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Metabolism of γ-hydroxybutyrate to D-2-hydroxyglutarate in mammals: further evidence for D-2-hydroxyglutarate transhydrogenase

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Abstract

γ-Hydroxybutyratic acid (GHB), and its prodrugs 4-butyrolactone and 1,4-butanediol, represent expanding drugs of abuse, although GHB is also used therapeutically to treat narcolepsy and alcoholism. Thus, the pathway by which GHB is metabolized is of importance. The goal of the current study was to examine GHB metabolism in mice with targeted ablation of the GABA degradative enzyme succinic semialdehyde dehydrogenase ($SSADH^{-/-}$ mice), in whom GHB persistently accumulates, and in baboons intragastrically administered with GHB immediately and persistently. Three hypotheses concerning GHB metabolism were tested: (1) degradation via mitochondrial fatty acid β-oxidation; (2) conversion to 4,5-dihydroxyhexanoic acid (a putative condensation product of the GHB derivative succinic semialdehyde); and (3) conversion to D-2-hydroxyglutaric acid (D-2-HG) catalyzed by D-2-hydroxyglutarate transhydrogenase (a reaction previously documented only in rat). Both D-2-HG and 4,5-dihydroxyhexanoic acid were significantly increased in neural and nonneural tissue extracts derived from $SSADH^{-/-}$ mice. In vitro studies demonstrated the ability of 4,5-dihydroxyhexanoic acid to displace the GHB receptor ligand NCS-382 (IC_{50} = 38 μmol/L), although not affecting $GABA_B$ receptor binding. Blood and urine derived from baboons administered with GHB also accumulated D-2-HG, but not 4,5-dihydroxyhexanoic acid. Our results indicate that D-2-HG is a prominent GHB metabolite and provide further evidence for the existence of D-2-hydroxyglutarate transhydrogenase in different mammalian species.

1. Introduction

γ-Hydroxybutyratic acid (GHB), a 4-carbon hydroxy acid derived from γ-aminobutyratic acid (GABA) in brain and periphery, manifests broad pharmacological activity, including altered dopamine release and tyrosine hydroxylase activity, in addition to a number of known (and putative) receptor interactions [1]. GHB was developed as an analogue of GABA for the induction of anesthesia in humans, but

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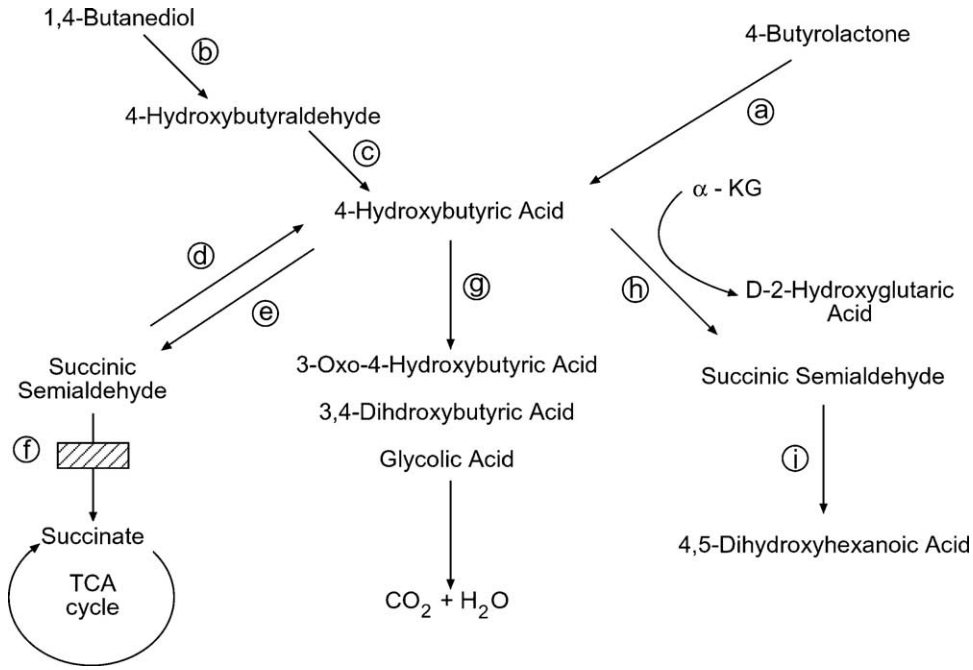

