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RESEARCH PAPER

Brain Extracellular γ-hydroxybutyrate Concentrations are Decreased by L-lactate in Rats: Role in the Treatment of Overdoses

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ABSTRACT

Purpose L-lactate represents a potential treatment for GHB overdose by inhibiting GHB renal reabsorption mediated by monocarboxylate transporters. Our objective was to assess the dose-dependence of L-lactate treatment, with and without D-mannitol, on GHB toxicokinetics/toxicodynamics (TK/TD).

Methods Rats were administered GHB 600 mg/kg i.v. with L-lactate (low and high doses), D-mannitol, or L-lactate (low dose) with D-mannitol. GHB-induced sleep time and GHB plasma, urine and brain extracellular fluid (ECF) concentrations (by LC/MS/MS) were determined. The effect of L-lactate and D-mannitol on the uptake and efflux of GHB was assessed in rat brain endothelial RBE4 cells.

Results L-lactate treatment increased GHB renal clearance from 1.4 ± 0.1 ml/min/kg (control) to 2.4 ± 0.2 and 4.7 ± 0.5 ml/min/kg after low and high doses, respectively, and reduced brain ECF AUC values to 65 and 25% of control. Sleep time was decreased from 137 ± 12 min (control) to 91 ± 16 and 55 ± 5 min (low and high L-lactate, respectively). D-mannitol did not alter GHB TK/TD and did not alter L-lactate's effects on GHB TK/TD. L-lactate, but not D-mannitol, inhibited GHB uptake, and increased GHB efflux from RBE4 cells.

Conclusions L-lactate decreases plasma and brain ECF concentrations of GHB, decreasing sedative/hypnotic effects.

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KEY WORDS lactate · microdialysis · monocarboxylate transporter · overdose · γ-hydroxybutyrate

ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AUC	area under the plasma concentration-time curve
BBB	blood–brain barrier
CHC	α-cyano-4-hydroxycinnamate
Cl_r	renal clearance
ECF	extracellular fluid
GABA	gamma-aminobutyric acid
GHB	γ-hydroxybutyrate
MCT	monocarboxylate transporter
TK/TD	toxicokinetics/toxicodynamics

INTRODUCTION

γ-hydroxybutyrate (GHB) is an endogenous metabolite of γ-aminobutyric acid (GABA) and a putative neurotransmitter or neuromodulator with its own receptor (1,2). GHB is used therapeutically, currently marketed under the trade name Xyrem ® (Jazz Pharmaceuticals, Palo Alto, CA) for narcolepsy with cataplexy and is also available as a treatment for alcohol withdrawal in Europe (3). However, the illicit use of GHB overshadows its therapeutic potential.

The illicit use and abuse of GHB and its precursors 1,4 butanediol and γ-butyrolactone (which are rapidly converted into GHB in the body) has been widely documented. Body builders use these compounds for their anabolic steroid-like effects (4). GHB is also used as a club drug due to its euphoric effects, and in drug-facilitated sexual assault because of its sedative/hypnotic and amnesic effects (5,6). As the use and abuse of GHB has increased, so have cases of GHB overdose and death (7,8). GHB toxicity is characterized by dizziness,

respiratory depression, vomiting, coma, and death (9). There are currently no approved treatments for GHB toxicity other than supportive care.

GHB is a substrate for the proton-coupled monocarboxylate transporter 1 (MCT1), and also the isoforms MCT2 and MCT4 (10,11). MCT1 and MCT2 are found throughout the body, including the kidney in the renal proximal tubules and the vascular endothelium at the blood–brain barrier (BBB) and can transport substrates in a bidirectional manner depending on proton concentration gradients (12,13). Endogenous substrates for MCTs include lactate, pyruvate, and ketone bodies such as β-hydroxybutyrate and acetoacetate (14). Recent findings indicate that L-lactate inhibits MCT1-mediated reabsorption of GHB in the renal proximal tubule, increasing the renal clearance and reducing the sedative/hypnotic effects of GHB (15), suggesting that L-lactate may be a potential treatment for GHB overdose.

The sedative/hypnotic effects of GHB seen during toxicity and overdose are due to its effects in the brain, mediated (at least in part) by the $GABA_B$ receptor (16,17). Recent studies in our laboratory used brain microdialysis to measure free GHB concentrations in rat frontal cortex, indicating that GHB exhibits MCT-mediated uptake into the frontal cortex, which would be saturated at high GHB concentrations (18). In addition, GHB concentrations at the *offset* of its sedative/hypnotic effect (return of righting reflex, RRR) are equivalent across doses in frontal cortex ECF, plasma, and whole brain (19), indicating a relationship between GHB concentrations at these sites and sedative/hypnotic effects. The actual GHB concentrations in plasma, brain frontal cortex or frontal cortex ECF differed from one another, but the higher plasma concentrations were not due to plasma protein binding, since binding is negligible for GHB (20). Since MCT1 is present on brain microvessel endothelial cells at the BBB, we hypothesized that MCT1 inhibition with L-lactate would alter GHB brain distribution and serve as an additional site of action in treating GHB overdose.

The use of D-mannitol as an osmotic diuretic and its effects on the BBB have been well-documented (21). Previous work suggested that combining D-mannitol with Lactated Ringer's injection could increase the renal clearance and decrease the sleep time of GHB (1,000 mg/kg), when Lactated Ringer's injection alone or D-mannitol alone had no effect on the TK/TD of GHB (15). The mechanism underlying this reported additive effect on GHB TK is unknown. We further explored this interaction by studying the effects of L-lactate and D-mannitol alone and in combination on GHB brain ECF concentrations *in vivo* using brain microdialysis, and on the TK/TD of GHB. In addition, the effects of these treatments on GHB transport at the BBB were further explored *in vitro* by characterizing the uptake and efflux of GHB from cultured rat brain endothelial cells.

