 min, 5%B at 0-4 minutes, 0%B at 4-5 minutes with an injection volume of 2ul. The retention time was observed at 1.64, with βHB at 102.9/58.8 (m/z). MultiQuant software (AbSciex, Redwood, CA) was used to quantify the peaks against a 6-point-standard curve. Acylcarnitines were measured by standard methodology (Lee et al., 2016). 60 µl of 3N HCl in n-butanol was added to extracted samples, incubated for 15 min at 65°C and then dried under LN_2. Butylated acylcarnitines were reconstituted in 100 µl of mobile phase acetonitrile/water/formic acid (H_2O:CH_3CN:HCOOH, 80:19.9:0.1 v/v%). Samples were vortexed, transferred to a centrifuge filter, spun, and transferred to an injection vial. Acylcarnitines were analyzed on an API 3200 (AB Sciex) operated in positive ion mode employing a precursor ion scan for m/z 85, which was generated as a characteristic product ion of a butyl ester of acylcarnitine species. Quantitation of acylcarnitines was achieved by the Chemoview (AB Sciex) application. Metabolite concentrations were normalized to protein content (**Table S2**).

RNA sequencing

RNA was isolated from brain and liver of two *T. truncatus* samples using the RNeasy Mini Kit (Qiagen). Single Molecule, Real-Time next generation sequencing was used to generate and analyze full-length cDNA sequences. The processing and sequencing of RNA as well as bioinformatic analysis was performed by Novogene Corporation Inc. (Sacramento, CA) (**Table S3**).

RESULTS AND DISCUSSION

In order to determine the presence or absence of ketogenesis in odontocete cetaceans, we obtained samples of liver from multiple species (*Tursiops truncatus*, *Delphinus delphis*, *and Grampus griseus)* collected following stranding and measured the concentration of βHB by LC/MS/MS in comparison to mouse liver serving as a positive control. We would expect the stress associated with stranding to further potentiate the release of ketogenic precursors even in animals consuming a ketogenic diet. While mouse liver contains abundant βHB, as expected, we could not find evidence of βHB from any odontocete cetacean liver (**Fig. 1B**, **Table S2**). The direct comparison of livers from stranded dolphins collected in the field and mice under well controlled laboratory conditions certainly contains caveats. Regardless, we were unable to observe any βHB in any of the dolphin livers. Additionally, a recent study utilizing untargeted metabolomics did not observe βHB in the serum of fasted dolphins (Houser et al., 2021). These data show that, consistent with interpretations inferred from genomic sequencing, odontocete cetaceans are unable to generate βHB.

Dolphins consume a protein rich ketogenic diet but do not produce ketone bodies. How then can they sustain high rates of hepatic fatty acid β-oxidation without recycling CoA via ketogenesis? To gain insight into this, we profiled liver acylcarnitines as a measure of protein and lipid intermediary metabolism. Consistent with a loss of βHB, odontocete cetacean livers did not contain appreciable βHB-carnitine esters, further demonstrating a lack of functional ketogenesis (**Fig. 1B**). Interestingly, odontocete cetacean livers contained surprisingly low concentrations of long even chain (C16 or greater) acyl-carnitine species such as palmitoylcarnitine and oleoylcarnitine in comparison to mouse liver, again with similar caveats described above (**Fig. 1C**). However, odontocete cetacean liver exhibited markers of protein catabolism such as isovalerylcarnitine and methylmalonylcarnitine (**Fig. 1D**). These data suggest that odontocete cetaceans do not rely heavily on hepatic fatty acid β-oxidation like other mammals and therefore have obviated their requirement for the hepatic CoA recycling facilitated by ketogenesis.

