

L-Ornithine and Phenylacetate Synergistically Produce Sustained Reduction in Ammonia and Brain Water in Cirrhotic Rats

Nathan A. Davies,^{1*} Gavin Wright,^{1*} Lars M. Ytrebø,² Vanessa Stadlbauer,¹ Ole-Martin Fuskevåg,³ Claudia Zwingmann,⁴ D. Ceri Davies,⁵ Abeba Habtesion,¹ Stephen J. Hodges,¹ and Rajiv Jalan¹

Treatment of hyperammonemia and hepatic encephalopathy in cirrhosis is an unmet clinical need. The aims of this study were to determine whether L-ornithine and phenylacetate/phenylbutyrate (administered as the pro-drug phenylbutyrate) (OP) combined are synergistic and produce sustained reduction in ammonia by L-ornithine acting as a substrate for glutamine synthesis, thereby detoxifying ammonia, and the phenylacetate excreting the ornithine-derived glutamine as phenylacetylglutamine in the urine. Sprague-Dawley rats were studied 4 weeks after bile duct ligation (BDL) or sham operation. Study 1: Three hours before termination, an internal carotid sampling catheter was inserted, and intraperitoneal saline (placebo), OP, phenylbutyrate, or L-ornithine were administered after randomization. BDL was associated with significantly higher arterial ammonia and brain water and lower brain myoinositol ($P < 0.01$, respectively), compared with sham-operated controls, which was significantly improved in the OP-treated animals; arterial ammonia ($P < 0.001$), brain water ($P < 0.05$), brain myoinositol ($P < 0.001$), and urinary phenylacetylglutamine ($P < 0.01$). Individually, L-ornithine or phenylbutyrate were similar to the BDL group. In study 2, BDL rats were randomized to saline or OP administered intraperitoneally for 6 hours or 3, 5, or 10 days and were sacrificed between 4.5 and 5 weeks. The results showed that the administration of OP was associated with sustained reduction in arterial ammonia ($P < 0.01$) and brain water ($P < 0.01$) and markedly increased arterial glutamine ($P < 0.01$) and urinary excretion of phenylacetylglutamine ($P < 0.01$) in each of the OP treated groups. **Conclusion: The results of this study provide proof of the concept that L-ornithine and phenylbutyrate/phenylacetate act synergistically to produce sustained improvement in arterial ammonia, its brain metabolism, and brain water in cirrhotic rats. (HEPATOLOGY 2009;50:155-164.)**

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Hepatic encephalopathy (HE) incorporates a spectrum of mental disturbances observed in patients with liver disease, ranging from minimal effects on quality of life to coma and death.¹ Hyperammonemia is considered central to the pathogenesis of

HE, with arterial ammonia levels correlating with severity of intracranial hypertension and prediction of deaths from cerebral herniation in acute liver failure commensurate with an increase in brain delivery and uptake of ammonia.^{2,3} In cirrhosis, induction of hyperammonemia has been shown to be associated with deterioration in neuropsychometric tests, worsening of brain osmolytes, and

Abbreviations: BDL, bile duct ligation; HD, high-protein/ammoniogenic; HE, hepatic encephalopathy; ¹H-NMR, proton nuclear magnetic resonance; LOLA, L-ornithine, L-aspartate; OP, L-ornithine, phenylacetate; SEM, standard error of the mean.

From the ¹Liver Failure Group, Institute of Hepatology, University College London, UK; ²The Department of Anaesthesiology, University Hospital of Northern Norway and University of Tromsø, Norway; ³The Department of Clinical Pharmacology, University Hospital of Northern Norway and University of Tromsø, Norway; the ⁴Center of Research, Department of Medicine, University of Montreal, Quebec, Canada; and the ⁵Division of Surgery, Oncology, Reproductive Biology and Anaesthetics, Imperial College London, UK.

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*Dr. N. Davies and Dr. G. Wright have contributed equally to this paper and are joint first authors.

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Address reprint requests to: Rajiv Jalan, Institute of Hepatology, University College London, 69-75 Chenies Mews, London WC1E 6H, UK. E-mail: r.jalan@ucl.ac.uk; fax: (44) 207-380-0405.

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Potential conflict of interest: UCL has filed patents surrounding the use of L-Ornithine and Phenylacetate for the treatment of hyperammonemia and hepatic encephalopathy which has been licensed to Ocera Therapeutics.

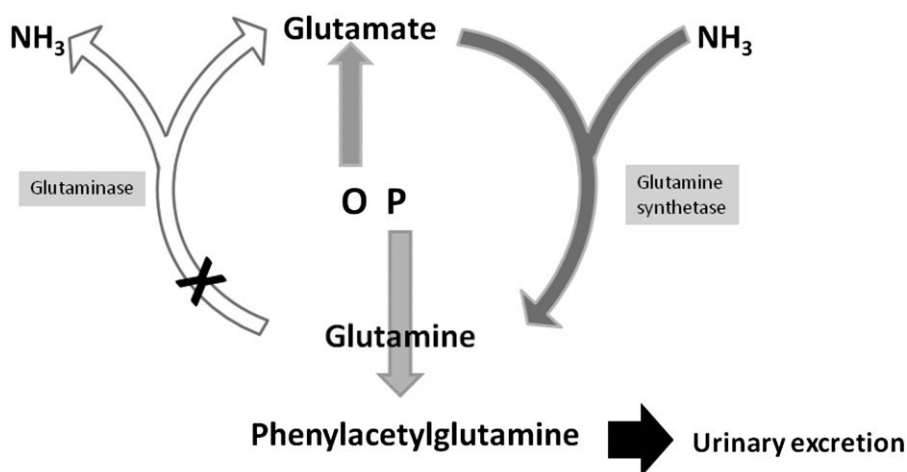


Fig. 1. Schematic demonstrating the glutamate-glutamine cycling and the hypothesized mechanism of action of the synergy between L-ornithine and phenylacetate (OP) in reducing ammonia concentration in liver failure. L-ornithine acts as a substrate for glutamine synthetase, thereby detoxifying ammonia into glutamine. Phenylacetate combines with the glutamine that is generated, excreting it as phenylacetylglutamine.