MATERIALS AND METHODS

Chemicals

γ-hydroxybutyrate (sodium salt) was obtained from NIDA (Rockville, MD). Ketamine, xylazine, buprenorphine, and carprofen were purchased from Butler Schein (Melville, NY). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, MA). Sodium L-lactate and alpha-cyano-4-hydroxycinnamate (CHC) were purchased from Sigma-Aldrich (St. Louis, MO), and D-mannitol was obtained from Fisher Scientific (Pittsburgh, PA). For LC/MS/MS analysis, formic acid was purchased from Sigma-Aldrich (St. Louis, MO), deuterated GHB (GHB-d6) was purchased from Cerilliant (Round Rock, TX), and HPLC-grade acetonitrile, methanol, water, and acetic acid were purchased from Honeywell Burdick and Jackson (Morristown, NJ).

Animals and Surgery

All animal procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (280–320 g) were used in all studies and surgeries were performed as previously described (19). Briefly, rats were anesthetized with ketamine/xylazine (90/9 mg/kg), implanted with jugular and femoral vein cannulae, and then mounted in a stereotaxic frame (Harvard Apparatus, Holliston, MA). Microdialysis guide cannulae (CMA 11 guide cannulae, CMA Microdialysis, North Chelmsford, MA) containing dummy probes were implanted in the frontal cortex (AP + 3.2 mm and ML± 2.5 mm from Bregma, and DV −0.5 mm from dura (22). The cannula was fixed in place and buprenorphine (0.05 mg/kg s.c.) and 0.9% saline (6 ml s.c.) were administered immediately post-surgery, and carprofen 5 mg/kg s.c. was administered daily for 2 days post-surgery. Rats were allowed at least 6 days for recovery prior to microdialysis probe implantation and conduct of the experiment.

Microdialysis Probe Implantation, Retrodialysis, and Sample Collection

Microdialysis probes (CMA11, CMA Microdialysis, North Chelmsford, MA) were prepared as described by manufacturer's instructions, and implanted 24 h prior to each experiment, which allows the blood–brain barrier to reform post-implantation (23). Experiments were conducted in metabolic cages using awake and freely moving rats with *ad libitum* access to water. The experimental procedure is shown in Fig. 1. On the day of the experiment, microdialysis probes were perfused with aCSF at a flow rate of 2 μl/min and allowed to stabilize for 2 h. Microdialysate fractions

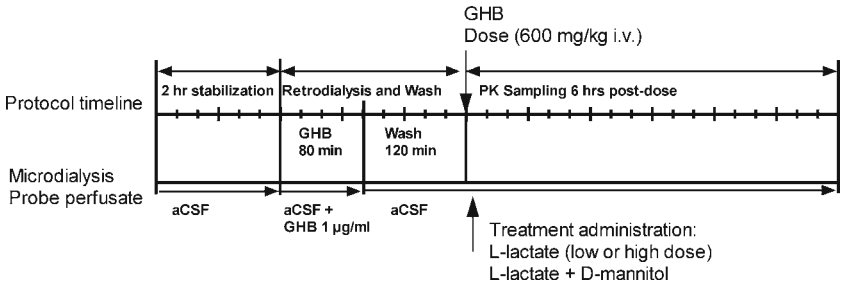

Fig. 1 The experimental timeline to study the effects of L-lactate and D-mannitol on GHB TK/TD in rats. All L-lactate and D-mannitol treatments were administered 5 min after the GHB dose.

were collected every 20 min. After stabilization, the probe was perfused with GHB 1 µg/ml in aCSF for 80 min to calculate *in vivo* probe recovery of GHB for each rat using the established retrodialysis method (24). After retrodialysis, blank aCSF was perfused through the probe for 80 min to remove any residual GHB (washout). Although GHB is endogenous, no GHB was detected in the final 2 washout samples. Rats were then administered GHB (600 mg/kg i.v., same GHB dose in all studies) and corresponding treatments were administered as described below. Plasma and dialysate samples were collected for 6 h post-dose, and times of loss of righting reflex and return to righting reflex (LRR and RRR) were recorded for each rat. Six hours after GHB administration, rats were sacrificed and the probe tracks were stained with dye. Brains were collected to confirm probe location and to ensure probe track condition. Blood samples were centrifuged at 1,000 g for 15 min at 4°C, and plasma, urine, and dialysate samples were stored at −80°C until analysis. Rat exclusion criteria included highly inflamed probe tracks as determined by visual observation, poor retrodialysis recovery **(< 5%),** and GHB concentration-time profiles with concentrations that were greater than 3 standard deviations from group means. Following these criteria resulted in the exclusion of at least 2, but no more than 4, rats in each treatment group. Data presented are from rats that met the criteria.

Experimental Study Design

The Effects of L-lactate on GHB Brain ECF Concentrations and Sleep-Time

To determine the dose-dependent effects of L-lactate administration on GHB brain ECF concentrations and sleep-time, rats ($n=3$/group) were prepared as described above and were administered GHB 600 mg/kg i.v. combined with L-lactate as an i.v. bolus followed by an i.v. infusion (low dose, bolus 66 mg/kg, infusion 302.5 mg/kg/h; high dose, bolus 330 mg/kg, infusion 605 mg/kg/h) 5 min after GHB administration. This lactate dose was calculated to elevate steady-state blood lactate concentrations from 1.5 to 2.5 mM for the low dose and above 4 mM for the high dose. Data from rats administered GHB 600 mg/kg alone was previously published (18) and used here for comparison purposes.

Effects of Mannitol on L-lactate Administration and GHB TK/TD

The goal of this study was to determine if the administration of D-mannitol altered the effects of L-lactate on GHB TK/TD. Rats were prepared as above ($n=3$/group) and administered either D-mannitol (500 mg/kg i.v.) alone or combined with L-lactate low dose (bolus 66 mg/kg, infusion 302.5 mg/kg/h) 5 min after administration of GHB 600 mg/kg i.v.. Times for LRR and RRR were recorded and kinetic parameters determined; values were compared with rats treated with GHB alone and GHB + L-lactate low dose.

