Obligate role for ketone body oxidation in neonatal metabolic homeostasis

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Running title: Neonatal lethality of SCOT-deficient mice
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To compensate for the energetic deficit elicited by reduced carbohydrate intake, mammals convert energy stored in ketone bodies to high-energy phosphates. Ketone bodies provide fuel particularly to brain, heart, and skeletal muscle in states that include starvation, adherence to low-carbohydrate diets, and the neonatal period. Here, we use novel Oxct1-/− mice, which lack the ketolytic enzyme succinyl-CoA 3-oxo-transferase (SCOT), to demonstrate that ketone body oxidation is required for postnatal survival in mice. While Oxct1-/− mice exhibit normal prenatal development, all develop ketoacidosis, hypoglycemia, and reduced plasma lactate concentrations within the first 48h of birth. In vivo oxidation of 13C-labeled β-hydroxybutyrate in neonatal Oxct1-/− mice, measured using NMR, reveals intact oxidation to acetoacetate, but no contribution of ketone bodies to the tricarboxylic acid cycle. Accumulation of acetoacetate yields a markedly reduced β-hydroxybutyrate:acetoacetate ratio of 1:3, compared to 3:1 in Oxct1+ littermates. Frequent exogenous glucose administration to actively suckling Oxct1-/− mice delayed, but could not prevent lethality. Brains of newborn SCOT-deficient mice demonstrate evidence of adaptive energy acquisition, with increased phosphorylation of AMPKα, increased autophagy, and 2.4-fold increased in vivo oxidative metabolism of 13C-glucose. Furthermore, 13C-lactate oxidation is increased 1.7-fold in skeletal muscle of Oxct1-/− mice, but not in brain. These results indicate the critical metabolic roles of ketone bodies in neonatal metabolism, and suggest that distinct tissues exhibit specific metabolic responses to loss of ketone body oxidation.

INTRODUCTION

Transition from the intrauterine to the extrauterine environment incurs a marked shift in nutrient delivery and energy metabolism. A continuous pipeline replete with glucose and lactate, but calorically reduced in lipid, is replaced by a reduced-carbohydrate, high-fat milk diet that is cyclically interrupted by periods of nutrient deprivation (1-3). High energy-requiring organs like heart and skeletal muscle are poised to meet the energetic demands of this new nutrient environment because they are endowed with enzymatic machinery that avidly generates high-energy phosphates from oxidative metabolism of fatty acids and lactate (4). Unlike cardiomyocytes and skeletal myocytes, most neurons oxidize fatty acids poorly, and therefore remain dependent on hepatic gluconeogenesis to support energetic needs (5-8). However, because newborn brain comprises 10% of body weight, and requires up to 60% of total body energy expenditure, maintenance of energetic homeostasis in the nervous system requires allocation of multiple fuels for metabolic homeostasis in the neonatal period. The rate of ketone body extraction by human neonatal brain is up to 40-fold higher than adult brain. Furthermore, ketones uniquely contribute to maturation within the nervous system (1-3,9-16).

Most ketogenesis occurs in the liver, and is driven primarily by rates of fatty acid oxidation (FAO). Mitochondrial FAO-derived acetoacetyl-CoA (AcAc-CoA) and acetyl-CoA together serve as the primary ketogenic substrates. Ketogenic reactions are sequentially catalyzed by HMG-CoA synthase 2 and HMG-CoA lyase, generating acetoacetate (AcAc), which is converted to D-β-hydroxybutyrate (βOHB) in an NAD+/NADH-coupled redox reaction catalyzed by βOHB dehydrogenase. AcAc and βOHB diffuse into the bloodstream, and are delivered to ketolytic organs.
in which they are exceptionally energy-efficient substrates (12,17-22). Within mitochondria of ketolytic organs, βOHB is oxidized back to AcAc in a reaction catalyzed by βOHB dehydrogenase. AcAc receives a CoA moiety from succinyl-CoA, generating AcAc-CoA in a reaction catalyzed by succinyl-CoA:3-oxoacid CoA transferase (SCOT, EC 2.8.3.5), encoded by nuclear Oxct1. This enzyme is not expressed in liver (12,23). Mitochondrial AcAc-CoA thiolase catalyzes conversion of AcAc-CoA to acetyl-CoA, which is terminally oxidized within the tricarboxylic acid cycle.

Reports of approximately 20 individuals who harbor homozygous or compound heterozygous OXCT1 loss-of-function mutations (Online Mendelian Inheritance in Man #245050) indicate that a functional allele is required for ketone body oxidation, and as such patients typically present in infancy with spontaneous ketoacidosis (24-31). Numerous single nucleotide polymorphisms have been identified within the human OXCT1 locus, but functional significance has been ascribed to relatively few of them.