increase in brain water.⁴ The severity of HE in cirrhosis correlates with plasma ammonia levels, confirming that ammonia lowering is an important goal for the treatment of HE. Therapy of hyperammonemia and HE in patients with liver disease remains an unmet clinical need because the current ammonia-lowering therapies for HE have not been conclusively proven to be of value.^{5,6}

Studies of interorgan metabolism in liver failure have suggested that when the ammonia-removing capacity of the liver is reduced, the muscles, gut, and the kidneys interact to try and maintain ammonia levels.⁷⁻¹⁰ Glutamine acts as both a sink for excess ammonia, by ammonia combining with glutamate to produce glutamine (through the enzyme glutamine synthetase), or as a source for ammonia release (through the enzyme glutaminase).¹¹ Skeletal muscle glutamine synthetase activity is normally low, but has been shown to be up-regulated in liver failure.^{7,12} Therefore, supplying glutamate to the large mass of skeletal muscle, increasing glutamine production, can be an alternative therapeutic target for ammonia detoxification.

Because glutamate is not readily transported into muscle cells, glutamate can be provided to the muscle as L-ornithine, consuming ammonia to produce glutamine. This mode of delivery is used by the agent L-ornithine L-aspartate (LOLA), which has been used for the treatment of the HE. However, although some randomized controlled trials with LOLA have reported reductions in plasma ammonia and improved psychometric test scores,^{13,14} recent observations suggest that the reduction in plasma ammonia with administration of LOLA may be associated with a later increase in ammonia once LOLA is discontinued.¹⁵ This is likely attributable to a significant rise in glutamine levels, which eventually becomes a source for ammoniagenesis by the kidney and gut through the effects of glutaminase.⁸ This preliminary observation will have to be confirmed in future studies.

Phenylacetate (administered as the pro-drug phenylbutyrate), which has been used to treat the hyperammonemia of urea cycle enzyme deficiencies,¹⁶ can effectively remove excess glutamine to make phenylacetylglutamine, which can be excreted by the kidneys, thereby removing it as a substrate for ammoniagenesis. However, unlike urea cycle enzyme deficiency in which the liver is normal and usually the glutamine concentrations are markedly elevated, in cirrhosis the liver is dysfunctional and the plasma glutamine concentrations are variable, usually normal or mildly elevated,^{4,8,9,15,16} making it unlikely that phenylacetate alone would be a useful treatment for HE. These observations have led to the search for a novel ammonia-lowering therapy, combining L-ornithine and phenylacetate/phenylbutyrate (OP),¹⁵ for the treatment of hyperammonemia and HE. OP has the advantage that any ammonia trapped as glutamine (derived from the administered L-ornithine) will not be available for later return to the circulation, resulting in net removal and reduction in ammonia concentration (Fig. 1).

The aims of this proof of concept study were to determine whether the combination of L-ornithine and phenylacetate/phenylbutyrate are synergistic in reducing hyperammonemia by L-ornithine acting as a substrate for glutamine synthesis, thereby detoxifying ammonia and phenylacetate, excreting the ornithine-derived glutamine as phenylacetylglutamine in the urine. To test our hypothesis,¹⁵ we chose our well-characterized bile duct ligated (BDL) rat model of cirrhosis because it exhibits characteristic hyperammonemia, alterations in brain osmolytes, and cytotoxic brain edema.¹⁷ The first part of the study was a 3-hour study to determine whether L-ornithine and phenylbutyrate were synergistic in reducing ammonia in this model compared with L-ornithine or phenylbutyrate alone. The second part of the study was designed to determine whether administration of OP

Table 1. Longitudinal Changes (Over 3 hours) in the Plasma Ammonia, Ornithine, Glutamate, and Glutamine

	Hours	Sham	BDL (HD)	BDL (HD) + O	BDL (HD) + P	BDL (HD) + OP
Ammonia	0	59 ± 9	202 ± 20 ^a	218 ± 31	211 ± 20	232 ± 19
	1		211 ± 16	232 ± 44	300 ± 40	220 ± 24
	3	57 ± 7	198 ± 24	217 ± 14	256 ± 21	151 ± 10*
Ornithine	0	54 ± 12	51 ± 5	48 ± 4	46 ± 2	68 ± 14
	1		42 ± 2	448 ± 65*	49 ± 6	387 ± 35**
	3	57 ± 9	66 ± 2	114 ± 20	45 ± 2	239 ± 82
Glutamate	0	109 ± 15	151 ± 7	256 ± 63	139 ± 11	135 ± 6
	1		126 ± 9	205 ± 74	207 ± 44	320 ± 55
	3	115 ± 14	165 ± 19	172 ± 14	174 ± 30	212 ± 53
Glutamine	0	517 ± 32	605 ± 40	487 ± 62	633 ± 25	442 ± 62
	1		576 ± 25	663 ± 41	651 ± 35	663 ± 41
	3	524 ± 26	670 ± 11	649 ± 52*	516 ± 58	735 ± 67*

Data are expressed as mean ± standard error of mean (SEM). Symbols represent; ^a $p < 0.001$ compared to Sham group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to baseline (T = 0 hours) for that group. Concentrations of ammonia, ornithine, glutamate, and glutamine are expressed in $\mu\text{mol/L}$.

Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylbutyrate; P, Phenylbutyrate; and O, L-ornithine, HD, hyperammonemic diet.

achieved sustained reduction in ammonia over a 10-day period.

Materials and Methods

All animal experiments were conducted according to Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley rats (body weight 230-280 g) were used (Charles River Laboratories UK Ltd.). All rats were housed in the unit and given free access to standard rodent chow and water, with a light/dark cycle of 12 hours, at a temperature of 19°C to 23°C and humidity of approximately 50%.

Animal Model

BDL. Under anesthesia (diazepam 1 mg/kg intravenously, followed by Hypnorm 150 $\mu\text{L/kg}$ intramuscularly, Janssen Pharmaceutica, Belgium), all rats underwent BDL to induce biliary cirrhosis, or a sham operation as described previously.¹⁸ In study 1, the BDL rats were administered a high-protein/ammoniogenic (HD) diet for 7 days before inclusion in the study. The diet consisted of a liquid rodent feed (BioServe, Frenchtown, NJ) and a tailor-made mixture mimicking the amino acid composition of hemoglobin molecule (4 g/kg/

day Nutricia, Cuijk, The Netherlands) as described previously,^{4,9} mixed with commercially available gelatin to prevent sedimentation. As described previously, this regimen produces chronic hyperammonemia.¹⁹ In the BDL animals in study 2, the animals were not administered this ammoniogenic diet before or during the study period to allow longer duration experiments without the effect of the variability that may be induced by the diet and allow survival of animal into the 5th week so that at least a 10-day treatment could be achieved. These animals became spontaneously hyperammonemic; however, ammonia concentration was lower in this group of animals at the time of sacrifice compared with the animals in study 1 by approximately 20 $\mu\text{mol/L}$ (Tables 1 and 2).

Study Design

The following two studies were performed sequentially.

Study 1. Four weeks after surgery, the BDL animals were randomized into four groups. Three hours before termination, intraperitoneal injections of L-ornithine (0.3 g/kg), phenylbutyrate (0.3 g/kg), combined L-ornithine (0.3 g/kg) and phenylbutyrate (0.3 g/kg), or saline (placebo) were administered. The sham animals were

Table 2. Longitudinal Changes (Over 10 Days) in Plasma Ammonia, Ornithine, Glutamate, and Glutamine

	Sham-Operated	BDL	BDL + OP 6 Hours	BDL + OP 3 Days	BDL + OP 5 Days	BDL + OP 10 Days
Ammonia	67 ± 6	186 ± 20*	76 ± 3†	69 ± 17‡	91 ± 24†	87 ± 23†
Ornithine	60 ± 35	51 ± 54	497 ± 56†	322 ± 43†	1293 ± 395‡	1313 ± 279‡
Glutamate	101 ± 21	169 ± 21	132 ± 14	256 ± 79	196 ± 27	238 ± 75
Glutamine	490 ± 25	525 ± 36	1193 ± 117†	1198 ± 291†	1297 ± 139†	1224 ± 143†

Data are expressed as mean ± standard error of mean (SEM). Symbols: * $P < 0.001$ compared with sham-operated control rats; † $P < 0.01$ and ‡ $P < 0.001$ compared with BDL control rats. Concentrations of ammonia, ornithine, glutamate, and glutamine are expressed in $\mu\text{mol/L}$.

Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylbutyrate.

treated in the same manner but administered saline intraperitoneally. The final study groups were: (1) Sham-operated + saline (n = 7); (2) BDL (HD) + saline (n = 7); (3) BDL (HD) + ornithine (n = 7); (4) BDL (HD) + phenylbutyrate (n = 8); and (5) BDL (HD) + OP (n = 8).

On the day of the experiment, after anesthesia and immediately before the administration of study medication, a left internal carotid catheter (0.96 outer diameter Portex fine-bore polythene tubing, Scientific Laboratory Supplies Ltd, Nottingham, UK) was inserted as previously described.²⁰ In brief, the procedure involved a mid-line cervical insertion with isolation and catheterization of the right carotid artery (post-clamping). The catheter was held in place for the duration of the study by both proximal and distal holding sutures. Once in place, blood sampling was undertaken. The catheter was kept patent by a heparinized saline block. As per protocol, the rats were allowed free access to food and water for 3 hours postintervention in a temperature-controlled environment and were then sacrificed by exsanguination under anesthesia (Hypnorm 200 $\mu\text{L}/\text{kg}$ intraperitoneally). Blood was withdrawn from the descending aorta and immediately put into ice-cold heparin/ethylenediamine-tetra-acetic acid-containing tubes (until full exsanguination), centrifuged at 1300g at 4°C for 10 minutes, and the plasma collected and stored at -80°C until assayed. Urine samples were collected at the time of sacrifice by direct bladder puncture, and snap-frozen and also stored at -80°C . Brain samples, were also collected as described later.