Fig. 1. Metabolic interrelationships of GHB, 4-butyrolactone, and 1,4-butanediol. Enzyme reactions include (*a*) serum lactonase; (*b*) alcohol dehydrogenase; (*c*) aldehyde dehydrogenase; (*d*) SSA reductase; (*e*) GHB dehydrogenase; (*f*) SSADH, site of the block in heritable human and murine SSADH deficiency; (*g*) fatty acid β-oxidation spiral; (*h*) D-2-hydroxyglutarate transhydrogenase; and (*i*) an uncharacterized reaction forming 4,5-dihydroxyhexanoic acid from succinic semialdehyde. Succinic semialdehyde also derives from GABA (and succinic semialdehyde may also contribute to GABA production), primarily in the central nervous system, in a reversible reaction catalyzed by GABA transaminase (not shown). α-KG indicates α-ketoglutarate; TCA, tricarboxylic acid.

early animal studies revealed unwanted side effects [2]. Renewed interest in GHB has occurred, however, in relation to its potential as a treatment for alcohol and opiate dependence and narcolepsy-associated cataplexy, as an illicit drug of abuse, and as an agent to facilitate acquaintance sexual assault [3]. Because of its capacity to induce euphoria, short-term amnesia, and sedation at high concentrations, the use of illicit GHB is expanding [4]. Unlike GHB (a controlled substance in the United States), the GHB prodrugs 4-butyrolactone and 1,4-butanediol (Fig. 1), which rapidly convert to GHB in the body, are widely accessible and uncontrolled substances, and may be potentially substituted for GHB in instances of illicit consumption [2].

Despite expanding clinical and illicit consumption, the pathways by which GHB is metabolized remain largely unexplored. Less than 2% of ingested GHB in humans is excreted unchanged in the urine, suggesting considerable metabolism [5], yet the major GHB metabolite(s) remains unknown. Walkenstein and coworkers [6] were among the first to suggest the β-oxidation of GHB (Fig. 1). In addition, urine derived from succinic semialdehyde dehydrogenase (SSADH)-deficient patients has variably shown metabolites consistent with β-oxidation, including glycolic, 3-oxo-4-hydroxybutyric, and 3,4-dihydroxybutyric acids [7]. GHB may also be metabolized to succinic semialdehyde (SSA) with stoichiometric conversion of 2-ketoglutarate to D-2-hydroxyglutaric acid (D-2-HG), in the reaction catalyzed by D-2-hydroxyglutarate transhydrogenase, an $NAD(P)^+$-independent reaction [8] (Fig. 1). Furthermore, the presence of 4,5-dihydroxyhexanoic acid has been observed in the urine of SSADH-deficient patients, a metabolite presumably deriving from further metabolism of succinic semialdehyde (Fig. 1) [9]. At present, then, putative sequences for GHB metabolism may be represented by (1) conversion to SSA with further metabolism via the Krebs cycle (producing carbon dioxide and water, and perhaps the major metabolic pathway) [10], transamination to GABA [11,31,32], or conversion to 4,5-dihydroxyhexanoic acid; (2) degradation via β-oxidation; and (3) conversion to succinic semialdehyde by D-2-hydroxyglutarate transhydrogenase, with concomitant generation of D-2-HG.

GHB accumulates supraphysiologically in heritable human SSADH deficiency, a defect in the GABA degradative pathway (Fig. 1) [12], and in the corresponding gene-ablated murine model [13-16]. Understanding the sequelae of GHB metabolism could have important treatment ramifications for SSADH-deficient patients and those ingesting GHB therapeutically. Accordingly, we have begun to map the mammalian metabolism of GHB. To achieve our objectives, we first evaluated GHB metabolism in SSADH-deficient ($SSADH^{-/-}$) mice, followed by metabolic studies in baboons receiving short- and long-term administration of GHB [17]. In addition, the physiological significance of 4,5-dihydroxyhexanoic acid remains unknown, as it is not detected in other biological systems. Structural similarities with GHB raised the possibility that 4,5-dihydroxyhexanoic acid could compete for GHB binding. Accordingly, we tested the hypothesis that 4,5-dihydroxyhexanoic acid might

be a ligand for either the high-affinity GHB or the $GABA_B$ receptors [1,25]. The current report summarizes our findings, presented earlier in abstract form [17].