Adverse consequences of ketoacidosis are well-appreciated, and physiological states that increase ketone body turnover have been extensively analyzed. Nonetheless, experimental models to date have not definitively revealed whether loss of ketone oxidation can be energetically tolerated, particularly at the tissue level, and the metabolic adaptations to ketolytic insufficiency are unknown. In this study, we use novel Oxct1−/− mice to examine the metabolic roles of ketone body oxidation in the neonatal period, and the adaptations to its absence.

**EXPERIMENTAL PROCEDURES**

**Animals.** Oxct1−/− C57BL/6 mouse embryonic stem (ES) cells (clone EPD0082-1-CO2) were acquired through the NIH knockout mouse project (KOMP) consortium. The targeting sequence inserts two transcriptional terminators (pA) within intron 5 of the Oxct1 locus, which is upstream of sequence that encodes critical catalytic SCOT residues (Fig. 1A) (32,33). Genotyping was performed using primer sets schematized in Fig. 1A and listed in Table S1. ES cells were microinjected into embryonic day 3.5 (E3.5) C57BL/6 blastocysts, and four chimeric mice (determined by tail biopsy PCR) were obtained. Transmission of the targeted allele was achieved by breeding chimeric males to C57BL/6 wild-type females. Heterozygote (Oxct1+/−) progeny were then crossed to generate Oxct1−/− mice. All mice were maintained on standard polysaccharide-rich chow diet (Lab Diet 5053) and autoclaved water ad libitum. Lights were off between 1800-0600. All P0 litters were obtained at 0900 and tissues/blood were harvested mid-morning. All experiments were performed using protocols approved by the Animal Studies Committee at Washington University.

**Plasma metabolite and insulin measurement.** Measurement of plasma glucose, AcAc, βOHB, and triglycerides were performed using biochemical assays coupled to colorimetric substrates (Wako), as previously described (34). Blood glucose was measured in duplicate using a glucometer (Aviva). Measurement of plasma insulin was performed by ELISA (Millipore) as previously described (34).

**Gene expression analysis.** Quantification of gene expression was performed using real-time RT-qPCR using the ‘ΔΔCt’ approach as described, normalizing to Rpl32, using primer sequences listed within Table S1 (34).

**Immunoblot.** Immunoblots, using protein lysates from neonatal brain, heart, and quadriceps/hamstring muscles to detect Oxct1/SCOT (rabbit anti-SCOT, Proteintech Group, Inc.) were performed as described (34). Detection of phospho-AMPKα [p-AMPKα (Thr172)] and total AMPKα were performed as previously described (35). Microtubule associated protein 1 light chain 3 (LC3) was detected using rabbit polyclonal anti-LC3 (Novus Biologicals, #NB100-2220) and donkey anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare, NA9340). Band intensities were densitometrically quantified using QuantityOne software (Bio-Rad).

**Measurement of in vivo substrate utilization.** P0 mice were injected intraperitoneally with 10 μmol/g body weight of either sodium [2,4-13C2]βOHB, [1-13C]glucose, or sodium [3-13C]lactate (Cambridge Isotope Laboratories). Because P1 Oxct1−/− mice are hypoglycemic and hypolactatemic, 13C-isotope injections were supplemented in these animals with either 20 μmol/g naturally-occurring glucose, for [1-
13C]glucose studies, or 10 μmol/g naturally-occuring lactate, for [3,13C] lactate studies, to ensure tissue delivery of total and 13C-labeled substrate would remain equal among genotypes. After the indicated incubation durations in minutes, neonatal mice were killed by decapitation, and tissues were rapidly freeze-clamped in liquid N2. Neutralized perchloric acid extracts were profiled using gradient heteronuclear single quantum correlation (gHSQC) 13C-edited proton NMR measured at 11.75T. Quantification of integrals of carbon 2 of 13C-taurine (a normalizing metabolite whose tissue concentrations were constant across conditions, and which is not enriched by administration of these substrates) and 13C-glutamate (carbon 4) were all performed as previously described (34). Signals were collected from extracts dissolved in 300 μL D2O + 1 mM trimethylsilyl propionate (TSP), loaded into high-precision thin-walled 5 mm tubes (Shigemi, Inc.).

Statistical analyses. Analyses were performed using GraphPad software (Prism), using tests described within the text.

RESULTS
Ketolytic deficiency in Oxct1−/− mice. To determine the energetic role of ketone bodies in the neonatal period, Oxct1−/− mice were generated on the C57BL/6 genetic background (Fig. 1A-B). Genotyping analysis of Oxct1+/− X Oxct1−/− live progeny from 12 litters examined on postnatal days 0-1 (P0-P1) indicated that progeny are born in a Mendelian ratio: 19 +/+, 40 +/-, and 25 −/− mice ($\chi^2$=0.5, p = 0.78). Body weights among +/+, +/-, and −/− mice did not vary on P0 or P1; gastric milk spots were observed with equal frequency within each genotype; and no gross anatomic or behavioral abnormalities were observed in P0-P1 Oxct1−/− mice (Table 1). As expected, Oxct1 mRNA and SCOT protein were reduced ≈50% in P0 Oxct1−/− mice, and absent in Oxct1−/− mice (Fig. 1C-D).