Study 2. BDL animals were randomized into five groups. All animals were sacrificed 4.5 to 5 weeks after the initial surgery. Study medications included intraperitoneal injection of combined L-ornithine (0.3 g/kg) and phenylacetate (0.3 g/kg), or saline (placebo) in two divided doses administered 8 hours apart. The sham animals were administered saline intraperitoneally for 10 days before sacrifice. The final study groups were: (1) Sham-operated + saline (n = 7); (2) BDL + saline (n = 7); (3) BDL + OP for 6 hours (n = 4); (4) BDL + OP for 3 days (n = 6); (5) BDL + OP for 5 days (n = 6); and (6) BDL + OP for 10 days (n = 6). As per protocol, the rats sacrificed by exsanguination under anesthesia (Hypnorm 200 $\mu\text{L}/\text{kg}$ intraperitoneally). Blood, urine, and brain samples were collected as described previously.

Arterial Ammonia, Ornithine, Glutamate, and Glutamine

Two hundred fifty microliters plasma was deproteinized with 20 mg dry sulfosalicylic acid and the amino acid concentrations analyzed by high-performance liquid chromatography as previously described.²¹ A further 900

μL plasma was deproteinized with 90 μL trichloroacetic acid for measurement of plasma ammonia concentrations. The substrate concentrations were measured by using a COBAS (COBAS INTEGRA, Roche, UK).²²

Brain Water

Immediately after death, the whole brain was rapidly removed, and 50-mm² samples were dissected from the frontal cortex (gray matter). Brain tissue water content was determined using a previously described dry weight technique.^{23,24}

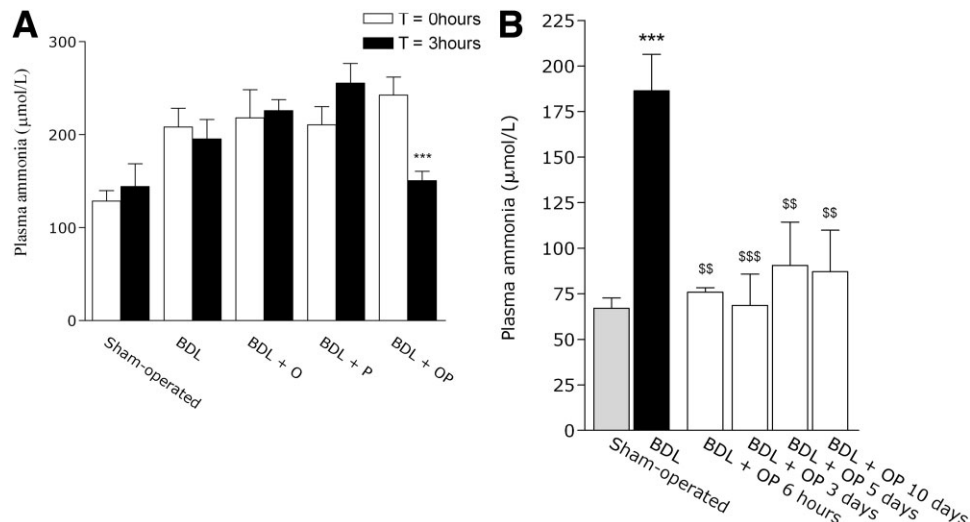
Brain Proton Magnetic Resonance Spectroscopy

Snap-frozen cortical brain samples were processed and analyzed by proton nuclear magnetic resonance (¹H-NMR) with a previously described technique using the brain tissue from animals in study 1.²⁵ ¹H-NMR spectra were recorded on a Bruker WB 360 spectrometer using a 5-mm quadrupole nuclear probe (QNP), 100 to 200 accumulations, repetition time 16 seconds, spectral width 3,623 Hz, data size 16 K, zero filling to 32 K. Chemical shifts were referenced to lactate at 1.33 ppm. Total metabolite concentrations ($\mu\text{mol}/\text{g}$ ww) were analyzed from ¹H-NMR spectra of perchloric acid extracts using (trimethylsilyl) propionic-2,2,3,3d₄-acid as external standard.²⁶

Urinary Phenylacetylglutamine

The urine concentration of phenylacetylglutamine was measured using liquid chromatography tandem mass spectrometry, based on a modified method that has been previously described.²⁷ In brief, the urine samples were prepared by adding 50 μL 0.1mol/L phosphate buffer solution (pH 2.4), 50 μL 60 $\mu\text{mol}/\text{L}$ internal standard, 3-(4-hydroxyphenyl) propionic acid (Sigma-Aldrich, Steinheim, Germany), and 50 μL urine in a 4.5-mL polypropylene tube (Sarstedt, Germany). To the same tube, 1 mL tert-butyl methyl ether was added as the extractant. The tubes were capped, mixed by shaking for 1 minute, and centrifuged at 1,700g for 180 seconds. Seven hundred fifty microliters supernatant (in a clean polypropylene tube) was evaporated to dryness under a stream of nitrogen at 40°C. The residue was then reconstituted in 100 μL mobile phase. Samples were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) using Waters Acquity UPLC system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to Waters Micromass Quattro Premier XE benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). The chromatography was performed on a 100 \times 2.1 mm Waters Acquity BEH C₁₈ 1.7-mm column. The mobile phase consisted of

Fig. 2. Changes in arterial ammonia. (A) Reduction in arterial ammonia concentration from baseline and at 3 hours after administration of OP (** $P < 0.001$) compared with BDL controls and at 3 hours were no different from sham controls. The BDL animals in this study were fed an additional ammonia-genic diet 1 week prior to the study. (B) Sustained reduction in arterial ammonia after administration of OP for 6 hours ($^{**}P < 0.01$), 3 ($^{***}P < 0.001$), 5 and 10 days ($^{***}P < 0.01$, respectively) compared with hyperammonemic BDL controls ($^{***}P < 0.001$ vs sham-operated controls). Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate/phenylbutyrate; P, phenylbutyrate; and O, L-ornithine.



