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Effect of Chronic γ -hydroxybutyrate (GHB) Administration on GHB- Induced Respiratory Depression

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Abstract

Background.— γ -hydroxybutyrate (GHB) has a high potential for illicit use; overdose of this compound results in sedation, respiratory depression and death. Tolerance to the hypnotic/sedative and electroencephalogram effects of GHB occurs with chronic GHB administration; however, tolerance to respiratory depression has not been evaluated. GHB toxicodynamic effects are mediated predominantly by GABA_B receptors. Chronic treatment may affect monocarboxylate transporters (MCTs) and alter the absorption, renal clearance and brain uptake of GHB.

Objectives.—To determine effects of chronic GHB dosing on GHB toxicokinetics, GHB-induced respiratory depression, and MCT expression.

Methods.—Rats were administered GHB 600 mg/kg intravenously daily for 5 days. Plasma, urine and tissue samples and respiratory measurements were obtained on days 1 and 5. Plasma and urine were analyzed for GHB by LC/MS/MS and tissue samples for expression of MCT1, 2 and 4 and their accessory proteins by QRT-PCR.

Results.—No differences in GHB pharmacokinetics or respiratory depression were observed between days 1 and 5. Opposing changes in MCT 1 and MCT4 mRNA expression were observed in kidney samples on day 5 compared to GHB-naïve animals and MCT4 expression was increased in the intestine.

Conclusions.—The lack of tolerance observed with GHB-induced respiratory depression, in contrast to the tolerance reported for the sedative/hypnotic and electroencephalogram effects, suggests different GABA_B receptor subtypes may be involved in different GABA_B-mediated toxicodynamic effects of GHB. Chronic or binge users of GHB may be at no less risk for fatality from respiratory arrest with a GHB overdose than with a single dose of GHB.

Introduction

Use of γ -hydroxybutyrate (GHB) and its precursors has been recognized as a significant issue in the US, several European countries, and Australia (1–6) with manifestations including sedation, coma, and respiratory depression, which can result in GHB-associated fatality (1, 4, 6). GHB is used clinically to treat narcolepsy as well as alcohol withdrawal. In the treatment of narcolepsy, GHB has been shown to reduce cataplexy attacks on a daily and weekly basis as well as improve a number of narcolepsy-associated symptoms such as nocturnal awakening and daytime sleepiness (7). GHB has also been shown to significantly reduce alcohol intake, as well as relapses into heavy drinking (8). GHB is marketed as Xyrem® in the U.S., Canada and Europe for the treatment of narcolepsy (9). In Germany, GHB is an approved anesthetic and is marketed as Somsanit® (9). Additionally, GHB is approved for the treatment of alcohol withdrawal in Austria and Italy, under the tradename Alcover® (9). Despite the number of approved clinical indications, the therapeutic use of GHB has been overshadowed by its high abuse potential. Despite the number of approved clinical indications, the therapeutic use of GHB has been overshadowed by its abuse potential even though no cases of intoxication and coma have been described during its clinical use. Case reviews of GHB overdose indicate that those who use GHB as “street drug” and not under clinical prescriptions often do so chronically or engage in ‘binge’ use of GHB, in which high doses of GHB are ingested for several days at a time (10, 11).

The toxicokinetics of GHB involve its transport by monocarboxylate transporters (MCTs), which govern many aspects of GHB toxicokinetics, including its oral absorption, renal reabsorption, and brain uptake (12–16). The first four members of the MCT family require a chaperone protein in order to be trafficked to the plasma membrane (17). There are two chaperone proteins, basigin and embigin, which differ in their expression and affinity for the different MCTs (18). Basigin is more widely expressed and is the preferred chaperone for MCT1, 3 and 4 while embigin has a higher affinity for MCT2 (18). Both proteins contain a single transmembrane domain, share a conserved glutamate residue, have an intracellular C-terminus and a large glycosylated extracellular domain (18). Administration of MCT inhibitors in rats has been demonstrated to increase GHB renal and oral clearance and inhibit the brain uptake of GHB, making these inhibitors a potential treatment strategy for GHB overdose (13, 16, 19, 20). Prolonged exposure of MCT substrates, including lactate and butyrate, to cells which express MCT1 results in up-regulation of this transporter in vitro (21, 22). These data suggest chronic administration of GHB in vivo may have a similar effect on MCT expression, potentially leading to alterations in its own toxicokinetics upon repeated dosing. Results of studies examining the effect of chronic use of GHB, where GHB is administered every day for several days, on total plasma clearance have reported decreases of 20% or no change (23, 24); however, renal clearance was not evaluated.