L-lactate Pharmacokinetics in the Presence and Absence of GHB

In conjunction with the above studies, we characterized L-lactate plasma concentrations after a saline bolus, a saline bolus and infusion, and the administration of GHB 600 mg/kg, to determine if GHB administration or a saline infusion alters baseline L-lactate plasma concentrations. Rats were implanted with jugular and femoral cannulae and after 5 days of recovery, GHB or saline (vehicle) was administered and blood samples were collected over 6 h. Plasma was obtained and stored at −80°C until L-lactate concentrations were measured. L-lactate was measured in plasma samples obtained from rats with microdialysis probes implanted that were treated with GHB alone, GHB + L-lactate (low and high doses), and GHB + L-lactate (low dose) + D-mannitol. Lactate concentrations were measured using the 1500 Sport Lactate Analyzer (YSI Inc, Yellow Springs, OH).

Plasma, Microdialysate and Urine Sample Preparation and LC/MS/MS Analysis

Plasma, microdialysate and urine samples were all prepared as previously described (19,25). Briefly, plasma (50 µl) was combined with 5 µl of GHB-d6 (1 mg/ml) and GHB stock solution (or double-distilled water for samples). Acetonitrile was added to plasma samples to precipitate proteins and the

samples centrifuged. Double-distilled water was added to the supernatant and sample extraction was performed using Bond Elut SAX cartridges (Varian, Palo Alto, CA), prepared as described previously (19). Samples were reconstituted in 1.25 ml 0.1% formic acid in double-distilled water and 5% acetonitrile. For rats in the high dose lactate group and the low dose L-lactate + D-mannitol group, a separately validated modification of the sample preparation protocol was incorporated, omitting the solid-phase extraction step to simplify plasma sample preparation (detailed in (17)). Briefly, plasma proteins were precipitated using 0.1% formic acid in acetonitrile, samples were vortexed, and then centrifuged at 10,000 g for 20 min. Supernatant was then evaporated under a stream of nitrogen gas and samples were reconstituted in aqueous mobile phase. Microdialysis samples were diluted with aCSF to bring concentrations within the range of the standard curve. GHB-d6 (5 µl of 5 µg/ml) was added to 35 µl of microdialysate sample or standard and injected directly onto the LC/MS/MS. Urine samples (50 µl) were prepared for analysis by adding GHB-d6 (10 µl of 200 µg/ml), double-distilled water and acetonitrile; samples were centrifuged and the supernatant was collected for LC/MS/MS analysis.

For all LC/MS/MS analyses, an Agilent 1100 series HPLC with an online degasser, binary pump and autosampler (Agilent Technologies, Palo Alto, CA) linked to a PE Sciex API triple-quadrupole tandem mass spectrometer with a turbo ion spray (Applied Biosystems, Foster City, MA) was used. HPLC conditions, mass spectrometer parameters, linear calibration ranges and accuracy/precision are detailed in (19).

GHB Cell Uptake Studies

The immortalized rat brain capillary endothelial cell line (RBE4) was kindly provided by Professor P. Couraud (Institut Cochin, Paris). RBE4 cells (passages 39–44) were cultured as described previously (18). 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. RBE4 culture media was 1:1 alpha-minimum essentials medium/Hams F10 nutrient mixture supplemented with L-glutamine (2.0 mM), geneticin (300 µg/ml), human recombinant fibroblast growth factor (1 ng/ml), gentamicin (50 µg/ml), and 10% v/v qualified fetal bovine serum (FBS). Cells were passaged with 0.25% trypsin/EDTA and seeded on individual collagen-coated 35×10 mm wells for uptake and efflux studies, conducted 7 days post confluence (the time needed for formation of tight junctions)(26).

Uptake Studies

For lactate inhibition studies in RBE4 cells, cells were washed and equilibrated for 30 min at 37°C with uptake buffer containing 138 mM NaCl, 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM D-glucose, and 20 mM HEPES (pH7.4). Cells were then equilibrated to room temperature for 5 min and subsequently incubated for 15 s with 10 mM ^{3}H-GHB alone or with 1, 5, or 10 mM L-lactate or 10 or 100 mM D-mannitol. Previous work indicated that 15 s is within the time of linear uptake of GHB in RBE4 cells (18). A concentration of 10 mM GHB was used in these studies since it is similar to the high concentrations of GHB observed in rats after 600 mg/kg GHB i.v., and 1, 5, and 10 mM concentrations of L-lactate were used, as 1 mM approximates baseline L-lactate concentrations, 5 mM is a high L-lactate concentration reached after dosing (see Fig. 3), and 10 mM represents a very high L-lactate plasma concentration. The D-mannitol concentrations were chosen since 10 mM represents a low D-mannitol concentration, and 100 mM is a maximal concentration calculated to be reached after infusion of 1 M D-mannitol (a clinical dosing regimen), assuming D-mannitol only distributes into extracellular fluid. After incubation, cells were washed 3 times with ice-cold buffer and lysed with 0.5 ml 1.0 N NaOH for 60 min at room temperature. After lysis, NaOH was neutralized with 0.5 ml 1.0 N HCl. [^{3}H]-GHB accumulation was determined by liquid scintillation counting, and protein concentration was measured using bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. GHB uptake was normalized per mg of protein.

Efflux Studies

The time course of GHB efflux and the effects of treatments on GHB efflux were determined. Confluent RBE4 cells on individual collagen-coated wells were incubated with 10 mM [^{3}H]-GHB for 30 min at 37°C and subsequently allowed to equilibrate to room temperature (5 min). GHB-containing uptake buffer was removed and 1 ml uptake buffer was applied for 15 and 30 s and 1, 2, 5, and 10 min. At the designated time point, the uptake buffer was removed and the reaction was stopped with 3 washes of ice-cold uptake buffer, followed by cell lysis using 1.0 N NaOH. 1 N HCl was added to neutralize the NaOH and samples were aliquoted for liquid scintillation counting and analysis of protein expression using the bicinchoninic acid assay. The amount of remaining intracellular GHB was normalized per mg of protein. The effects of 100 mM D-mannitol, 10 mML-lactate (alone and combined), 3 and 15 mM GHB with or without 10 mML-lactate, and 2.5 mM α-cyano-4-hydroxycinnamate (CHC) (negative control) on the amount of GHB efflux were determined by applying these solutions (prepared in uptake buffer) for 0.5 and 2 min after removal of GHB-containing uptake buffer (onset of efflux). Three separate studies were performed with triplicate determinations in each study.