50% methanol in 10 mmol/L aqueous formic acid with a flow rate of 0.2 mL/min (isocratic). The phenylacetylglutamine (PAG) standard was supplied by Bachem (Bubendorf, Switzerland). The method showed good linearity and reproducibility, with a correlation coefficient (r) of greater than 0.99 and a coefficient of variation of less than 5%.

Statistics

Data are expressed as mean \pm standard error of the mean (SEM). Significance of difference was tested with Newman-Keuls multiple comparison test or two-way analysis of variance; $P < 0.05$ was taken to be statistically significant. Software used included Microsoft Excel 2007 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

All rats continued to gain weight after surgery. From the final weight taken immediately before termination, BDL rats (mean \pm SEM; 320 g \pm 14), were not significantly different from sham-operated controls (mean \pm SEM; 350 g \pm 19). The systemic hemodynamics in the BDL animals were well maintained, as previously shown.^{17,20} All rats were alive after injection of study medication in each of the study groups.

Arterial Ammonia

Study 1. Three hours after the administration of OP, the arterial ammonia levels were significantly reduced by 45% from the baseline value, from a mean \pm SEM of 232 \pm 19 to 151 \pm 10 ($P < 0.001$) (Fig. 2A). The ammonia level was already reduced at 1 hour after administration of OP but did not reach statistical significance at

this time (Table 1). The arterial ammonia levels achieved at the end of the 3-hour period in the OP animals was not significantly different from that of the sham animals. No significant change in ammonia was observed in the animals treated with placebo, L-ornithine, or phenylbutyrate alone (Table 1, Fig. 2A).

Study 2. Bile duct ligation induced significant hyperammonemia ($P < 0.001$) compared with sham-operated controls (Fig. 2B). After administration of OP, there was sustained significant reduction in arterial ammonia at 6 hours ($P < 0.01$) and at 3 ($P < 0.001$), 5, and 10 days ($P < 0.01$) compared with BDL controls.

Ornithine, Glutamate, and Glutamine

Study 1. There was a significant increase in the plasma ornithine concentration of the BDL rats administered either L-ornithine ($P < 0.05$) or OP ($P < 0.05$) (Table 1). This initial increase started to decline by 3 hours as the L-ornithine was metabolized. After the increased circulating levels of ornithine and its later metabolism in the L-ornithine and OP-treated BDL rats, an initial rise (at 1 hour) of glutamate was observed that then fell (at 3 hours), associated with a progressive rise in glutamine (from 1 hour), reaching statistical significance by T = 3 hours ($P < 0.05$) (Table 1). No significant change in ornithine level was observed in the phenylbutyrate alone group; the glutamine level at 3 hours was lower, but this did not reach statistical significance. The changes in glutamate concentrations were not statistically significant in any of the groups.

Study 2. There was a significant and sustained increase in the ornithine concentration in the all of the OP-treated animals compared with the placebo-treated groups. Ornithine concentration was markedly higher in

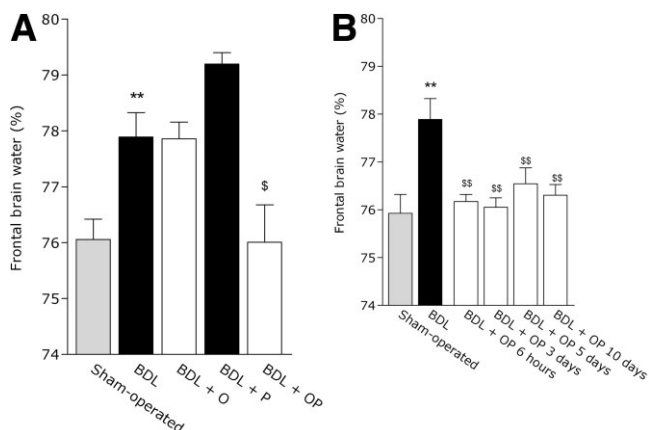


Fig. 3. Frontal cortex brain water content. (A) Significantly higher water content in the frontal cortex of BDL rats (** $P < 0.01$) compared with sham-operated controls. Administration of OP resulted in a significant reduction in brain water ($^{\$}P < 0.05$) compared with BDL controls. The BDL animals in this study were fed an additional ammoniagenic diet 1 week before the study. (B) Sustained reduction in frontal brain water content (%) after 6 hours' and 3, 5, and 10 days' ($^{\$}P < 0.01$, respectively) administration of OP compared with BDL controls with an established high water content (** $P < 0.01$). Abbreviations: sham-operated, sham-control; BDL, bile duct ligation; OP, L-ornithine, phenylacetate/phenylbutyrate; O, L-ornithine; P, phenylbutyrate.

the animals treated for 5 and 10 days compared with the animals treated for 3 days (Table 2). Similarly, glutamine concentrations were significantly higher in the OP-treated animals, reaching twice the concentrations observed in the placebo-treated group. However, this was not significantly different between the animals treated for 3 days compared with the animals treated for 5 or 10 days. The changes in glutamate concentration were not significantly different between any of the OP-treated groups compared with the placebo-treated group.