Chronic administration of GHB or the GHB precursor γ -butyrolactone (GBL) to mice or rats induces tolerance to the hypnotic/sedative effect of GHB (23–29), as well as the EEG effects of GHB (23), indicating alteration of GHB toxicodynamics by chronic use. GABAergic mechanisms, including GABA_B agonism, have been implicated in many of the behavior effects of GHB including the sedative and anesthetic effects (30). The mechanism of GHB tolerance has not been systematically studied, it has been shown that chronic

administration of a GHB precursor, 1,4-butanediol, resulted in cross-tolerance with GHB and (±)-baclofen, another GABA_B agonist (31). After 2–3 weeks without 1,4-butanediol administration sensitivity to GHB and (±)-baclofen was restored, demonstrating that the tolerance was reversible (31). Chronic gamma-butyrolactone (GBL), a compound that is rapidly metabolized to GHB *in vivo*, administration in mice resulted in altered GABA binding in brain tissue. After 12 weeks of oral GBL there was a significant reduction in B_{max} for a high and low affinity site for GABA in the cerebral cortex, striatum and cerebellum (32). This information suggests that GABA_B receptor regulation may play a role in GHB tolerance, but further investigation is needed to define the mechanism for GHB tolerance. A recent report of known GHB-associated fatalities indicated that the most common cause of mortality was cardiorespiratory depression (6). While respiratory depression has been reported in both fatal and non-fatal GHB overdose cases, tolerance to this pharmacodynamic endpoint, which is also mediated through GABA_B receptors (33) has not been evaluated. The objectives of the present study were to determine if chronic GHB administration results in (1) tolerance to respiratory depression, important in the toxicity and deaths produced by GHB overdoses, and (2) changes in the toxicokinetics, and (3) alterations in MCT expression, important for the absorption, renal clearance and tissue uptake of GHB.

Methods

Chemicals and Reagents.

Sodium GHB used in this experiment was provided by the National Institute on Drug Abuse (NIDA).

Animals and Animal Surgery.

Male Sprague-Dawley rats (Envigo, Indianapolis, IN) weighing 270–330 g were used for all experiments. Animals were housed under controlled temperature and humidity with an artificial 12 hour light/dark cycle and food and water were available *ad libitum*. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University at Buffalo. Animals were allowed to acclimate to their environment for one week prior to surgical implantation of jugular vein cannulae under anesthesia with ketamine/xylazine. Cannulae were flushed daily with 40 IU/mL heparinized saline to maintain patency. Animals were allowed a minimum of 72 hours for recovery from surgery before drug administration.

Toxicokinetic/toxicodynamic experiments.

Rats were administered GHB 600 mg/kg intravenously daily for 5 days. GHB was administered as a 300 mg/mL solution in sterile water through the jugular vein cannula. Our intention with choosing 600 mg/kg for this study was to use a reasonably high dose, without risking lethality in the rat (LD₅₀ for GHB in rats is 1750 mg/kg). A dose of 600 mg/kg is high enough to obtain significant respiratory depression in order to assess whether chronic dosing produces changes in respiratory depression. Our aim was to mimic high chronic recreational dosing. On Day 1 and Day 5, GHB toxicokinetics and toxicodynamics were evaluated. Respiration was measured using plethysmography as previously described (33).

Briefly, rats were placed in plethysmography chambers 1 hour prior to GHB administration and allowed a 45 minute acclimation. Following acclimation, 5 baseline measurements were taken over 15 minutes, then GHB was administered at time 0. Respiratory measurements were obtained at 2.5, 5, 10, 15, 20, 25, and 30 minutes, and every 15 minutes thereafter for 6 hours. Blood samples were taken at 3, 10, 20, 30, 60, 120, 150, 180, 210, 240, 270, and 300 minutes after GHB administration. Urine samples were also collected at intervals up to 6 hours. The parameter of respiratory frequency (rate) was used to determine effects of chronic GHB administration on respiratory depression, as we have previously identified change in this parameter as the primary effect of GHB on respiration (33). Twenty-four hours following the administration of GHB on day 5, animals were sacrificed by aortic exsanguination under isoflurane and kidney, brain, and intestine were collected and snap-frozen in liquid nitrogen. For a control group, GHB-naïve animals were similarly sacrificed and kidney, brain, and intestine collected. All samples were stored at -80°C until analysis.