Data and Statistical Analysis

To calculate the relative *in vivo* recovery of GHB from the dialysis probe, retrodialysis recovery was used as described previously (19). The recovery of GHB from the probe was calculated using the equation below:

$$Recovery_{Probe} = \frac{C_{perfusate} - C_{dialysate}}{C_{perfusate}} \quad (1)$$

where $C_{perfusate}$ is the perfusate GHB concentration (inlet) and $C_{dialysate}$ is the dialysate GHB concentration (outlet). To determine GHB concentrations in frontal cortex ECF, dialysate GHB concentrations after i.v. administration of GHB were divided by the experimentally determined GHB probe recovery.

The area under the concentration-time curve (AUC) for GHB in plasma and brain ECF was calculated by the log-linear trapezoidal method using Phoenix WinNonlin 6.0 (Pharsight Corp., Mountain View, CA). Time-averaged renal clearance was calculated by dividing the total amount of GHB excreted in urine (Ae,inf) by the GHB plasma AUC. Overall unbound brain ECF/plasma partition coefficients for GHB ($K_{p,u,u}$) were calculated by dividing the brain GHB ECF AUC by the GHB plasma AUC. GHB is not bound to plasma proteins, so plasma concentrations represent unbound concentrations (20). Sleep time (minutes) was determined as the difference between RRR and LRR. GHB concentrations in ECF and plasma at RRR were calculated using noncompartmental methods. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Inc., San Diego, CA). Statistical differences between group means were assessed using one-way ANOVA with nonparametric analysis (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

RESULTS

The Effects of L-lactate and D-mannitol Administration on GHB TK/TD

Toxicokinetics

Plasma and frontal cortex extracellular GHB concentration-time profiles collected during *in vivo* microdialysis after administration of GHB 600 mg/kg alone or in the presence of two L-lactate dosing regimens (low dose, bolus 66 mg/kg, infusion 302.5 mg/kg/h and high dose, bolus 330 mg/kg, infusion 605 mg/kg/h) are shown in Fig. 2. In addition, the osmotic diuretic D-mannitol was administered alone or combined with low dose L-lactate (Fig. 2). When low dose L-lactate was administered, GHB plasma concentrations were substantially reduced beginning 90 min after GHB administration (Fig. 2a). L-lactate also elicited a decline in GHB frontal cortex ECF concentrations 180 min post-GHB administration (Fig. 2b). The administration of D-mannitol alone did not significantly alter GHB plasma or frontal cortex ECF concentration-time profiles, although a trend towards higher GHB ECF concentrations was observed. The combined administration of D-mannitol with L-lactate did not alter the effect of L-lactate alone on GHB plasma or ECF concentration-time profiles. Administration of the high L-lactate dose resulted in a substantial decrease in both plasma and ECF GHB concentrations. Plasma GHB concentrations in the presence of the high lactate dose were lower than other treatment groups 90 min post-GHB administration (Fig. 2a), and average ECF GHB concentrations were lower immediately (midpoint of 10 min, during the first fraction collected (Fig. 2b), although changes were not statistically different.

Noncompartmental analysis of the GHB plasma and brain ECF concentration-time profiles after the administration of L-lactate as low or high doses, D-mannitol alone, or combination of low-dose L-lactate and D-mannitol are shown in Table I. Administration of low-dose L-lactate increased GHB time-averaged renal clearance and reduced GHB plasma AUCs with a trend towards lower ECF AUCs. This was reflected in similar brain:blood partition coefficients, as seen with GHB alone. The high L-lactate dose resulted in an even greater increase in GHB time-averaged renal clearance, as observed by the high fraction of the GHB dose excreted in urine, which reduced GHB plasma AUCs and significantly reduced GHB ECF AUCs and GHB brain:blood partitioning. D-mannitol alone did not significantly alter GHB renal clearance or GHB plasma and ECF AUCs.

Toxicodynamics

The low dose of L-lactate significantly reduced sleep-times after GHB administration from 137 min to 90 min (Table I, $p<0.05$). Plasma and brain ECF concentrations of GHB at RRR were unchanged with the administration of this L-lactate dose. The high dose of L-lactate resulted in higher plasma concentrations of GHB at RRR ($p<0.05$), but ECF GHB concentrations at RRR were unchanged. D-mannitol alone did not significantly alter GHB-induced sleep times compared to GHB alone. D-mannitol combined with L-lactate reduced sleep times from 137 min to 99 min, similar to the effect of L-lactate alone (Table I, $p<0.05$). Brain plasma and ECF concentrations of GHB at RRR when D-mannitol was administered alone or with L-lactate were higher but not significantly different than those of GHB alone.