2. Methods

2.1. Subjects

2.1.1. $SSADH^{-/-}$ mice

Development of $SSADH^{-/-}$ mice has been described [13]. Mutant (n = 6-9) and wild-type animals (n = 6-9) were age-matched (12-17 days old). For preparation of tissue extracts, mice were anesthetized with avertin and killed. Tissues (liver, brain, and kidney) were rapidly removed and frozen immediately on dry ice. Extracts were prepared by homogenization in Tris-HCl buffer (pH 8.0), rapidly deproteinized, and the extracts clarified by centrifugation followed by neutralization. Samples were stored at −80°C before analysis. Protein concentration was determined by the Bradford method using bovine serum albumin as standard. All metabolite contents were normalized to protein content or creatinine concentration for urine studies.

2.2. Baboons

Subjects were 4 adult male baboons (*Papio anubis*) that weighed 27 to 35 kg. Baboons had long-term indwelling intragastric (IG) catheters surgically implanted [18] and had completed behavioral studies to evaluate the effects of short- and long-term GHB administration. Complete description of these studies and the procedures used are reported [19,20]. For IG dosing, GHB sodium salt (Sigma-Aldrich, St Louis, MO) was dissolved in distilled water. Short-term doses (32-320 mg/kg) were infused at 15 mL/min IG. Blood samples were collected under ketamine anesthesia at 15, 30, 60, 120, 240, and 360 minutes after infusion. Long-term dosing consisted of continuous slow (0.3-0.4 mL/min over 24 hours) IG infusion of GHB solutions (320 or 750 mg/kg per day). Blood samples were collected between 8 and 10 AM under ketamine anesthesia after 1 to 4 weeks of long-term administration. In all cases, approximately 5 mL of blood was obtained using vaccutainers with a lithium heparinized tube. Samples were immediately centrifuged at 3200 rpm for 12 minutes, and then plasma was retrieved and transferred to 2 separate labeled polypropylene tubes. Urine samples were collected in the first 2, 6 to 8, and at 24 hours after short-term GHB dosing in clean cage pans from unanesthetized baboons. Urine samples were transferred to 2 separate labeled polypropylene tubes and frozen. All samples were frozen until analysis. All animal studies were approved by the Institutional Animal Care and Use Committee at the respective institutions.

2.3. Studies of GHB β-oxidation in $SSADH^{-/-}$ mice

We were unable to collect sufficient urine from geneablated mice to assess metabolites indicative of GHB β-oxidation by routine gas chromatography–mass spectrometry (GC-MS). Alternatively, we evaluated secondary markers for evidence of β-oxidation in these animals. We quantified free fatty acids and total triglycerides in extracts of liver derived from $SSADH^{-/-}$ mice spectrophotometrically, hypothesizing that significant disruption in the metabolism of these intermediates would occur if GHB were undergoing substantial β-oxidation (free fatty acids, Wako Chemicals, Neuss, Germany; triglycerides, Stanbio Laboratory, Boerne, TX). Similarly, we assessed free carnitine and acylcarnitine levels, and free fatty acids in liver and sera derived from $SSADH^{-/-}$ mice by tandem mass spectrometry to ascertain if GHB interfered with acylcarnitine metabolism [21].

2.4. Stable isotope dilution GC-MS

Levels of D- and L-2-HG in plasma and urine from rats and baboons, and tissues derived from $SSADH^{-/-}$ mice, were quantified by stable isotope dilution GC-MS as previously described [22,23]. 4,5-Dihydroxyhexaonic acid is not commercially available, which necessitated synthesis. For the isotopic material, deuterated methyl iodide was used for introduction of deuterium. A Grignard reaction was carried out with deuterated methyl iodide on acrolein to generate [1,1,1-d_3]3-buten-2-ol. This was condensed with triethyl orthoacetate to produce [6,6,6-d_3]hex-4-enoic acid ethyl ester, which upon saponification with KOH/EtOH and acidification generated the free 4,5-dihydroxyhexanoic acid. Oxidative ring closure with meta-chloroperbenzoic acid/Amberlyst produced the internal lactone. The stable isotope dilution assay for 4,5-dihydroxyhexanoic acid was similar to that for GHB [22], with the exception of the extraction protocol. For 4,5-dihydroxyhexanoic acid, a single extraction with 4-mL ethyl acetate was performed, and quantification was achieved with positive chemical ionization GC-MS, based upon the ions *m*/*z* 220 and *m*/*z* 223 (internal standard).