To gain a better understanding of the link between genomic structure and systemic physiology, we sequenced RNA from two brains and two livers from *T. truncatus* via long read Single Molecule, Real-Time Sequencing (SMRT technology). We were unable to recover mRNA sequence of an *Hmgcs2* gene in *T. truncatus*. However, *T. truncatus* has retained the enzymes responsible for ketolysis and we recovered the succinyl-CoA:3-oxoacid-CoA transferase Oxct1 from *T. truncatus* brain. Oxct1 is required for ketone body utilization. In order to utilize βHB, it first must be oxidized to acetoacetate. In order to oxidize acetoacetate it must be esterified onto CoA. Oxct1 functions to transfer CoA from succinyl-CoA to acetoacetate to generate acetoacetyl-CoA. The mouse knockout of Oxct1 results in perinatal lethality, but this

lethality is mediated by ketoacidosis via an inability to clear acetoacetate and βHB rather than the loss of brain bioenergetics (Cotter et al., 2011; Cotter et al., 2013). Excess ketogenesis brought about by loss of insulin or the loss of Oxct1 is acutely life threatening given the change in blood pH brought on by high concentrations of these weak acids. Why then would an animal evolve to eliminate genes of ketone body synthesis while retaining genes of ketone body oxidation? Ketogenesis is not the only metabolic pathway that generates acetoacetate. We would suggest the retention of Oxct1 expression in the ketogenic incompetent *T. truncatus* is related to their requirement for acetoacetate catabolism from the breakdown of amino acids such as phenylalanine and tyrosine rather than ketone body catabolism *per se*.

The wealth of comparative genomic sequencing data has provided important insights into the evolution of unique adaptations across the animal kingdom (Huelsmann et al., 2019; Lopes-Marques et al., 2019). Dolphins have lost several important genes in lipid metabolism including *Acsm3*, *Dgat2l6*, *Fabp12*, *Pla2g2a*, and *Thrsp* largely associated with unique adaptations in skin. The loss of key ketogenesis genes such as *Hmgcs2* and *Bdh1* in odontocete cetaceans suggests divergent hepatic function as well. Dolphins clearly perform gluconeogenesis as they can maintain or increase glucose levels following starvation (Houser et al., 2021; Venn-Watson et al., 2011; Venn-Watson and Ridgway, 2007). Dolphins may utilize gluconeogenic substrates that do not require the coupling of high rates of fatty acid oxidation to subsequent pyruvate carboxylation or they have evolved another mechanism to recycle CoA such as a mitochondrial thioesterase or mitochondrial uncoupling.

Do ketone bodies play an essential role as an alternative oxidative substrate in nonhepatic tissues during prolonged fasting? One might argue there is little experimental evidence supporting this notion. The clinical presentation of Hmgcs2 mutations in humans and Hmgcs2 knockout mice support a predominant role for ketogenesis in support of hepatic fatty acid oxidation rather than systemic bioenergetics (Arima et al., 2021; Thompson et al., 1997). Both humans and mice with Hmgcs2 deficiency resemble inborn errors in long chain fatty acid oxidation, i.e. fasting-induced hypoketotic hypoglycemia, but not an obvious neurological energy crisis. Perhaps the predominant role for ketogenesis is to recycle Coenzyme A in the liver to enable high rates of fatty acid oxidation in hepatocytes with a limited capacity for TCA cycle oxidation of acetyl-CoA. Ketogenesis and ketolysis must be coordinated during fasting in humans to prevent ketoacidosis, but further experimentation is required to elucidate the bioenergetic role/requirement of ketone bodies in ketogenic competent mammals.

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Conflict of Interest

The authors have no competing financial interests.

Author Contributions

Conceptualization, M.J.W.; Methodology, S.S.; Investigation, J.C. and S.S.; Formal Analysis, all authors; Visualization, M.J.W.; Writing—Original draft, M.J.W; Writing—Reviewing and editing, all authors; Funding acquisition, M.J.W.