Brain Water

Study 1. There was a significant increase in the water content in the frontal cortex of saline-treated BDL rats compared with the corresponding sham-operated rats ($P < 0.01$; Fig. 3A). This increase in brain water in BDL rats was significantly attenuated by administration of OP, compared with the BDL animals administered placebo ($P < 0.05$), to values that were not significantly different from the sham-operated controls. Brain water was not significantly different in the BDL rats given L-ornithine or phenylbutyrate alone compared with the animals treated with placebo.

Study 2. With administration of OP, over 6 hours and 3, 5, and 10 days, there was a marked and significant reduction in frontal brain water content in each of the groups ($P < 0.01$, respectively) when compared with the placebo-treated animals, which had a higher brain water content compared with the sham animals ($P < 0.01$) (Fig.

3B). The brain water content in each of the OP-treated groups was not significantly different from the sham group.

Proton NMR Spectroscopy

Study 1. In BDL animals, brain glutamine was not different but the brain myo-inositol was lower ($P < 0.001$, Fig. 4), compared with sham-operated controls (Table 2). After administration of OP to BDL rats, there was a significant increase in brain myo-inositol levels ($P < 0.001$, Fig. 4). This resulted in a significant reduction in the glutamine/myo-inositol ratio ($P < 0.001$) to values that were not significantly different from those of sham-operated controls. After administration of L-ornithine or phenylbutyrate alone, there was no significant change in glutamine or myo-inositol levels (Fig. 4; Table 3).

Urinary Phenylacetylglutamine

Study 1. In the sham group and the BDL animals treated with placebo or L-ornithine alone, the urinary concentration of phenylacetylglutamine was not significantly different from zero. There was a significant increase in urinary phenylacetylglutamine excretion after admin-

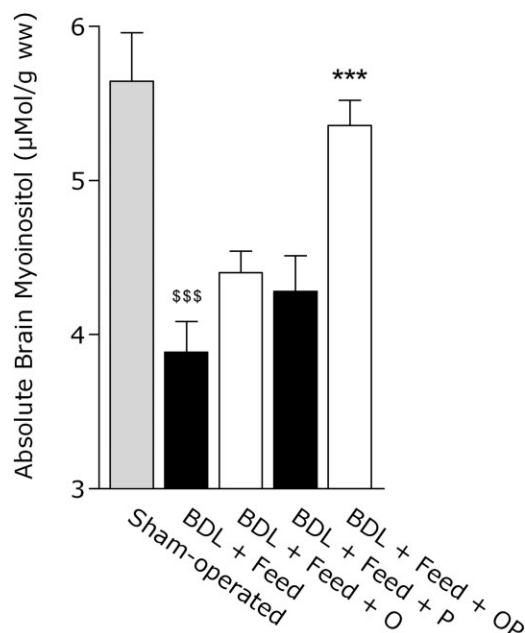


Fig. 4. Absolute brain myo-inositol levels: Bile duct ligation was associated with a significant reduction in brain myo-inositol level ($^{\$}P < 0.001$) compared with sham operated controls. Administration of OP to BDL rats significantly increased the myo-inositol level ($^{\$}P < 0.001$) compared with BDL controls, to levels that were no different from those of sham-operated animals. Administration of L-ornithine or phenylbutyrate alone had no statistically significant effect on myo-inositol levels compared with BDL controls. Abbreviations: sham-operated, sham-control; BDL, bile duct ligation; OP, L-ornithine, phenylbutyrate; O, L-ornithine; P, phenylbutyrate. The BDL animals in this study were fed an additional ammoniagenic diet 1 week before the study.

Table 3. Brain Osmolyte Profile as Analyzed by Proton Magnetic Resonance Spectroscopy

	Sham-Operated	BDL (HD)	BDL (HD) + O	BDL (HD) + P	BDL (HD) + OP
Glutamine ($\mu\text{mol/g ww}$)	4.4 \pm 0.2	5.1 \pm 0.2	4.8 \pm 0.2	4.8 \pm 0.2	5.1 \pm 0.2
Myo-inositol ($\mu\text{mol/g ww}$)	5.7 \pm 0.3	3.9 \pm 0.2*	4.4 \pm 0.1	4.3 \pm 0.2	5.4 \pm 0.2‡
Creatine ($\mu\text{mol/g ww}$)	7.4 \pm 0.1	6.6 \pm 0.3	6.8 \pm 0.3	6.8 \pm 0.2	7.5 \pm 0.2

Data are expressed as mean \pm standard error of mean (SEM) Symbols: * $P < 0.001$ compared with sham-operated control rats; ‡ $P < 0.001$ compared with BDL control rats. Abbreviations: BDL, bile duct ligation; HD: ammoniagenic diet administered 1 week before the study; OP, L-ornithine, phenylbutyrate; P, Phenylbutyrate; and O, L-ornithine.

istration of phenylbutyrate or OP to BDL rats compared with placebo-treated BDL and sham-operated control rats ($P < 0.05$; Fig. 5A).

Study 2. With administration of OP, over 6 hours and 3, 5, and 10 days, there was a significant increase in urinary phenylacetylglutamine excretion that increased with ongoing administration in each of the groups over the 10-day period ($P < 0.01$) when compared with the sham animals and the placebo-treated BDL control rats, in whom the excretion of phenylacetylglutamine was not significantly different from zero (Fig. 5B). The concentration of phenylacetylglutamine in the urine in the animals treated for 3, 5, and 10 days was approximately 20 times higher in the urine compared with that observed at the 3-hour time-point. In the acute study (study 1), the concentration of phenylacetylglutamine at the 3-hour time point was approximately 10 to 20 $\mu\text{mmol/L}$, which was increased to approximately 200 to 300 $\mu\text{mol/L}$ in the animals treated for 3, 5, and 10 days.

