L-Ornithine phenylacetate reduces ammonia in pigs with acute liver failure through phenylacetylglutamine formation: a novel ammonia-lowering pathway

Kristiansen RG, Rose CF, Fuskevåg OM, Mæhre H, Revhaug A, Jalan R, Ytrebø LM. L-Ornithine phenylacetate reduces ammonia in pigs with acute liver failure through phenylacetylglutamine formation: a novel ammonia-lowering pathway. Am J Physiol Gastrointest Liver Physiol 307: G1024–G1031, 2014. First published September 25, 2014; doi:10.1152/ajpgi.00244.2014.—Glycine is an important ammoniagenic amino acid, which is increased in acute liver failure (ALF). We have previously shown that L-ornithine phenylacetate (OP) attenuates ammonia rise and intracranial pressure in pigs suffering from ALF but failed to demonstrate a stoichiometric relationship between change in plasma ammonia levels and excretion of phenylacetylglutamine in urine. The aim was to investigate the impact of OP treatment on the phenylacetylglutamine pathway as an alternative and additional ammonia-lowering pathway. A well-validated and characterized large porcine model of ALF (portacaval anastomosis, followed by hepatic artery ligation), which recapitulates the cardinal features of ALF, was used. Twenty-four female pigs were randomized into three groups: 1) sham operated + vehicle, 2) ALF + vehicle, and 3) ALF + OP. There was a significant increase in arterial glycine concentration in ALF (P < 0.001 compared with sham), with a three-fold increase in glycine release into the systemic circulation from the kidney compared with the sham group. This increase was attenuated in both the blood and brain of the OP-treated animals (P < 0.001 and P < 0.05, respectively), and the attenuation was associated with renal removal of glycine through excretion of the conjugation product phenylacetylglutamine in urine (ALF + vehicle: 1,060 ± 166 μmol/l; ALF + OP: 27,625 ± 2,670 μmol/l; P < 0.003). Data from this study provide solid evidence for the existence of a novel, additional pathway for ammonia removal in ALF, involving glycine production and removal, which is targeted by OP.

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benzoate treatment involves the removal of glycine. Additionally, phenylacetate can conjugate with glycine to produce phenylacetylglycine, an alternative ammonia-lowering pathway that has not been explored in ALF (13). We hypothesize that the ammonia-lowering effect of OP observed in the ALF pigs involves stimulation of glycine production and removal.

Therefore, the aim of this study was to investigate the impact of OP treatment on the phenylacetylglycine pathway as an alternative and additional ammonia-lowering pathway in ALF.

MATERIALS AND METHODS

Study Outline

The study was performed in the Surgical Research Laboratory at the University of Tromsø (Norway) and was approved by the Norwegian Experimental Animal Board. A well-validated and -characterized large porcine model, which recapitulates the cardinal features of human ALF, including acute hyperammonemia and increased intracranial pressure (ICP), was used (32). The beneficial effects of OP treatment on these animals on ammonia levels and ICP were described in our previous paper (31). This paper uses the samples from the Norwegian Experimental Animal Board. A well-validated and -characterized large porcine model, which recapitulates the cardinal features of human ALF, including acute hyperammonemia and increased intracranial pressure (ICP), was used (32). The beneficial effects of OP treatment on these animals on ammonia levels and ICP were described in our previous paper (31).

Experimental Design

Three groups of pigs were fasted overnight with free access to water. The animals were fed with Combi Fri chow (Felleskjøpet, Trondheim, Norway). All animals were monitored for the internal standards (average of these two). The linear dynamic range [correlation coefficient (r²) > 0.99] for phenylacetylglycine was from 1 to 3,000 µM. Between-day coefficient of variation (%CV) for phenylacetylglycine was <10%; intraday precision values (CV) were all <6%. Hippuric acid. Stock solutions of hippuric acid (8.42 mmol/l) were prepared in methanol and stored at −70°C. Calibrators were prepared by serial dilution of the stock solution with synthetic urine in the range of 10–20 µM. Two quality controls (0.8 and 8 µM) were prepared in the same manner as calibrators.