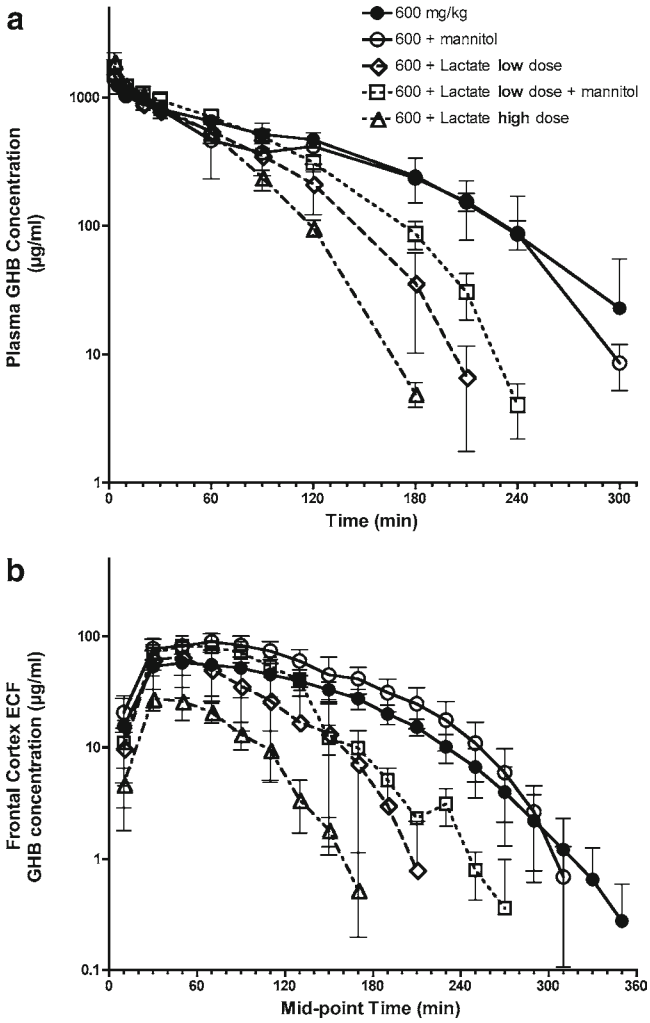

Fig. 2 GHB plasma (**a**) and brain ECF (**b**) concentration-time profiles after GHB administration in the presence and absence of L-lactate and D-mannitol treatments. L-lactate was administered 5 min after GHB dosing either as a low dose (bolus 66 mg/kg, infusion 300 mg/kg/h) or a high dose (bolus 330 mg/kg, infusion 605 mg/kg/h). A separate group of rats received D-mannitol 500 mg/kg 5 min after GHB dosing with or without the low dose of L-lactate. Data shown are mean ± SD, $n = 4$ for GHB alone, $n = 3$ for L-lactate and mannitol treatments.

Table I Plasma and Brain ECF Concentration-Time Profiles and Toxicodynamic Endpoints (Sleep Time, GHB Concentrations in Plasma and ECF at RRR) for Rats Administered 600 mg/kg GHB i.v Alone or with L-lactate, D-mannitol, or L-lactate and D-mannitol Combined Administered 5 Min after the GHB Dose

GHB dose (mg/kg)	Toxicokinetics					Toxicodynamics		
	Plasma AUC (mg*min/ml)	Brain ECF AUC (mg*min/ml)	Overall partition coefficient (brain AUC/plasma AUC)	GHB renal clearance (ml/min/kg)	F_e (mean ± SD)	Sleeptime (min)	GHB C_p at RRR (µg/ml)	GHB C_{ecf} at RRR (µg/ml)
600	123.7 ± 23	8.7 ± 3	0.070 ± 0.02	1.40 ± 0.1	0.29 ± 0.07	137.6 ± 12	373.2 ± 46	38.8 ± 16
600 + Lactate (low dose)	79.6 ± 16[a]	5.7 ± 3	0.069 ± 0.02	2.42 ± 0.2[a]	0.32 ± 0.03	90.7 ± 16[a]	339.4 ± 26	34.3 ± 15
600 + Mannitol	116.0 ± 9	13.2 ± 6	0.112 ± 0.04	1.60 ± 0.3	0.30 ± 0.04	128.7 ± 12	385.8 ± 59	62.9 ± 26
600 + Lactate (high dose)	78.9 ± 9[a]	2.1 ± 0.6[a]	0.027 ± 0.08[a]	4.68 ± 0.5[a,b]	0.60 ± .04[a]	55.3 ± 5[a,b]	544.7 ± 96[a]	23.3 ± 6
600 + Lactate (low dose) + Mannitol	107.4 ± 5	9.3 ± 3	0.087 ± 0.03	2.60 ± 0.4	0.45 ± 0.07	99.3 ± 7[a]	425.2 ± 30	55.4 ± 9

Lactate was administered 5 min after GHB dosing as a bolus of 66 mg/kg and infusion of 302.5 mg/kg/h. High-dose lactate was a bolus of 330 mg/kg and infusion of 605 mg/kg/h. A separate group of rats received a bolus of D-mannitol 500 mg/kg 5 min after GHB dosing with and without L-lactate (low dose). Data shown are mean ± SD, $n = 4$ for GHB alone, $n = 3$ for lactate and mannitol treatments

[a] $p < 0.05$ when compared to control

[b] $p < 0.05$ when compared to low dose lactate treatment

L-lactate Pharmacokinetics after GHB and L-lactate Administration

L-lactate concentrations were measured in rats administered a bolus of GHB or a bolus and infusion of saline to determine if GHB or a saline infusion altered L-lactate concentrations. GHB administration did not significantly alter baseline L-lactate concentrations (Data not shown). A saline bolus or a saline bolus plus infusion did not alter baseline L-lactate concentrations, and L-lactate plasma concentration-time data from the saline treatments and GHB 600 mg/kg i.v. were combined and compared to the effects of L-lactate administration .

The administration of the low dose of L-lactate (bolus 66 mg/kg, infusion 302.5 mg/kg/h) resulted in a moderate increase in plasma L-lactate concentrations, from baseline concentrations of 1.5 mM to a value of 2.5 mM. Plasma L-lactate concentrations slightly declined to 2 mM during the final 4 h of sampling. D-mannitol did not alter L-lactate plasma concentrations after the low dose, and the L-lactate plasma concentrations from both groups were combined. Administration of the high dose of L-lactate (bolus 330 mg/kg, infusion 605 mg/kg/h) elicited a rapid increase in plasma lactate concentrations to 6.5 mM, and remained above 4 mM for 90 min, after which concentrations declined to about 3 mM for the remainder of the study.