Independent measurements of plasma biochemical metabolites on P0 and P1 revealed marked and progressive hyperketonemia in Oxct1−/− mice, with total ketone body concentrations increasing to > 16 mM in Oxct1−/− mice on P1, consistent with accumulation of unmetabolized substrate (Table 1). An abnormally increased ratio of AcAc to βOHB is also consistent with a ketolytic lesion at the reaction catalyzed by SCOT: ligation of a CoA moiety to AcAc. Measurement of in vivo metabolism of 13C-βOHB in skeletal muscle of P0 Oxct1−/− mice, using 13C-edited proton NMR of extracts acquired 30 min after labeled substrate administration, confirmed the absence of 13C-enriched glutamate – a quantitative surrogate for procession of carbon through the tricarboxylic acid cycle – and accumulation of 13C-acetone and 13C-AcAc, products of 13C-βOHB that remain unmetabolized due to the absence of SCOT (Fig. 1E) (34,36). These findings were corroborated in P1 skeletal muscle and in P0 and P1 brain (Fig. S1). Taken together, these results indicate that (i) Oxct1−/− mice are ketolysis-deficient and (ii) ketone body oxidation in mice does not notably proceed through SCOT-independent pathways.

Ketoacidosis, hypoglycemia, and neonatal lethality of Oxct1−/− mice. Unlike P0-P1 progeny of Oxct1+/− X Oxct1+/− pairings, genotyping analysis of live progeny from 14 litters between P2-P10 yielded 34 +/+, 50 +/-, and zero −/− mice ($\chi^2$=24.7, p < 0.0001). No lethality phenotype was evident in heterozygotes ($\chi^2$=0.92, p = 0.34). In addition to hyperketonemia, hypoglycemia and reduced plasma lactate concentrations were also observed in P1, but not P0 Oxct1−/− animals. Significant differences in plasma insulin, free fatty acid, and triglyceride concentrations were not observed (Table 1). In particular, the rise in plasma free fatty acid concentrations that occurs after birth in association with suckling of high-fat milk was preserved in P1 Oxct1−/− mice (37,38).

While P1 Oxct1−/− mice exhibit marked hyperketonemia, relatively reduced plasma glucose and plasma lactate concentrations indicate that ketolytic insufficiency also results in diminution of other circulating metabolic fuels. To determine if suckling Oxct1−/− mice could be rescued from these metabolic abnormalities and prospective energetic deficiency, we performed metabolic resuscitation experiments using newborn progeny of Oxct1+/− X Oxct1−/− mice. Serial subcutaneous injections of 155 mM NaHCO3 ± 10% glucose were delivered to suckling neonates (50 μL every 3-6h; 7 injections were administered per 24h period). While NaHCO3 partially buffers the acidifying effects of ketone bodies, glucose provides an additional...
energetic substrate that stimulates insulin release, thereby inhibiting ketogenesis and prospectively curtailing ketoacidosis in suckling Oxct1<sup>−/−</sup> mice (39,40). As expected, survival analysis revealed that control suckling Oxct1<sup>−/−</sup> mice injected with 155 mM NaHCO<sub>3</sub> alone exhibited similar survival and weight course as un.injected suckling Oxct1<sup>+/+</sup> animals, indicating that partial buffering of the acidifying effects of ketone bodies had no impact on survival (Fig. 2A-B, n=20 pups/group). On the other hand, serial administration of NaHCO<sub>3</sub> + glucose markedly improved the survival and weight trajectory of suckling Oxct1<sup>−/−</sup> mice in the first 48h of life. However, by P3, Oxct1<sup>−/−</sup> animals maintained on the NaHCO<sub>3</sub> + glucose regimen had fallen off a normal growth curve, and all gradually died over an additional 2-3 days. No significant effects of the NaHCO<sub>3</sub> + glucose, or NaHCO<sub>3</sub> alone regimens were observed in Oxct1<sup>+/−</sup> or Oxct1<sup>/+</sup> animals.

To determine if the failure-to-thrive phenotype observed within NaHCO<sub>3</sub> + glucose resuscitated Oxct1<sup>−/−</sup> animals was attributable to an insufficiency of glucose uptake, plasma glucose concentrations were measured in resuscitated animals 30 and 180 min after NaHCO<sub>3</sub> + glucose administrations in P2 Oxct1<sup>+/−</sup>, Oxct1<sup>−/−</sup>, and Oxct1<sup>+/+</sup> mice. In addition, because SCOT-dependent ketone body metabolism within pancreatic β cells may influence glucose-stimulated insulin secretion, we also measured plasma insulin 30 min after NaHCO<sub>3</sub> + glucose administration in these mice (41,42). Our results indicate that post-injection glucose mobilization and plasma insulin concentrations were not reduced in Oxct1<sup>−/−</sup> mice (Fig. 3A-B). However, despite treatment with the resuscitation regimen, suckling Oxct1<sup>−/−</sup> mice still developed marked hyperketonemia (Fig. 3C). These results indicate that while systemic glucose utilization is intact in Oxct1<sup>−/−</sup> mice, the lethal metabolic consequences of ketolytic insufficiency in suckling mice are refractory to frequent glucose administration.