2.5. Binding studies of 4,5-dihydroxyhexanoic acid in isolated rat frontal cortex membranes

Using membrane preparations from rat frontal cerebral cortex, we evaluated the capacity of 4,5-dihydroxyhexanoic acid to displace 30 nmol/L [^{3}H]NCS-382, the high-affinity GHB receptor ligand [24]. This was performed essentially as described [24]. For competition studies with 4,5-dihydroxyhexanoic acid, 30 nmol/L [^{3}H]NCS-382 was used throughout as ligand for the high-affinity GHB receptor [1,25]. For the $GABA_B$ receptor, the ligand used was 5 nmol/L [^{3}H]-CGP54626 in all studies.

3. Results

3.1. Free fatty acids, triglycerides, and carnitine levels in liver and serum of $SSADH^{-/-}$ mice

Quantification of free fatty acids in tissue extracts of $SSADH^{-/-}$ and $SSADH^{+/+}$ mice (μmol/100 mg protein, n = 6 each genotype) revealed the following: liver, $SSADH^{-/-}$

Table 1
Metabolites in SSADH$^{-/-}$ and SSADH$^{+/+}$ mouse tissue extracts*

Metabolite	Tissue	SSADH$^{+/+}$	SSADH$^{-/-}$
D-2-HG	Brain	118 ± 9	523 ± 30
	Liver	26 ± 13	2939 ± 2241
	Kidney	17 ± 3	1099 ± 682
DHHA	Brain (cerebellum)	ND	102 ± 11
	Brain (cortex)	ND	134 ± 51
	Brain (hippocampus)	ND	138 ± 12
	Liver	110 ± 9	290 ± 40
	Kidney	380 ± 50	5140 ± 460

All values are expressed as mean ± SEM (nmol/100 mg protein); for all groups, n = 6 animals each, with the exception of 4,5-dihydroxyhexanoic acid in SSADH$^{+/+}$ brain regions (n = 7 each) and 4,5-dihydroxyhexanoic acid in SSADH$^{-/-}$ brain regions (n = 9 each). DHHA indicates 4,5-dihydroxyhexanoic acid; ND, not detected.

* $P < .001$, all means ± SEM for SSADH$^{-/-}$ mice compared with SSADH$^{+/+}$ mice (1-tailed Student t test)

3.8 ± 1.0 (SEM) and SSADH$^{+/+}$ 3.3 ± 0.7; kidney, SSADH$^{-/-}$ 2.3 ± 0.5 and SSADH$^{+/+}$ 1.9 ± 0.2. Quantification of triglycerides (mg/100 mg protein; n = 6 except for n = 4 in SSADH$^{-/-}$ kidney) revealed the following: liver, SSADH$^{-/-}$ 20.2 ± 4.5 and SSADH$^{+/+}$ 12.3 ± 1.9; kidney, SSADH$^{-/-}$ 64.0 ± 12.0 and SSADH$^{+/+}$ 95.1 ± 13.1 (P = NS between genotypes for all tissues, 2-tailed t test). There was no difference in free carnitine/acylcarnitine levels in serum for SSADH$^{+/+}$ and SSADH$^{-/-}$ mice quantified by tandem mass spectrometry, nor evidence for alterations of free fatty acids, carnitine, or glucose in liver extracts (data not shown). There was no difference in sera C_4OH carnitine levels between genotypes, which would be the expected carnitine analogue should GHB be esterified as the coenzyme A ester for metabolism via β-oxidation (eg, 4-hydroxybutyryl-coenzyme A) [26].

3.2. Conversion of GHB to 4,5-dihydroxyhexanoic acid and d-2-HG in SSADH$^{-/-}$ mice

The levels of 4,5-dihydroxyhexanoic acid and D-2-HG in extracts of tissues derived from SSADH$^{-/-}$ mice are shown in Table 1. D-2-HG concentrations were significantly elevated in brain, liver, and kidney, especially in peripheral tissues. A similar trend was observed for 4,5-dihydroxyhexanoic acid, with the most significant accumulation in kidney (Table 1), perhaps reflecting renal accumulation before excretion. There was no elevation of L-2-HG in any tissue of SSADH$^{-/-}$ animals as compared with SSADH$^{+/+}$ mice. 4,5-Dihydroxyhexanoic acid displaced [^{3}H]NCS-382 binding with an approximate IC_{50} of 38 μmol/L (Fig. 2), but did not displace high-affinity $GABA_B$ receptor ligands at concentrations up to 3 mmol/L.