EXTRACTION PROCEDURE. Pig urine samples were first diluted 10 times with synthetic urine before adding 0.1 M phosphate buffer (pH 2.4), 25 µl internal standard mix (20 µM N-benzoylglycine-2,2-d2 and 4-phenylbutyric-2,2,3,3-d4 acid)–50 µl sample in a 4.5-ml polypropylene tube (Sarstedt AG, Nümbrecht, Germany). To the same tube, 1 ml tert-butyl methyl ether:ethyl acetate (1:1) was added as the extractant. The tubes were capped, mixed by shaking for 1 min, and centrifuged at 1,700 g for 4 min. The supernatant (700 µl) was transferred to a second set of clean polypropylene tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was then reconstituted in 200 µl mobile phase and transferred to LC vials.

LC-TANDEM MS. Phenylacetylglycine was quantified using an Acquity I-Class ultra-performance LC (UPLC) system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to the Xevo TQ-S benchtop tandem quadrupole MS (Waters).

The chromatography was performed on a 2.1 × 100-mm Waters Acquity HSS T3 (C18) UPLC column, maintained at 50°C. The column was eluted isocratically with 0.1% aqueous formic acid:methanol (1:1) at 0.45 ml/min. Sample injection volume was 0.1 µl, and the injection interval was 2 min.

The mass spectrometer was operated in negative electrospray ion mode (ESI−) and spray voltage was 1.5 kV. The system was controlled by MassLynx version 4.1 software, desolvation gas temperature 500°C, source temperature 150°C, collision gas flow 150 l/h, cone gas pressure 3.5 mbar (argon), and ion energies 0.8 V for both quadrupoles. For quantitative analysis of phenylacetylglycine, multiple reaction monitoring (MRM) transition mass-to-charge ratio (m/z) 192 → 73.5 was monitored, and MRM transition m/z 180 → 136 (N-benzoylglycine-2,2-d2) and 166.8 → 90.8 (4-phenylbutyric-2,2,3,3-d4 acid) were monitored for the internal standards (average of these two). The linear dynamic range [correlation coefficient (r²) > 0.99] for phenylacetylglycine was from 1 to 3,000 µM. Between-day coefficient of variation (%CV) for phenylacetylglycine was <10%; intraday precision values (CV) were all <6%.

Hippuric acid. Stock solutions of hippuric acid (8.42 mmol/l) were prepared in methanol and stored at −70°C. Calibrators were prepared by serial dilution of the stock solutions with synthetic

...was verified manually during the laparotomy. Study medication was administered as a continuous infusion for the duration of the experiment, starting at T = 0 h and ending at T = 8 h. Physiological saline (9 mg/ml) was used as vehicle in the ALF and sham groups. All groups received an equal amount of fluids.

Measurements of Phenylacetylglycine and Hippuric Acid

Chemicals. Water was obtained from a Milli-Q Advantage system (Millipore SAS, Molsheim, France). Analytical-grade ethyl acetate was supplied by Merck (Darmstadt, Germany). Tert-butyl methyl ether, liquid chromatography (LC)-mass spectrometry (LC-MS)-grade formic acid, LC-MS-grade acetonitrile, and hippuric acid were obtained from Sigma-Aldrich (Steinheim, Germany). Phenylacetylglycine was purchased from Toronto Research Chemicals (Toronto, Canada). Deuterium-labeled internal standard for hippuric acid was purchased from C/D/N Isotopes (Pointe-Clair, Quebec, Canada).

Phenylacetylglycine. PREPARATION OF SERUM CALIBRATORS AND CONTROLS. Two sets of calibrators were prepared by serial dilution of the stock solutions with dialyzed serum or PBS in the range of 10–20 µM. Two quality controls (0.8 and 8 µM) were prepared in the same manner as calibrators.

Stock solutions of phenylacetylglycine (3.34 mmol/l) were prepared in methanol and stored at −70°C. Calibrators were prepared by serial dilution of the stock solution with synthetic urine in the range of 2,000–10 µM. Quality-control (QC) samples (0.08, 80, 800) for phenylacetylglycine were prepared in the same manner as calibrators.
urine in the range of 20–0.156 μM for hippuric acid. QC samples (0.8, 8 μM) of hippuric acid were prepared in the same manner as calibrators.