**Metabolic adaptations of Oxct1<sup>−/−</sup> mice.** To determine if the development of hypoglycemia and reduced plasma lactate concentrations result from metabolic adaptations to the loss of ketolytic capacity, we performed a series of experiments in neonatal Oxct1<sup>−/−</sup> mice and their littermates. First, we measured the abundance of the phosphorylated (active) form of the energy sensor/effector AMPKα in skeletal muscle, heart, and brain of untreated P0 Oxct1<sup>−/−</sup> mice and their P0 Oxct1<sup>+/+</sup> littermates. To ensure that measured responses of Oxct1<sup>−/−</sup> mice were not downstream consequences of ketoacidosis and hypoglycemia, P0 mice were harvested within three hours of birth. Protein lysates from skeletal muscle and heart of Oxct1<sup>−/−</sup> mice did not reveal evidence of AMPKα activation, but the phosphorylated form of AMPKα was 6.9±1.4-fold augmented in brain of Oxct1<sup>−/−</sup> mice, suggesting tissue-specific adaptation to the absence of ketolysis (n=7/group, p < 0.01, Fig. 4).

Second, we determined whether autophagy was altered in Oxct1<sup>−/−</sup> mice. Autophagy is an AMPK-activated self-degradative intracellular process in which organelles are recycled, prospectively in part for the purpose of energy conservation during periods of nutrient deprivation (43-45). Adaptation to extrauterine life requires normal autophagic progression (46). To determine if ketolytic deficiency in brain of P0 Oxct1<sup>−/−</sup> mice is accompanied by induction of autophagy, we measured a key biomarker of autophagic progression, the differential processing/migration of protein LC3 on SDS-PAGE (47). The relative abundance of the faster migrating form of LC3 (LC3-II/LC3-I ratio) was increased 2.0±0.2-fold (n=7/group, p < 0.001) in brain lysates of Oxct1<sup>−/−</sup> mice (Fig. 5). This result, which was not observed in skeletal muscle (data not shown), is consistent with a pro-autophagic response to energy deficiency in brain of Oxct1<sup>−/−</sup> mice.

Third, to determine if ketolytic insufficiency, and the associated ultimate development of reduced plasma glucose and lactate concentrations, were linked to increases in glucose and lactate utilization by tissues that normally oxidize ketones in Oxct1<sup>−/−</sup> mice, we used NMR to measure in vivo oxidative metabolism of independently administered <sup>13</sup>C-glucose or <sup>13</sup>C-lactate in brains and skeletal muscle of P0 and P1 Oxct1<sup>−/−</sup> mice and their littermates. Brain extracts obtained from P0 neonates 30 min after intraperitoneal administration of 10 μmol/g body weight <sup>13</sup>C-glucose revealed 2.4±0.2-fold increased accumulation of <sup>13</sup>C-enriched glutamate in brains of Oxct1<sup>−/−</sup> mice, compared to Oxct1<sup>+/+</sup> mice.
mice (from 3.6±0.8% to 8.5±1.7%; n=7/group, p = 0.002; Fig. 6A; no differences were observed between Oxct1+/+ and Oxct1−/− mice, which are combined and represented as Oxct1+). As expected, at the time of tissue harvest, tissue 13C-enrichment of glucose (35.3% and 41.3% in Oxct1+ and Oxct1−/− mice, respectively, p = 0.51) and total blood glucose concentrations were also equivalent between groups that received 13C-glucose, indicating that increased 13C-enrichment of glutamate could not be explained by increased delivery of 13C-substrate. These findings were corroborated in brain extracts of P0 animals collected 45 min after substrate injection: 13C-enrichment of glutamate was 2.8±0.3-fold increased in brains of Oxct1−/− mice (n=5/group, p = 0.02). Evidence for increased glucose utilization persisted in brains of Oxct1+/+ mice on P1, which exhibited 1.8±0.2-fold greater 13C-enrichment of glutamate in Oxct1−/− brains than by brains of Oxct1+ littermates, 30 min after substrate administration (n=7/group, p = 0.02; Fig. 6A). Because P1 Oxct1+/+ mice are hypoglycemic, this experiment was performed by administering a cocktail of 13C- and naturally-occurring glucose (see Experimental Procedures), which prevented disproportionately high delivery of labeled glucose to brains of Oxct1−/− mice. Taken together, these results are consistent with a higher rate of glucose oxidation in brains of Oxct1−/− mice on both P0 and P1, which possibly contributes to the ultimate development of hypoglycemia.

Unlike brain of Oxct1−/− mice, evidence for increased glucose oxidation was not observed in skeletal muscle, which after 30 min of 13C-glucose administration revealed only scant 13C-enrichment of glutamate in Oxct1+ (1.4±0.2%) or Oxct1−/− mice (1.9±0.3%). After 45 min, 13C-enrichment of glutamate increased equally to ~3% in Oxct1+ and Oxct1−/− mice. Similar results were observed in muscle of P1 mice.