Discussion

The results of this study demonstrate a synergy between L-ornithine and phenylacetate in reducing arterial ammonia levels in BDL rats to values that were not significantly different from sham-operated controls. This reduction in ammonia was associated with a significant reduction in brain glutamine/myoinositol ratio, which was associated with a normalization of brain water. These effects of OP were sustained during a 10-day period of administration with respect to both a marked reduction in ammonia and also in brain water without any evidence of a rebound increase in ammonia. According to the proposed hypothesis, the reduction in ammonia was associated with an increase in arterial glutamine and urinary phenylacetylglutamine.

The observed reduction in ammonia in the OP-treated animals was associated with an initial increase in circulating ornithine at 1 hour in both the OP-treated and L-ornithine-treated rats, which at 3 hours was substantially reduced. In accordance with the first part of the hypothesis, an increase in the glutamine at 3 hours was also observed. These changes in glutamine levels are consistent

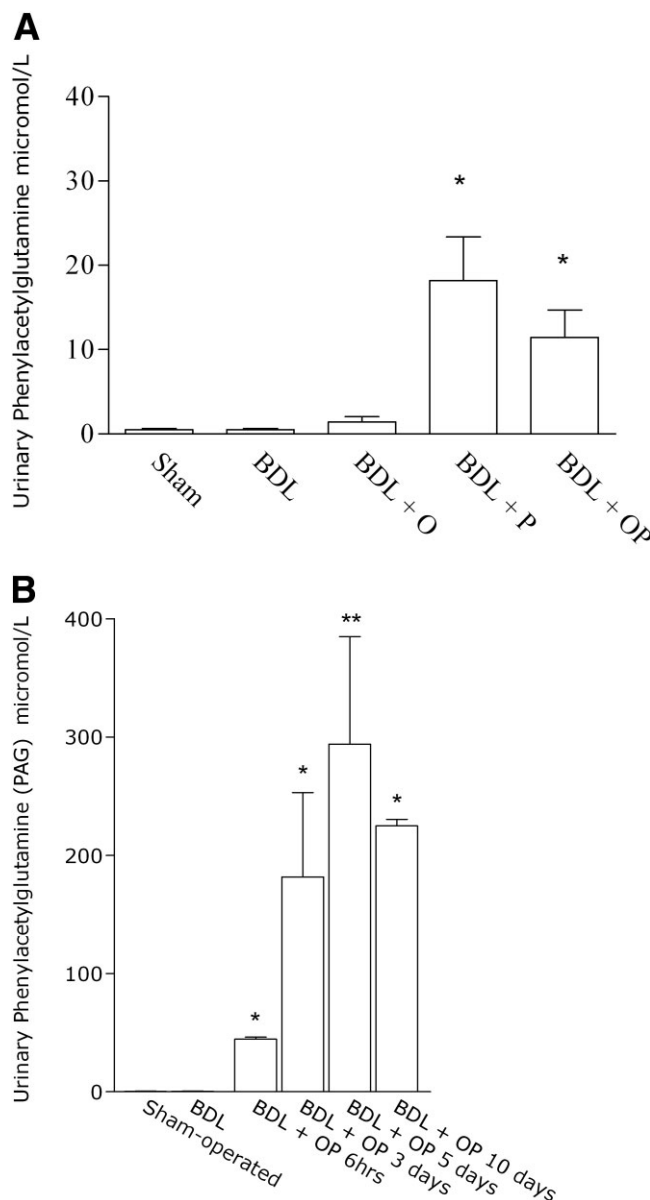


Fig. 5. Urinary phenylacetylglutamine excretion. (A) Significant increase in urinary phenylacetylglutamine after administration of OP and phenylbutyrate to BDL rats ($*P < 0.05$) compared with both sham-operated and BDL administered placebo or L-ornithine. The BDL animals in this study were fed an additional ammoniagenic diet 1 week prior to the study. (B) Profound and progressive increase in phenylacetylglutamine with OP administration over 6 hours and 3, 5, and 10 days ($*P < 0.01$, respectively), peaking at 5 days compared with BDL controls. Abbreviations: sham-operated, sham-control; BDL, bile duct ligation; OP, L-ornithine, phenylacetate/phenylbutyrate; O, L-ornithine; P, phenylbutyrate.

with previous observations of increased skeletal muscle glutamine synthetase activity and the effect of administration of LOLA to devascularized rats.⁷ The variability in the levels of arterial glutamine observed in the different groups may reflect the fact that the reactions at the 3-hour time point are not at a steady state. In the BDL animals treated with OP for up to 5 and 10 days, the ornithine levels continued to increase to twice the values seen in the animals treated for up to 3 days, but the glutamine levels remained similar in the animals treated for 6 hours or 3 days compared with the animals treated for 5 or 10 days, indicating perhaps that the doses of ornithine used in OP could be reduced. This will need to be tested in future studies. The demonstration of phenylacetylglutamine in the urine of animals treated with phenylacetate/phenylbutyrate and OP supports the hypothesis that glutamine can be conjugated and excreted as phenylacetylglutamine by administration of phenylbutyrate.¹⁶ Taken together, the data suggest that an increase in glutamine alone as observed in the L-ornithine-treated animals, or an increase in phenylacetylglutamine alone as observed in the phenylbutyrate-treated animals, do not result in a lowering of ammonia. However, the administration of OP, which results in an increase in both glutamine and urinary phenylacetylglutamine, does lower ammonia significantly and indicates clearly a synergy between L-ornithine and phenylacetate/phenylbutyrate in reducing ammonia.