Effects of L-lactate and D-mannitol on GHB Uptake *In Vitro*

Since MCT1 is highly expressed at the BBB (13), we examined the effects of L-lactate on the uptake of 10 mM GHB in RBE4 cells (which also expresses MCT1, (27)) as an *in vitro* model of the rat BBB. This concentration of GHB was used since it is near the K_m for uptake *via* MCT1 at pH7.4 (Morse *et al.* 2012), and is similar to GHB plasma concentrations after the i.v. administration of 600 mg/kg GHB. When L-lactate was applied at a concentration of 5 or 10 mM, the uptake of 10 mM GHB was inhibited to 74% and 64% of control (Fig. 3a, $p<0.05$). When D-mannitol was added at concentrations of 10 and 100 mM acutely (15 s prior to GHB) or for a longer period (30 min preincubation at 37°C), the uptake of 10 mM GHB was not changed (Fig. 3b).

GHB Efflux and Effects of L-lactate, D-mannitol, and GHB *In Vitro*

To further study the transport of GHB at the BBB, we conducted efflux studies in RBE4 cells to determine if D-mannitol or L-lactate altered GHB efflux. The time-course for GHB efflux from RBE4 cells is shown in Fig. 4a. The time-points of 0.5 and 2 min after the onset of efflux were chosen to study the effects of treatments on GHB efflux from RBE4 cells. The addition of 100 mM D-mannitol increased the efflux of GHB at 0.5 min such that the remaining intracellular GHB was 78% of control ($p<0.05$ compared to control); there was no effect at 2 min. The addition of 10 mM L-lactate resulted in a significant increase in GHB efflux (remaining intracellular GHB was 55% of control, $p<0.05$), and combining D-mannitol with L-lactate also resulted in increased GHB efflux (intracellular GHB remaining was 38% of control, $p<0.05$). To determine if GHB alone could increase its own efflux, we added unlabeled GHB at concentrations of 3 and 15 mM and observed increased GHB efflux (intracellular GHB remaining was 56% and 47% of control, $p<0.05$), which also was further increased when L-lactate was added with GHB (all to less than 38% of control, $p<0.05$). The MCT inhibitor CHC produced a modest but significant increase in GHB efflux at 0.5 min, but not at 2 min.

DISCUSSION

L-lactate and GHB are both substrates for MCT1, a transporter present in the renal proximal tubule that is largely responsible for GHB renal reabsorption. Previous studies demonstrated that L-lactate increases GHB renal clearance and reduces GHB-induced sleep time (15,19,20). The work here extends the previous findings to study the dose-dependent effects of L-lactate administration on GHB TK/TD *in vivo* in rats, including effects on GHB frontal cortex ECF concentrations, and characterizes the mechanisms underlying GHB transport in an *in vitro* model of the rat BBB. Our results demonstrate that L-lactate administration dose-dependently increased GHB renal clearance with higher doses resulting in greater reductions in GHB plasma and frontal cortex ECF concentrations and reduced GHB sleep time.

Since MCT1 is present at the BBB (13) and is largely responsible for GHB brain uptake (18,28), we hypothesized that L-lactate administration would inhibit GHB distribution into the brain. Our results indicated that a low dose of L-lactate (66 mg/kg bolus, 302.5 mg/kg/h infusion) increased GHB renal clearance and reduced GHB exposure as indicated by plasma and brain ECF AUCs, but the overall brain:blood partition coefficient was not changed (Table I). This indicates no change in GHB transport at the BBB, and that GHB brain concentrations correlate to plasma concentrations, as demonstrated previously (18,19). The plasma and brain ECF GHB concentrations at RRR were unchanged from GHB alone, consistent with previous findings that plasma and brain GHB concentrations correlate with RRR (18,19). The high dose of L-lactate (330 mg/kg bolus, 605 mg/kg/h infusion) resulted in a significant