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Figure

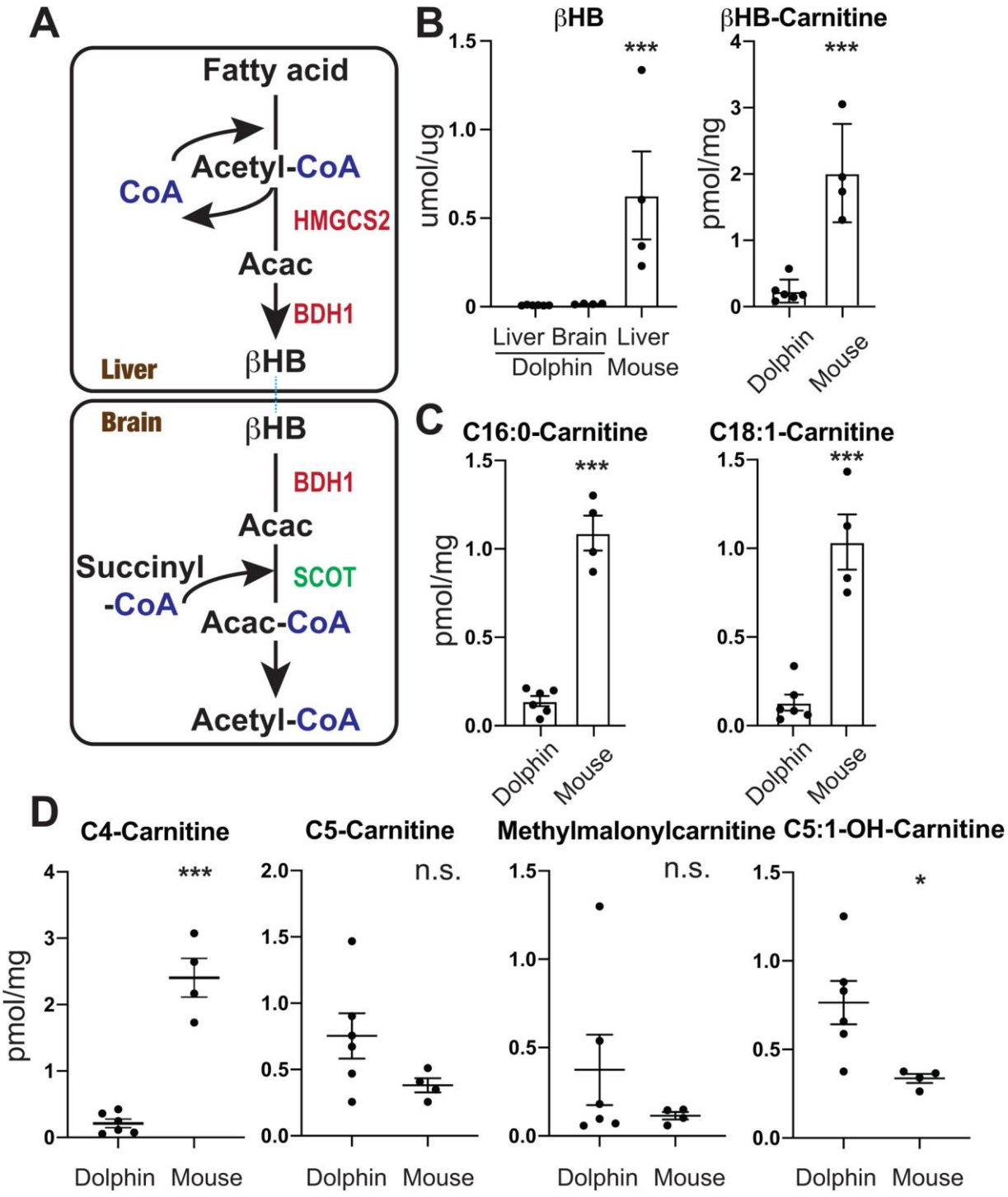

Figure 1. *T. truncatus* does not generate βHB and suppresses hepatic fatty acid oxidation. **a**) Schematic representation of ketone body metabolism with genes mutated in red and present in green in odontocete cetaceans. **b**) Liver βHB and βHB-carnitine, **c**) long chain acylcarnitines and **d**) additional acylcarnitines in livers. *T. truncatus* (n=6) and mice (n=4). * $p<0.05$, *** $p<0.01$ βHB, βhydroxybutyrate, CoA, Coenzyme A, Acac, acetoacetate.

Table S1.

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Table S2.

Click here to download Table S2

Table S3.

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