To obtain surrogates of in vivo lactate oxidation in neonatal mice, we measured 13C-glutamate in brains and skeletal muscles of P0 animals 30 min after administration of 10 μmol/g 13C-lactate. In Oxct1+ and Oxct1−/− mice, 13C-labeling of glutamate in brain was greater through 13C-lactate than that delivered through 13C-glucose. Nonetheless, there was no difference between brains of Oxct1+ and Oxct1−/− mice (11.1±3.0% and 12.0±3.7%, respectively, n=5/group, p = 0.84). Conversely, 13C-enrichment of glutamate from lactate was enhanced in skeletal muscle of P0 Oxct1−/− mice by 1.7±0.2-fold, compared to skeletal muscle of Oxct1+ mice (7.9±0.8% and 4.5±0.7%, respectively, n=5/group, p = 0.02; Fig. 6B). As expected, 13C-enrichment of substrate was not greater in skeletal muscle of P0 Oxct1−/− mice. Increased lactate oxidation rates did not persist to P1 in skeletal muscle, which were 5.5±0.4% and 5.0±0.8% in P1 Oxct1+ and Oxct1−/− mice, respectively (n=5/group, p = 0.6; Fig. 6B). Taken together, these results indicate (i) increased oxidation of lactate in skeletal muscle of P0 but not P1 Oxct1−/− mice and (ii) adaptation of both neonatal brain and skeletal muscle to ketogenic insufficiency.

**DISCUSSION**

Introduction to the extrauterine environment creates new metabolic and energetic demands. Studies of metabolic flux and ketone turnover have indicated that ketone bodies serve an important role in neonatal rodents and humans (1-3,9,37,38). This is the first study to use a genetic model to demonstrate that oxidation of ketones is required for postnatal survival in mice. Oxct1−/− mice exhibit no evidence of terminal ketone body oxidation, and develop ketoacidosis, indicating that the SCOT pathway is required for ketolysis in mice. Furthermore, plasma glucose and lactate levels become depleted in Oxct1−/− mice after the first 24h of extrauterine life. Increased energetic consumption of glucose and lactate are observed, starting prior to the depletion of these metabolites in plasma, suggesting that increased consumption, at least in part, contributes to their depletion.

While reduced nutrient ingestion could also contribute to ketoacidosis, hypoglycemia and hypolactatemia, our results, together with those of prior investigators, strongly suggest that these abnormalities occur in actively suckling neonatal Oxct1−/− mice. First, because mice and rats (unlike humans) possess very little white adipose stores at birth, neonatal ketogenesis – robust in Oxct1−/− mice – is dependent on suckling (37,38,48). Second, plasma free fatty acid concentration, a reporter of milk intake and a key determinant of ketogenesis, exhibited increases in P1 versus P0 Oxct1−/− mice, comparable to the increase observed in Oxct1+ littermates. Plasma triglyceride concentrations were also not reduced in Oxct1−/−
mice. While ingested milk volumes were not formally quantified, $Oxct1^{+/−}$ mice exhibited gastric milk spots with equal frequency to their littermates on P0 and P1. Thus, these studies indicate that loss of ketone oxidation in postnatal mice provokes a metabolic state that ultimately proves lethal, despite access to metabolic fuels through milk.

Reduced plasma glucose and lactate concentrations in P1 $Oxct1^{−/−}$ mice are prospectively explained by increased oxidation of glucose and lactate, but hypoglycemia and hypolactatemia also may occur due to insufficient biosynthesis. During the rodent suckling period, biosynthesis of glucose and lactate are driven by hepatic gluconeogenesis and extrahypheatic biosynthesis. During the rodent suckling period, biosynthesis of glucose and lactate are driven by hepatic gluconeogenesis and extrahypheatic biosynthesis, respectively (3). Altered mitochondrial redox potential of $Oxct1^{−/−}$ mice could contribute to prospective synthetic deficiencies. In neonatal $Oxct1^{+}$ mice, plasma $β$OH:AcAc ratio is 3:1, consistent with reported ratios in ketotic states (11,39). In hyperketonemic $Oxct1^{−/−}$ mice, this ratio is markedly reduced to 1:2 on P0, due to oxidation of $β$OH to, and no further than, AcAc, which significantly reduces mitochondrial [NAD$^+$]/[NADH] ratio, particularly in extrahypheatic tissues. Altered mitochondrial redox potential could in turn impair flux through the malate-aspartate shuttle, whose function is important for glycolysis, the source of lactate. In turn, curtailed lactate production deprives the liver of a key gluconeogenic substrate, via the Cori cycle. Furthermore, in P1 $Oxct1^{−/−}$ mice, plasma concentrations of $β$OH and AcAc accumulate to very high concentrations, while remaining in a ratio of 1:3, which may evoke extrahypheatic and hepatic impairments of the tricarboxylic acid cycle and fatty acid oxidation. Oxidative impairment within liver disrupts gluconeogenesis (3,49-51). Emergence of attenuated extrahypheatic oxidative capacity was evident in $Oxct1^{−/−}$ mice on P1, when glucose oxidation in brain, and lactate oxidation in skeletal muscle, were both reduced in $Oxct1^{−/−}$ mice, relative to P0 $Oxct1^{+}$ mice.