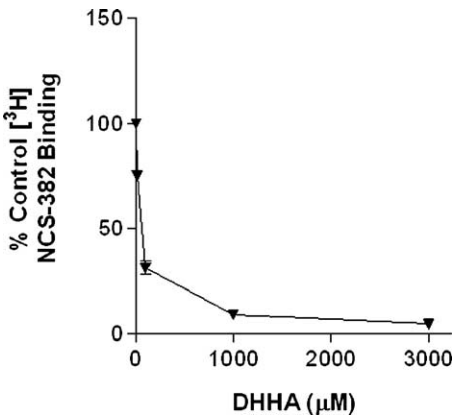

Fig. 2. Displacement of 30 nmol/L [^{3}H]NCS-382 binding from isolated rat cortex membranes by 4,5-dihydroxyhexanoic acid (0-3000 μmol/L). The data represent the mean of determinations on 2 occasions, with each time point run in triplicate. Binding protocols were as described in Methods.

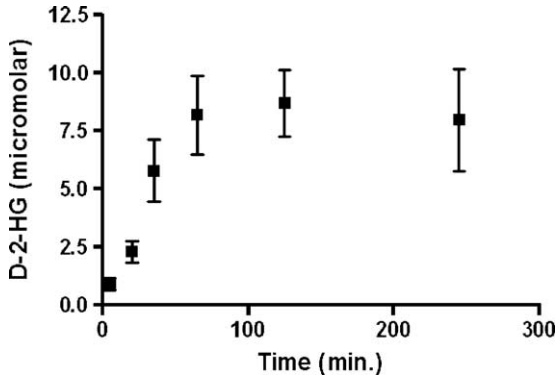

Fig. 3. D-2-Hydroxyglutarate in blood derived from baboons administered a single short-term bolus of 320 mg/kg GHB. Blood was obtained from a saphenous vein under ketamine anesthesia. Data are depicted as mean ± SD for n = 4 animals, with 0 time point representing the value obtained in animals receiving vehicle.

3.3. Metabolic transformations of GHB intragastrically administered to baboons

Blood D-2-HG accumulated after short-term administration of a single intragastric bolus of 320 mg/kg GHB, reaching a plateau at about 60 minutes (Fig. 3). There was no increase in L-2-HG. Higher D-2-HG concentrations were observed during long-term administration of GHB (320 or 750 mg/kg per day; blood D-2-HG, 14-101 μmol/L, n = 4; water vehicle, 0.9 ± 0.5 μmol/L). The urine of baboons persistently administered with GHB (750 mg/kg) contained D-2-HG, without accumulation of L-2-HG (D-2-HG, 465 ± 274 mmol/mol creatinine [n = 4]; vehicle, 1.9 ± 0.6 [n = 4]; $P < .001$, 1-tailed Student t test). 4,5-Dihydroxyhexanoic acid was detected in baboon urine, but it was not significantly different from vehicle; conversely, 4,5-dihydroxyhexanoic acid was undetectable in all baboon plasma samples.

4. Discussion

Despite its expanding role as therapeutic agent and drug of abuse, surprisingly little is known about the metabolism of GHB. These metabolic sequences possess important ramifications for clinical utility and efficacy, forensic investigation, as well as the mode of action of GHB in its broad neuromodulatory activity [2]. In the current report, we sought to fill this gap in our knowledge, using as a

springboard a model system (SSADH$^{-/-}$ mice) in which GHB accumulates to supraphysiological levels.

Several reports suggest that β-oxidation of GHB occurs [6,7,27,28]. This observation is supported by the frequent accumulation of dicarboxylic acids in the urine of SSADH-deficient patients [29]. In earlier in vitro studies, however, we found that extracts of mitochondria derived from rat liver and kidney metabolized GHB primarily to intermediates of the tricarboxylic acid cycle (Fig. 1) [10,30]. It is likely (although unproven) that the major metabolic transformation of GHB is via conversion to succinic semialdehyde and entry into the Krebs cycle [31,32], a process blocked in SSADH$^{-/-}$ mice. Although direct analysis of urine derived from SSADH$^{-/-}$ mice was unfeasible, we found no evidence for secondary disruption of fatty acid or triglyceride metabolism, nor any perturbation of the carnitine/acylcarnitine cycle in physiological fluids and tissues derived from SSADH$^{-/-}$ mice, leading us to conclude that, at least in this model system, β-oxidation is not a major pathway for GHB disposal.