RNA isolation and qPCR analysis.

For total RNA extraction, around 80 –100 mg of kidney, brain and small intestine tissue was homogenized and total RNA was extracted using RNeasy® Mini Kit (Qiagen Inc, Valencia, CA), according to the manufacturer's instructions. The RNA was quantified by spectrophotometry (Nanodrop Technologies, DE) and the sample purity was assessed by measuring OD 260/280 and 260/230 ratios. RNA integrity was analyzed by visual inspection of 18S and 28S rRNA bands after electrophoresis on an agarose gel. cDNA was synthesized from 250 ng of total RNA using a master mix containing AffinityScript Reverse Transcriptase (Agilent Tech Inc, Santa Clara, CA). The primers for MCT, MCT2, MCT4, basigin and embigin were commercially obtained (Qiagen). qPCR was performed with RT²; SYBR® Green qPCR Mastermix (Qiagen), primers and cDNA. Alien® QRT-PCR Inhibitor Alert was used as an external control for normalization. Non-reverse transcriptase controls were carried out without cDNA. Amplification was run on a CFX96 Connect™ (BioRad) thermal-cycler (95°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds and 60°C for 30 seconds, then to 72°C for 1 minute). The PCR primers efficiencies ranged from 90% to 110%. The cycle at which each sample crossed a fluorescence threshold, C_t , was determined, and the samples were run in triplicate and were averaged. Analyses of real-time PCR were performed using the comparative C_t method.

Cell Culture

The immortalized rat brain capillary endothelial cell line (RBE4) was kindly provided by Professor P. Couraud (Institut Cochin, Paris). RBE4 cells were cultured as described previously (Roiko et al. 2013). Briefly, cells were grown at 37°C with 5% CO_2 on Type-I collagen-coated flasks, and media was changed every 2–3 days. The culture medium was 1:1 α -minimum essentials medium/Hams F10 nutrient mixture supplemented with L-glutamine (2.0 mM), geneticin (300 $\mu\text{g}/\text{ml}$), human recombinant fibroblast growth factor (1 ng/ml), gentamicin (50 $\mu\text{g}/\text{ml}$), and 10% V/V fetal bovine serum (FBS). Cells were passaged using 0.25% trypsin/EDTA and induction studies began when cells were 7 days post confluent. Cells were incubated with vehicle, 1, 10 or 100 mM GHB in RBE4 complete media for up to 24 hours. RNA was obtained from the cells by first washing the cells twice with 10 mL of 1X sterile PBS, then the cells were removed from the flask by scraping in 5 mL 1X sterile

PBS. Samples were spun down ($1000 \times g$ for 3 min) and the resulting pellet was then added to 350 μ L RNA lysis buffer and mixed by inversion. This solution was then passed through a 27 gauge needle using a 1 mL syringe 3–4 times and 350 μ L RNA dilution buffer was added and mixed by inversion. Samples were then further processed using the SV Total RNA isolation kit and RNA concentrations were measured by spectrophotometry (Nanodrop Technologies). mRNA for MCT1 was quantified as described for RNA isolated from tissues.

Sample analysis.

GHB plasma and urine GHB concentrations were determined using previously validated LC/MS/MS assays (33, 34).

Data/statistical analysis.

Noncompartmental analysis of toxicokinetic data was performed using WinNonLin 5.2 (Palo Alto, CA). Clearance (Cl) was determined as Dose/AUC, where AUC represents the area under the plasma concentration-time curve from 0– ∞ . Renal clearance (Cl_R) was calculated at A_e/AUC , where A_e represents the total amount of GHB excreted in the urine. Area below the effect curve (ABEC) for respiratory frequency was calculated using WinNonLin. Paired t-tests were used to determine statistically significant differences between mean toxicokinetic and toxicodynamic parameters on days 1 and 5. Student's t-tests were used to compare MCT and ancillary protein mRNA expression in animals following 5 days of dosing compared to expression in control (GHB-naïve) animals.