An additional abnormality of ketone metabolism may also occur in $Oxct1^{−/−}$ mice. In humans, up to 37% of AcAc is spontaneously decarboxylated to acetone (52). Therefore, with accumulation of high concentrations of AcAc, it is likely that acetone also accumulates in normally ketolytic tissues of $Oxct1^{−/−}$ mice, a notion supported by our NMR findings. Acetone is disposed through breath and urine, but a significant proportion of acetone can also be used as a substrate for anabolic and catabolic processes through SCOT-independent pathways (52). High circulating concentrations of acetone may also cause central nervous system depression.

It is important to note that the precise roles of neonatal ketone metabolism differ between mice and humans. Humans are born at a more mature point in development, and as indicated above, at the time of birth, body fat percentage is higher in humans than in rats and mice (37,48,53-55). In these neonatal rodents, ketogenesis is driven by suckling, rather than fasting (37,38). Moreover, due to increased fat/carbohydrate ratio, rodent milk is more ketogenic than most human milk and infant formulas (3,16). It is likely for these reasons that two features of $Oxct1^{−/−}$ mice are distinct from those of SCOT-deficient humans: (i) autosomal recessive $Oxct1$ mutations in humans are compatible with life (albeit with aggressive nutritional support), while analysis of >100 litters of $Oxct1^{−/−} × Oxct1^{+/+}$ pairings has yet to reveal any male or female $Oxct1^{−/−}$ neonate that spontaneously survives past 48h of extrauterine life and (ii) $Oxct1^{−/−}$ mice develop hypoglycemia, unlike most SCOT-deficient humans. Despite these differences, deficiencies of ketone metabolism in neonatal mice bear significant relevance to human infant metabolism. First, physiological ketosis (1-2 mM) occurs in the early neonatal period in both humans and mice (1,2,11,56,57). Second, human infants are particularly prone to develop hyperketonemia, and thus, increased energetic utilization of ketones, in response to relatively short periods of nutrient deprivation, due to adipose lipolysis (3,9,11,14,58). Consequences of common viral illnesses can rapidly trigger ketosis. Third, human milk exhibits significant variations of macronutrient content distribution, even within the same mother over different collections (59,60). Therefore, it is plausible that energetic crisis and/or ketoacidosis could emerge in infants with unsuspected ketolytic insufficiency. Current newborn screening regimens do not detect individuals with isolated ketolytic disorders, who exhibit ketosis but normal acylcarnitine and organic acid profiles (61). Therefore, a small subset of cases which ultimately receive a diagnosis of Sudden Infant Death Syndrome...
(SIDS) may actually be attributable to SCOT deficiency.