In all species examined, we found that D-2-HG represented a prominent GHB metabolite. For SSADH$^{-/-}$ mice, this accumulation was pronounced in peripheral tissues (Table 1). In baboon, GHB administration resulted in D-2-HG accumulation in both blood and urine. Therefore, our results indicate that D-2-hydroxyglutarate transhydrogenase is active in central nervous system and periphery of all species studied (Fig. 1). This enzyme was partially purified and characterized by Kaufman and coworkers [8], but its nucleotide sequence has not been reported. D-2-hydroxyglutarate transhydrogenase catalyzes a reaction converting GHB to succinic semialdehyde with stoichiometric conversion of α-ketoglutarate to D-2-HG, an NAD(P)$^+$-independent reaction (Fig. 1). The biological significance of D-2-hydroxyglutarate transhydrogenase remains unknown; however, although potentially detoxifying GHB, this reaction produces another probable neurotoxin (eg, D-2-HG), the biochemical hallmark of isolated D-2-hydroxyglutaric aciduria [33]. We have recently verified that D-2-hydroxyglutarate dehydrogenase gene mutations are responsible for heritable D-2-hydroxyglutaric aciduria [34], an abnormality associated with neurological abnormalities. In vitro studies suggest that D-2-HG mediates neurotoxicity through activation of *N*-methyl-D-aspartate receptors [35,36]. These observations suggest that consumption of GHB could potentially induce secondary toxicity linked to production of D-2-HG.

The first suggestion that 4,5-dihydroxyhexanoic acid might represent a GHB derivative came from Brown and coworkers [9] who detected this intermediate in the urine of SSADH-deficient patients. Four stereoisomers of 4,5-dihydroxyhexanoic acid may exist in solution, and it is unknown which species is predominantly active in mammalian central nervous system. Previous studies [37,38] revealed that succinic semialdehyde reacts with pyruvate in isolated beef heart particulate preparations to form 5-hydroxy-4-ketohexanoic and 5-keto-4-hydroxyhexanoic acid isomers, a conversion dependent upon thiamine pyrophosphate. NAD(P)H-linked reduction of these precursors would generate the stereoisomeric forms of 4,5-dihydroxyhexanoic acid. Although the precise physiological significance (if any) of 4,5-dihydroxyhexanoic acid remains undefined, we have now demonstrated that it is a GHB receptor ligand, although further functional analyses are needed to assess its specificity for the GHB receptor. The affinity of 4,5-dihydroxyhexanoic acid, however, for the GHB receptor is low (eg, the IC_{50} of the high-affinity GHB receptor antagonist NCS-382 is <1 μmol/L [39]), and it may be possible that concentrations of this intermediate in the brain of SSADH$^{-/-}$ mice are insufficient to produce a significant biological activity. Conversely, preliminary data indicate that 4,5-dihydroxyhexanoic acid also inhibits electron transport chain function in vitro [40]. These observations combine to suggest that 4,5-dihydroxyhexanoic acid might be active in the neuropathology of SSADH deficiency. Although 4,5-dihydroxyhexanoic acid merits further examination as a potential biomarker for ingestion of GHB, its absence in baboon physiological fluids after GHB administration argues against its use for toxicology screening associated with GHB consumption.

Whether any of the GHB metabolites (D-2-HG, 4,5-dihydroxyhexanoic acid) detected in the current study represents toxicological or pharmacological effects remains to be determined, although it is clear that D-2-HG production is associated with the ingestion of GHB. In addition, it remains unknown if D-2-HG accumulates to levels in brain sufficient to induce neurotoxicity after GHB ingestion [35]. Further studies in animals will be required to determine whether the circulating $t_{1/2}$ of D-2-HG is significantly longer than that of GHB (approximately 30-45 minutes). In conclusion, our studies provide evidence for the existence of D-2-hydroxyglutarate transhydrogenase beyond the rat [41] and suggest that this enzyme linked to GHB metabolism may be ubiquitous in mammalian species.

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