Results

GHB plasma concentrations on days 1 and 5 are presented in Figure 1A. As seen in Table 1, chronic GHB administration resulted in no significant differences in GHB total or renal clearance on day 5 compared to day 1. These data concur with previous *in vivo* studies indicating little effect of chronic GHB dosing on GHB plasma concentrations. The expression of MCT1 decreased with chronic administration of GHB in the kidney, while the expression of MCT2, MCT4, basigin and embigin all increased, shown in Table 2. There were no statistically significant expression changes in the brain for MCT1 (Table 2); however, *in vitro* studies, shown in Figure 3, indicated that there was an increase in MCT1 expression in RBE4 cells after a 24 hour incubation with GHB. The lack of a statistically significant increase in MCT1 mRNA in Table 2 may be related to the unusually high standard deviation obtained for MCT1 after chronic GHB dosing. When RBE4 cells were incubated for longer time periods (up to 96 hours) the mRNA expression of MCT1 returned to control levels (data not shown). As with the kidney, there were statistically significant increases in the mRNA expression of MCT2, MCT4, basigin and embigin in the brain. An interesting finding in the current study was an increased expression of MCT1, MCT4 and basigin mRNA in the intestine, even with IV administered GHB. There was a decrease in the expression of embigin in the intestine and no change in MCT2 expression.

When GHB was administered intravenously for 5 days at a dose of 600 mg/kg, no significant changes were observed in breathing frequency on day 1 and day 5, as shown in

Figure 2. As well, there was no difference in the area beneath the effect curve (ABEC) for these groups (Table 1).

Discussion

Although GHB use remains a significant concern, there still exists no specific treatment for GHB overdose. Due to the role of MCTs in GHB renal reabsorption, increasing GHB renal clearance and inhibiting brain uptake via administration of MCT inhibitors represents a potential treatment strategy for GHB intoxication (13, 19, 35). Recent studies demonstrate the ability of MCT inhibitor administration to increase GHB oral clearance and decrease GHB brain:plasma partitioning as well, further supporting the potential of this therapeutic strategy (16, 20). If changes in MCT transporter expression may occur in chronic GHB users, the efficacy of this treatment strategy in this population may differ from that in acute overdose.

The lack of change in renal clearance may be explained by the opposing changes observed in the expression of MCTs in the kidney (Table 2); the decrease in MCT1 expression in the kidney may be compensated for by the increase in MCT4 expression and/or in the expression of basigin, the ancillary protein necessary for MCT1-mediated transport. Previous studies also demonstrate no change in brain extracellular fluid (ECF) concentrations of GHB in rats following a similar chronic dosing regimen, suggesting no alteration of MCT-mediated brain uptake of GHB (24, 25). Our data are also in agreement with these findings, as we observed no significant changes in brain MCT1, the primary isoform expressed at the blood-brain barrier (36), with chronic dosing. Our in vitro studies with rat brain endothelial RBE4 cells suggested that mRNA of MCT1 increases with 24-hr exposure to concentrations of GHB that would be present in plasma samples (1 and 10 mM), although significant changes did not occur after shorter time exposures. Exposures to GHB after IV administration would only occur over a 4–6 hour period due to its rapid elimination from the body, so based on the cell culture studies, no changes would be expected over this time frame.

The changes in MCT1 and MCT4 expression in the intestine suggest that although systemic GHB toxicokinetics may not be altered with prolonged administration, oral absorption may be affected, which is particularly relevant considering GHB is orally ingestion and that oral administration would allow much higher GHB concentrations in the GI tract than that of the current study. Although clinical studies indicate no change in the oral clearance of GHB following chronic administration in humans (37, 38), these studies were performed using low, therapeutic dosages of GHB. Based upon the current data, alteration in GHB intestinal transport with chronic use cannot be ruled out, and evaluation of the effect of chronic high-dose GHB on MCT expression and GHB toxicokinetics is warranted.

Although preclinical studies demonstrate a lack of change in GHB brain ECF concentrations with chronic dosing, tolerance to the sedative effect of GHB has been consistently reported (26, 32, 39). However, in the current study we observed a lack of tolerance to the toxicodynamic effect of respiratory depression. This finding is particularly interesting considering both endpoints are known to be due to action at GABA_B receptors (33, 40).

Unlike GABA_A receptors, about which it is known that many receptor subtypes exist, less is known about the heterogeneity of GABA_B receptors (41, 42). However, variants of the GABA_B receptor 1 subunit have been identified, two of which (1a and 1b) were determined to be present in rat brain with differential regional distribution (43). Additionally, recent data demonstrate the extent of the effects of GABA_B receptor modulators to vary in different brain regions, suggesting that subtypes of GABA_B receptors expressed in various regions are functionally distinct (44, 45). Studies also support different interaction of each subunit variant with other intracellular proteins, including those responsible for cell surface expression (46). Therefore, differences observed in the effect of chronic GHB dosing on tolerance to different toxicodynamic endpoints are possibly due to GHB action at different GABA_B receptor subtypes in different areas of the brain undergoing differential regulation.