While our studies did not directly measure prospective changes in glycolytic flux, or metabolic shifts within myocardium (the particularly low mass of neonatal mouse heart, ~10 mg, prevented tractable NMR approaches), these results indicate both toxic effects of and metabolic adaptation to ketolytic insufficiency in the neonatal period. Future experiments will definitively determine the relative contributions of ketoacidosis, hypoglycemia, and energy deficiency to lethality of Oxct1-/- mice. The ability to further determine the energetic roles of ketone bodies in mouse models will be best supported through future studies in which (i) a lower-fat non-ketogenic nutrient formula replaces mother’s milk in the neonatal period and/or (ii) additional genetic approaches selectively induce a ketolytic defect within individual tissues. Such studies will ultimately permit determination of energetic and disease-modifying roles of ketolysis in neonates and adults in conditions that include nutrient deprivation, maintenance on low-carbohydrate diets, and diabetes. Finally, with increasing utilization of low- and very low-carbohydrate diets in clinical trials for conditions including adult obesity, pediatric and adult epilepsy, and malignancies of the central nervous system, it is important to consider the metabolic and clinical consequences of latent ketolytic defects that are revealed later in life, possibly caused by single nucleotide polymorphisms within the Oxct1 locus – case reports describe marked variations of tolerance to ketogenic milieu (62-64). These studies indicate the critical metabolic role that ketone body oxidation serves, and adaptations to its absence in the reduced carbohydrate nutrient environment of the neonatal period.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Ketolysis-deficient *Oxct1<sup>−/−</sup>* mice. (A) Targeting strategy for the *Oxct1* locus in ES cells. WT, wild-type locus; Null, loss-of-function allele. Arrows indicate locations of genotyping PCR primers. See Table S1 for a list of primer sequences. (B) PCR genotyping of *Oxct1<sup>−/−</sup>* P0 mice. NTC, no template control. (C) Expression of *Oxct1* mRNA in heart of P0 *Oxct1<sup>+/+</sup>, Oxct1<sup>+/−</sup>, and *Oxct1<sup>−/−</sup>* mice, determined by RT-qPCR; n=5/group. (D) Immunoblot of SCOT and Actin using protein lysates from brain, heart, and quadriceps/hamstrings skeletal muscle of P0 *Oxct1<sup>+/+</sup>, Oxct1<sup>+/−</sup>, and *Oxct1<sup>−/−</sup>* mice. (E) 13C-edited proton NMR spectrum (2.1-2.5 ppm, relative to chemical shift of TSP internal standard) from quadriceps/hamstrings of P0 *Oxct1<sup>+/+</sup> and *Oxct1<sup>−/−</sup>* mice that had been injected with [2,4-13C2]β-OHB 30 min prior to collection of tissues and generation of extracts. 13C-glutamate is a reporter of tricarboxylic acid flux of a 13C-labeled substrate that is selectively absent in extracts from *Oxct1<sup>−/−</sup>* mice. C2 and C4 correspond to carbon position.
Fig. 2. Metabolic resuscitation of Oxct1−/− mice. (A) Kaplan-Meier curves for neonatal mice in the untreated, treated with 155 mM NaHCO3 alone, or treated with 155 mM NaHCO3 + 10% glucose (glc) states. Subcutaneous injections were performed every 3-6h, starting within 3h of birth, with 7 injections over a 24h period. Oxct1+/+ and Oxct1+/− mice showed no differences and were pooled (noted as Oxct1+). Within the first 90h of life, no statistically significant differences were observed among treated Oxct1+ mice, untreated Oxct1− mice, and Oxct1+/− mice treated with NaHCO3 + glucose. Likewise, no statistically significant difference was observed between Oxct1− mice treated with NaHCO3 alone and untreated Oxct1− mice. However, addition of glucose to the NaHCO3 regimen significantly improved survival of Oxct1− mice in the first 90h of life (p < 0.001 by log-rank test, n=20/group). (B) Relative weights of animals within the genotype and treatment groups described within panel A. *** p < 0.001 by two-way ANOVA with post-hoc Bonferroni testing, independently compared to each of the other treatment groups.

Fig. 3. Normal glucose mobilization, but refractory hyperketonemia in glucose-resuscitated suckling Oxct1−/− mice. (A) Blood glucose concentrations measured on P2 within neonatal mice treated with the NaHCO3 + glucose resuscitation regimen every 3-6h. Measurements were taken independently 30 and 180 min post-administration of NaHCO3 + glucose. (B) Plasma insulin concentrations within resuscitated mice, 30 min after NaHCO3 + glucose administration. NS, not significant. (C) Total plasma ketone concentration (βOHb + AcAc) within resuscitated mice, 30 min after NaHCO3 + glucose administration. For all comparisons, Oxct1+/+ and Oxct1+/− mice showed no differences and were pooled (noted as Oxct1+). n=7/group; *** p < 0.001 by Student’s t-test.

Fig. 4. Increased phosphorylation of AMPKα within brain of Oxct1−/− mice. Immunoblots of lysates from untreated animals. Skeletal muscle (A), heart (B), and brain (C) protein lysates from P0 Oxct1+/+ and Oxct1−/− mice for phospho-AMPKα (p-AMPKα), total AMPKα, SCOT, and Actin. (D) Densitometric quantification of p-AMPKα/AMPKα ratio in brain lysates. ** p < 0.01 by Student’s t-test.

Fig. 5. Enhanced autophagy in brain of Oxct1−/− mice. (A) Immunoblots of P0 brain protein lysates for LC-3 and Actin. (B) Densitometric quantification of LC3-II/LC3-I ratios in brain lysates. *** p < 0.001 by Student’s t-test.

Fig. 6. Tissue-specific adaptations to ketolytic insufficiency. (A) Accumulation of 13C-glutamate carbon #4 (C4) in brains of P0 (left) and P1 (right) mice injected with [1-13C]glucose, measured by 13C-edited proton NMR. n=7/group. (B) Accumulation of 13C-glutamate in skeletal muscle of P0 (left) and P1 (right) mice injected with [3-13C]lactate, measured by 13C-edited proton NMR. n=5/group; **, p < 0.01 by Student’s t-test.
Table 1. Plasma metabolite concentrations in P0-P1 mice.

βOHB, β-hydroxybutyrate; AcAc, acetoacetate; TG, triglycerides; FFA, free (non-esterified) fatty acids; n=6-10/group; **, p < 0.01 compared to +/-; †, p < 0.01 compared to +/+; aa, p < 0.01 and a, p < 0.05 compared to same genotype at P0. Data presented are mean±SEM; comparisons are two-way ANOVA with Bonferroni post-hoc testing.