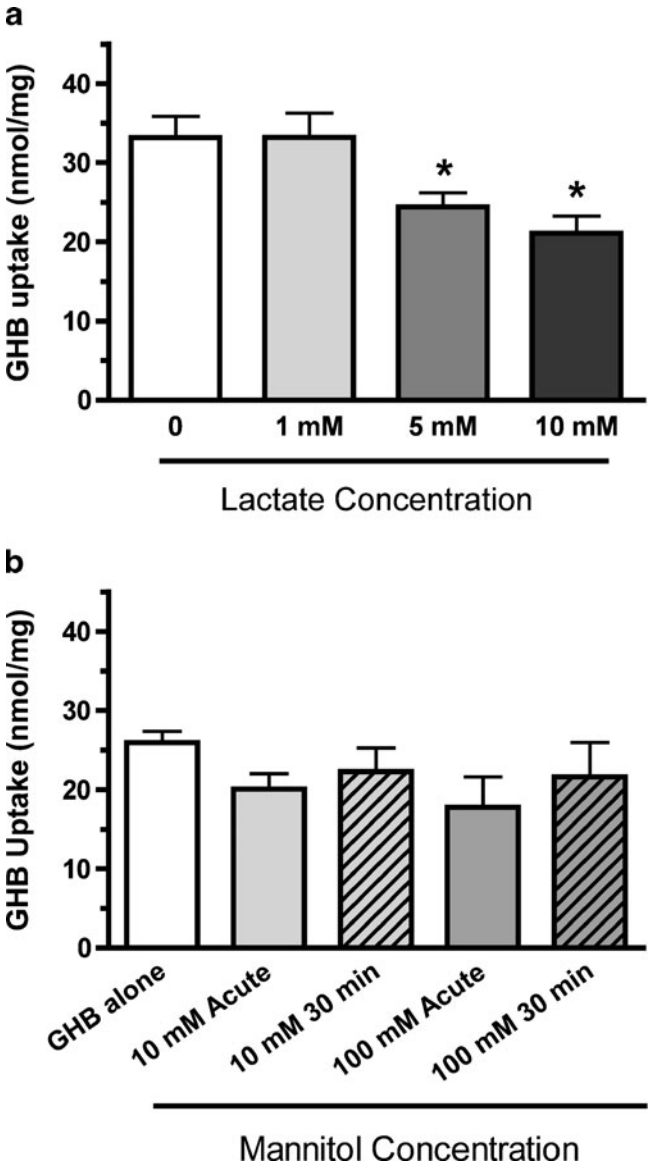

Fig. 3 (**a**) Effects of L-lactate (1, 5, and 10 mM) on the uptake of GHB (10 mM) in RBE4 cells. L-lactate 5 and 10 mM reduced the uptake of GHB to 74% and 64% of control, respectively ($p < 0.05$). Data represent mean ± SEM of three sets of studies conducted in triplicate. (**b**) Effects of D-mannitol (10 and 100 mM) on the uptake of GHB (10 mM) in RBE4 cells. D-mannitol was either applied acutely with GHB (acute) or cells were pre-incubated for 30 min at 37°C with mannitol (chronic). No significant differences in the uptake of GHB were observed. Data represent mean ± SEM of three sets of studies conducted in triplicate.

increase in GHB renal clearance and in the total fraction of the GHB dose excreted in urine (Table I). The GHB plasma AUC was not significantly reduced beyond that of the low L-lactate dose. However, the brain ECF AUC was significantly reduced, and GHB blood–brain partitioning was significantly lower as well, suggesting that GHB transport at the BBB was altered in the presence of the high L-lactate dose. This resulted in a significant reduction in GHB sleep time. The ECF GHB concentration at RRR was not changed; however, the plasma GHB concentration at RRR was significantly higher, consistent with the hypothesis that GHB concentrations in the frontal cortex ECF are more closely connected with GHB's effects in the brain.

The low dose of L-lactate administered in this study increased plasma L-lactate concentrations by 1 mM, which remained fairly stable throughout the 6 h sampling period. The high dose of L-lactate produced plasma L-lactate concentrations of approximately 5 mM above baseline which declined to 1.5 mM above baseline by 120 min. Recent findings from our laboratory indicated that GHB itself may alter L-lactate plasma concentrations when GHB is administered at high doses (17); however, this was not observed at the dose level used in the present study. In the present study, we observed a greater effect of L-lactate on renal elimination, while effects on BBB transport were only observed with the high L-lactate dose. These finding can be attributed to the differences in the pH gradients that are present in the renal tubules and at the BBB. MCT1 is dependent on the presence of a proton gradient and affinity of substrates for this transporter will vary, dependent on this gradient. The K_m for MCT1-mediated GHB transport shifts from 2.2 to 17 mM when the extracellular pH is raised from 6.5 to 7.4 (29). Similarly, while 2 mM L-lactate significantly inhibits GHB transport in rat kidney vesicles at pH

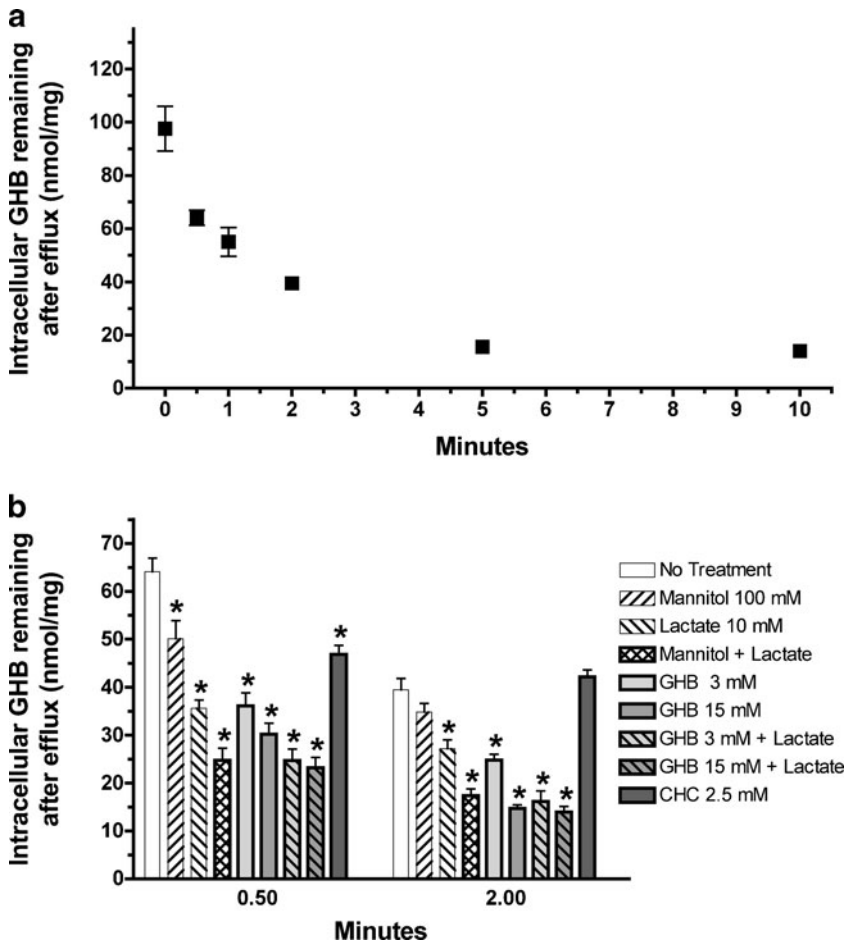

Fig. 4 (**a**) Time-course of GHB efflux in RBE4 cells. Cells were incubated with GHB (10 mM) for 30 min at 37°C. Efflux was characterized at room temperature by the removal of GHB and application of uptake buffer for the specified time points. Data represent the mean ± SEM of 3 experiments conducted in triplicate, plotted as intracellular GHB remaining after efflux. (**b**) Effects of GHB (3 and 15 mM), L-lactate (10 mM), D-mannitol (100 mM), and CHC (2.5 mM) on the efflux of GHB (10 mM) at 0.5 and 2 min. At 0.5 min, D-mannitol, L-lactate, GHB, and CHC, as well as L-lactate combined with D-mannitol or GHB increased the efflux of GHB compared to control. At 2 min, only L-lactate or GHB alone or L-lactate combined with D-mannitol or GHB increased the efflux of GHB. Data represent the mean ± SEM of 3 experiments conducted in triplicate, plotted as intracellular GHB remaining after efflux.