However, in the acute study (study 1), there was a lack of stoichiometry between the reduction in arterial ammonia concentration and the increase in urinary phenylacetylglutamine. Although this may be attributable to differences in conjugation pathways, as in humans, phenylacetylglutamine is the overwhelming renal excretion product of the reaction between phenylbutyrate and glutamine; however, in rats this association is less clear, because there are a number of additional pathways of phenylacetate metabolism.²⁸ It is more likely that the explanation may be related to kinetics of phenylacetylglutamine excretion. Phenylacetylglutamine is conjugated intracellularly and therefore requires time to be excreted from the system, as illustrated by a 20-fold increase in the concentration of urinary phenylacetylglutamine in the animals treated for 3 to 10 days. The measured high concentration of phenylacetylglutamine in the urine together with twice the concentration of arterial glutamine in the OP-treated animals explains in great part the observed reduction in ammonia. More detailed pharmacokinetic modeling studies will be necessary to determine the exact relationship between the reduction in ammonia, urinary excretion of phenylacetylglutamine, and the increase in glutamine concentration.

The results of ammonia reduction observed in the current study using OP has been confirmed in pigs with acute liver failure induced by hepatic devascularization, in which over an 8-hour period the ammonia concentration was reduced by approximately 300 $\mu\text{mol/L}$ compared with a group treated with L-ornithine, phenylbutyrate, or placebo, indicating synergy between ornithine and phenylacetate.²⁹ Importantly, the effect was observed in animals that were effectively anhepatic. Similar increases in urinary phenylacetylglutamine were also demonstrated. Arterial ammonia was also shown to be reduced by approximately 30% over a 3-day period in two patients treated with a combination of L-ornithine and phenylacetate, providing further proof for the hypothesis.¹⁵

The most important finding of this study was the observation that the elevated brain water observed in the BDL animals in both studies was reduced in the OP group to values that were not significantly different from those of the sham operated controls. Again, this effect was maintained throughout the period of administration, as was observed with ammonia lowering. Although there was an unexpected increase in brain water with the administration of phenylbutyrate alone, this was not statistically significant when compared with placebo-treated BDL controls. The effect of OP on the brain water in BDL animals is consistent with the observations in the porcine acute liver failure model, in which administration of OP prevented the rise in intracranial pressure to levels that were not significantly different from those of sham operated controls.²⁹ The reduction in arterial ammonia after OP in the two patients referred to previously was associated with an improvement in mental state,¹⁵ an observation that will have to be tested in appropriate clinical trials.

Several lines of investigation indicate that the cytotoxic brain edema observed in patients with hyperammonemia is associated with alterations in the brain osmolytes, which is characterized by an increase in brain glutamine/myoinositol ratio.³⁰⁻³² Accordingly, the glutamine/myoinositol ratio was significantly higher in the BDL animals compared with that observed in the placebo-treated BDL controls, which was reduced in the OP-treated animals to levels that were not significantly different from those of sham operated controls; largely because an increase in brain myoinositol levels was observed.^{4,32,33} In the groups treated with L-ornithine or phenylbutyrate alone, no significant changes in either glutamine or myoinositol were observed. It is likely that the effect of OP on the brain osmolytes is attributable to a reduction in arterial ammonia, which was recently shown in devascularized pigs to be associated with a reduction in extracellular brain ammonia.³⁰ From the pathophysiological perspective, this pre-

vention of reduction in myoinositol may attribute an increase in brain buffering capacity to the effects of other precipitants such as a further hyperammonemia or hyponatremia, stimuli that are known to increase brain swelling.^{32,34} Similar corrections in brain osmolytes have been induced by treatment of clinically overt HE with lactulose, which was principally shown to ameliorate reduction of brain myoinositol with liver failure rather than correct the rise in intracellular glutamine.³⁵ Although the observed modulation of brain myoinositol, which correlated with severity of HE, was observed after 1 week of lactulose, it is known that changes in levels of intracellular osmolytes such as myoinositol occur within hours,³⁶ as observed in this study. In rats given intraperitoneal inositol, the levels of myoinositol in the brain cortex rise by 33% in as soon as 2 hours.³⁶ This is likely to be associated with a very early and demonstrable increase in the transcription of brain Na⁺/myoinositol cotransporter in response to certain insults such as changes of osmotic gradient.³⁷

In conclusion, the results of this study provide support for our concept that combining L-ornithine with phenylbutyrate synergistically attenuates hyperammonemia by increasing ammonia detoxification into glutamine and eliminating the resultant glutamine as urinary phenylacetylglutamine. Furthermore, this ammonia-lowering effect is sustained over the 10-day period of administration. We also show that OP by reducing plasma ammonia modulates brain osmolytes, which is associated with a reduction in brain water. The data indicate that OP is likely to be beneficial for the treatment of hyperammonemia and HE, and as the individual components, that is, L-ornithine and phenylbutyrate, are already in use in humans, it is likely that these findings can be translated relatively quickly into appropriate clinical studies to determine dosing, exact mechanism of action, pharmacokinetics, safety, and efficacy.

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