<table>
<thead>
<tr>
<th>Oxct1 genotype and age</th>
<th>Weight, mg</th>
<th>βOHB, mM</th>
<th>AcAc, mM</th>
<th>Glucose, mg/dL</th>
<th>Insulin, ng/mL</th>
<th>FFA, mM</th>
<th>Lactate, mM</th>
<th>TG, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+, P0</td>
<td>1339±37</td>
<td>0.29±0.07</td>
<td>0.08±0.02</td>
<td>46±19</td>
<td>0.47±0.09</td>
<td>0.28±0.09</td>
<td>1.09±0.11</td>
<td>72.5±20.6</td>
</tr>
<tr>
<td>+/-, P0</td>
<td>1285±28</td>
<td>0.50±0.12</td>
<td>0.17±0.06</td>
<td>49±10</td>
<td>0.53±0.16</td>
<td>0.24±0.04</td>
<td>1.11±0.06</td>
<td>72.4±11.0</td>
</tr>
<tr>
<td>-/-, P0</td>
<td>1301±29</td>
<td>1.40±0.36</td>
<td>3.10±1.07</td>
<td>38±11</td>
<td>0.30±0.01</td>
<td>0.28±0.05</td>
<td>1.10±0.07</td>
<td>82.0±19.0</td>
</tr>
<tr>
<td>+/+, P1</td>
<td>1384±60</td>
<td>0.66±0.04</td>
<td>0.06±0.1</td>
<td>65±7</td>
<td>0.38±0.04</td>
<td>0.96±0.40</td>
<td>1.36±0.20</td>
<td>80.7±16.0</td>
</tr>
<tr>
<td>+/-, P1</td>
<td>1435±36</td>
<td>0.67±0.09</td>
<td>0.20±0.06</td>
<td>56±4</td>
<td>0.62±0.16</td>
<td>0.77±0.14</td>
<td>1.50±0.11</td>
<td>74.8±23.4</td>
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<tr>
<td>-/-, P1</td>
<td>1322±36</td>
<td>4.39±0.53</td>
<td>11.8±0.8</td>
<td>19±1**†a</td>
<td>0.42±0.06</td>
<td>1.14±0.30</td>
<td>0.67±0.04**†a</td>
<td>127.8±21.6</td>
</tr>
</tbody>
</table>
Cotter et al., Fig. 1

A

Oxct1 locus targeting construct

Homologous recombination

intron 4  

exon 5  
intron 5  
exon 6  
intron 6  

WT

- 1

- 2

- 3

exon 5  
intron 5  
intron 4  

Targeted transcriptional terminators (pA)

Null

B

PCR genotyping: tail biopsy

Genotype:  

WT  

Oxct1<sup>−/−</sup>  

Oxct1<sup>+/−</sup>  

NTC

Primer set: 1+

100 bp  

300 bp  

3  

2  

3  

2  

3  

2  

2,3

C

RT-qPCR: neonatal heart

Myocardial Oxct1 mRNA, relative abundance

Oxct1<sup>+/−</sup>  

Oxct1<sup>−/−</sup>  

Oxct1<sup>+/−</sup>

D

Immunoblots: neonatal tissue

WT  

Oxct1<sup>−/−</sup>  

Oxct1<sup>+/−</sup>

Brain  

Heart  

Skeletal muscle

SCOT  

Actin

E

13C-edited proton NMR

13C-[1]OHB substrate

[1]OHB (C2)  

[4]glutamate  

[4]acetate  

acetone  

AcAc
Cotter et al., Fig. 2

A

Percent survival

Hours

Oxct1−/−

Oxct1+/− HCO3

Oxct1+ No Rx

B

Fold weight change over P0

Days

Oxct1+ glc + HCO3

***

Oxct1+ No Rx

Oxct1− glc + HCO3

Oxct1− HCO3

Oxct1− No Rx
Cotter et al., Fig. 3

A

Blood glucose, mg/dL

0 100 200 300 400

30 min 180 min

Oxct1+ Oxct1−/−

B

Serum insulin, ng/mL

0.0 0.5 1.0 1.5

Oxct1+ Oxct1−/−

p = NS

C

Serum ketones, mM

0 4 8 12 16

Oxct1+ Oxct1−/−

***
Cotter et al., Fig. 4

A. Muscle immunoblot

- Oxct1^{+/+}
- Oxct1^{-/-}

B. Heart immunoblot

- Oxct1^{+/+}
- Oxct1^{-/-}

C. Brain Immunoblot

- Oxct1^{+/+}
- Oxct1^{-/-}

D. Brain

- p-AMPK/AMPK, arbitrary units

**Oxct1^{+/+} Oxct1^{-/-}**
Cotter et al., Fig. 6

A  
Substrate: $^{13}$C-glucose  
Tissue: Brain  

Age: P0  
% glutamate (C4) enrichment

Age: P1  
% glutamate (C4) enrichment

B  
Substrate: $^{13}$C-lactate  
Tissue: Muscle

Age: P0  
% glutamate (C4) enrichment

Age: P1  
% glutamate (C4) enrichment

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