Differences in tolerance for different pharmacodynamics effects have also been reported for opioids in rodents. Chronic morphine administration in both mice and rats results in significant tolerance developed for the analgesic effects of the drug without, or with a much reduced effect, tolerance for respiratory depression (47, 48). In rats, ventilation remains similar for opioid-tolerance and opioid-naïve animals (47). In mice, chronic morphine administration results in a 13-fold increase in the effective dose-50% (ED₅₀) for analgesia, while the ED₅₀ for inspiratory time was only increased 2- or 4-fold (48).

In summary, these data indicate little effect of chronic dosing of high GHB doses on GHB toxicokinetics, including total and renal clearances. A lack of tolerance to GHB-induced respiratory depression suggests that chronic or binge users of GHB are at no less risk for fatality from respiratory arrest in GHB overdose than with a single dose of GHB. Altogether, a lack of effect of chronic dosing on GHB toxicokinetics/toxicodynamics suggests that the effect of administration of an MCT inhibitor should not be affected by chronic dosing. Therefore, MCT inhibition may still represent a viable treatment option for GHB overdose in both the acute and chronic setting.

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Figure 1. GHB plasma concentrations following acute and chronic GHB administration. GHB 600 mg/kg was administered intravenously daily for 5 days and GHB toxicokinetics measured on days 1 and 5, representing acute and chronic GHB administration, respectively.

Figure 2. Respiratory rate following acute and chronic GHB administration. GHB 600 mg/kg was administered intravenously daily for 5 days and GHB toxicodynamics measured on days 1 and 5, representing acute and chronic GHB administration, respectively.

Figure 3. Fold-change in MCT1 mRNA expression in RBE4 cells from control.

Cells were incubated with vehicle, 1, 10 or 100 mM GHB in RBE4 complete media for up to 24 hours. Data are presented as mean $\pm$ S.D. (N =3).

Table 1.

Toxicokinetics/toxicodynamics of GHB following acute and chronic administration.

GHB 600 mg/kg	AUC (ug/ml*hr*10²)	Cl (mL/kg/min)	Cl_R (mL/kg/min)	ABEC (breaths)
Day 1	1013 (124)	6.00 (0.74)	1.89 (0.67)	4760 (410)
Day 5	986 (40.5)	6.09 (0.24)	1.64 (0.36)	4470 (1600)

AUC=area under the plasma concentration-time curve Cl=total clearance

Cl_R=renal clearance ABEC=area below the effect curve

GHB 600 mg/kg was administered intravenously daily for 5 days and toxicokinetics/toxicodynamic parameters measured on days 1 and 5, representing acute and chronic GHB administration, respectively.

Using paired t-tests, no statistically significant differences in toxicokinetic or toxicodynamic measures were determined between days 1 and 5.

Table 2.

Tissue mRNA expression in GHB-naïve rats and rats administered GHB 600 mg/kg IV daily for 5 days.

Protein	Tissue	Control	GHB 600 mg/kg x 5
MCT1	Kidney	1.22 (0.120)	0.387 (0.168) **
MCT2		1.13 (0.024)	3.21 (0.268) ***
MCT4		1.10 (0.022)	2.76 (0.377) **
Basigin		1.02 (0.011)	2.79 (0.254) ***
Embigin		1.03 (0.010)	2.60 (0.248) ***
MCT1	Brain	1.05 (0.008)	1.33 (0.571)
MCT2		1.02 (0.003)	1.41 (0.047) ***
MCT4		1.02 (0.011)	2.03 (0.066) ***
Basigin		1.01 (0.010)	1.28 (0.112) *
Embigin		1.02 (0.019)	1.50 (0.161) **
MCT1	Intestine	1.25 (0.087)	1.70 (0.153) *
MCT2		1.18 (0.026)	1.18 (0.089)
MCT4		1.21 (0.018)	3.18 (0.319) ***
Basigin		1.20 (0.054)	3.19 (0.237) ***
Embigin		1.26 (0.082)	1.01 (0.045) *

Control samples were taken from GHB-naïve animals. Data presented as mean $\pm$ SD fold change.

*
p<0.05,

**
p<0.01,

p<0.0001, compared to control, determined by student's t-test