5.5 (30), the IC_{50} for L-lactate inhibition of GHB uptake is 19.1 mM in red blood cells at pH7.4, with no inhibition observed when L-lactate concentrations are less than 5 mM (29). Therefore, L-lactate has a greater affinity towards inhibiting renal reabsorption in the renal proximal tubule lumen, where there is a pH gradient from lumen to blood. At the physiological pH of 7.4 present in blood perfusing the BBB, it would be expected that only very high concentrations of L-lactate would significantly inhibit MCT1-mediated GHB uptake into the brain, which is consistent with our current findings.

In vitro studies were conducted using RBE4 cells as an *in vitro* model of the rat BBB (31) to characterize the BBB transport of GHB. We previously characterized the time- and concentration-dependent uptake in RBE4 cells at pH 7.4 (18). Here, the uptake of 10 mM GHB was inhibited to 75% of control when L-lactate 5 mM was added, and inhibited to 64% of control with 10 mM L-lactate. When RBE4 cells were preincubated with GHB, both L-lactate and GHB increased GHB efflux from the cells. Since MCTs are bidirectional (32), these findings suggest that *trans* stimulation plays a role in increasing GHB efflux. The *in vivo* setting is likely complex, where multiple processes and transporters may be involved. It is probable that both the inhibition of GHB uptake and stimulation of GHB efflux play a role in the alteration of GHB BBB transport when high L-lactate concentrations are present.

The osmotic diuretic D-mannitol has been demonstrated to reduce intracranial pressure *via* increasing BBB permeability due to vasodilation and shrinkage of endothelial cells, thereby affecting BBB uptake (reviewed in (21)). Previous work from our laboratory had demonstrated that a bolus and infusion of 10% w/v D-mannitol reduced steady state plasma GHB concentrations and increased GHB renal and total clearances (20). Additionally, we previously reported that the co-administration of the osmotic diuretic D-mannitol, at the dose used in the present study (0.5 g/kg), enhanced the effects of Lactated Ringer's injection (containing 28 mM L-lactate) on GHB TK/TD *in vivo* by increasing the renal and total clearances of GHB following the administration of 1,000 mg/kg GHB i.v. (15). However, it was not clear by what mechanism D-mannitol enhanced the effects of Lactated Ringer's injection. In the same study, D-mannitol 0.5 g/kg i.v. did not significantly affect GHB TK, beyond the effects of L-lactate alone (60 mg/kg/h), when GHB was infused (208 mg/kg/h) to steady state concentrations of 0.62 mg/ml. Here, experiments were performed using brain microdialysis *in vivo* and with rat brain

endothelial RBE4 cells in order to gain mechanistic information regarding the effect of mannitol on GHB disposition and understand the controversial findings. D-mannitol itself had no significant effect on GHB TK/TD, and no effect on GHB TK/TD was observed after administration of D-mannitol with a low dose of L-lactate *in vivo*, as previously reported by Wang *et al* (15). The effects of Lactated Ringer's injection on GHB may be related to the higher dose of GHB administered in that study (1,000 mg/kg compared with 600 mg/kg in the present study), or to the use of L-lactate in the form of Lactated Ringer's injection, or possibly due to the larger volume of fluid administered when L-lactate was administered as Lactated Ringer's injection (300 μl/min). Therefore, further studies are needed to evaluate the effects of D-mannitol when administered in combination with Lactated Ringer's injection on GHB TK/TD. Our *in vitro* studies demonstrated that the uptake or efflux of GHB from rat brain endothelial RBE4 cells was not significantly altered by D-mannitol. The *in vitro* findings are not surprising, given the role of D-mannitol in increasing BBB permeability *via* opening of tight junctions rather than facilitating intracellular transport. GHB ECF concentrations did trend higher in the presence of D-mannitol compared to GHB alone, consistent with increased paracellular transport of GHB into the brain. However, based on our current studies, we were not able to confirm a substantial effect of mannitol on GHB TK/TD.

GHB overdose results in coma, sedation, respiratory depression, and death, most likely due to its actions at $GABA_B$ receptors in the brain (16). One potential treatment for GHB overdose is the administration of L-lactate to increase GHB's renal clearance. Data here suggest that if sufficiently high L-lactate concentrations are reached, GHB-induced sedative/hypnotic effects can be overcome through the inhibition of renal reabsorption as well as alterations in GHB transport at the BBB. The highest L-lactate concentrations obtained in this study (5 mM) are physiologically relevant values, since endogenous L-lactate plasma concentrations of 10 mM are reached after at least 20 min of moderate-intensity exercise (33). The exogenous administration of L- lactate has been demonstrated to have both inhibitory and stimulatory effects on respiration and may also result in electrolyte imbalances (34). As respiratory depression is the primary factor resulting in GHB fatality, it is critical to understand the impact of L-lactate on this measure. Previous work in our laboratory has demonstrated that L-lactate administered alone targeting an increase of 2 mM in plasma L-lactate concentrations results in no measurable effects on respiration (17) or electrolyte balance (15). Similarly, no adverse effects were measured when sodium lactate (1/6 M solution) was administered to humans co-administered GHB (35). These results suggest that treatment with L-lactate may represent a safe and efficacious therapeutic strategy for the treatment of GHB overdose.

In summary, we demonstrated that L-lactate dose-dependently increased GHB renal clearance and reduced GHB sedative/hypnotic effects. The use of microdialysis *in vivo* demonstrated that brain ECF GHB concentrations provided a direct correlation with the offset of GHB's sedative/hypnotic effect (RRR). Studies of GHB transport *in vitro* were consistent with the *in vivo* data suggesting alterations in BBB transport with high L-lactate concentrations. The findings here further support the role of L-lactate as a potential treatment for GHB overdose.

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