EXTRACTION PROCEDURE FOR HIPPURIC ACID. The extraction procedure for the urine samples was identical to the procedure for phenylacetylglycine, except that the samples were not diluted.

LC-TANDEM MS FOR HIPPURIC ACID. The chromatography was performed on a 2.1 × 100 mm Waters Acquity UPLC BEH C₁₈ column maintained at 50°C. The column was eluted isocratically with 0.1% aqueous formic acid:methanol (1:1) at 0.4 ml. The injection volume was 6 μL, and the time from injection to injection was 2 min. A triple quadrupole tandem mass spectrometer (Quattro Premier XE benchtop tandem quadrupole mass spectrometer; Waters), fitted with a Z-spray ion source, was used. The mass spectrometer was operated in ESI⁻, and spray voltage was 3 kV. The system was controlled by MassLynx version 4.1 software, desolvation gas temperature 350°C, source temperature 130°C, desolvation gas flow 800 l/hr, cone gas flow 40 l/hr, collision gas pressure 3.5 × 10⁻⁴ mbar (argon), and ion energies 1 V for both quadrupoles. For quantitative analysis, the following MRm transitions for hippuric acid and its deuterated analog transitions were used (bold transition is qualifier): m/z 178 → 77/134 and 180 → 136. The dwell time was set to 20 ms for each transition. The method showed good linearity and reproducibility, with r² > 0.99, and between-day CV for hippuric acid was <6% at three different days (six different samples); intraday precision values (CV) were all <6% (assaying six different samples, six times on the same day).

Analysis of Amino Acids in Blood and Brain Tissue

Blood. Amino acids were extracted by homogenizing 360 μL blood plasma with 36 μL 2 mmol/L norleucine and 40 μL 35% sulfosalicylic acid. Norleucine served as an internal standard. The suspension was centrifuged, and an aliquot of the supernatant was submitted to analysis. The concentration of glycine, glutamate, serine, and alanine was determined using a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, UK), equipped with a lithium citrate-equilibrated column. The signal was analyzed by Chroelone software (Dionex, Sunnyvale, CA) and compared with A9906 physiological amino acids (Sigma Chemical, St. Louis, MO).

Brain tissue. Glycine was extracted from the tissue samples by homogenizing ~200 mg tissue with 950 μL distilled water, 50 μL 20 mmol/L norleucine, and 100 μL 35% sulfosalicylic acid. Norleucine served as an internal standard. The mixture was centrifuged, and an aliquot of the supernatant was submitted to analysis, as described for measurements of amino acids in blood.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 21 for Windows (SPSS, Chicago, IL). Data are expressed as means ± SE. Two-way ANOVA was applied to test for differences within and between groups over time for arterial glycine and ornithine. Differences between the groups at T = 0 and T = 8 (sham vs. ALF and ALF vs. ALF-OP) were tested by using the Mann-Whitney U-test. The Spearman rank correlation test assessed r². P ≤ 0.05 was considered significant for all tests applied.

RESULTS

Glycine

Arterial glycine levels increased significantly over time, from T = 0 to T = 8 in the ALF group, with a two-fold increase at T = 8 h compared with the sham group (P < 0.001). This increase was significantly attenuated in the OP-treated group (P < 0.001; Fig. 1A). A significant correlation was found between arterial levels of glycine and ammonia (P < 0.001; r² = 0.67; Fig. 1B).

At T = 8, there was a significant attenuation of glycine release across the kidney in the OP-treated group compared with the ALF group. In the ALF group, at T = 8, there was a three-fold increase in glycine release into the systemic circulation from the kidney compared with the sham group. However, it did not reach statistically significant levels (P = 0.088; Fig. 2A). There were no significant intergroup differences at T = 8 for the VA difference of glycine across the portal-drained viscera (Fig. 2B) or hind leg (Fig. 2C).

A significant increase in glycine was found in the brain (frontal cortex) in the ALF group compared with sham group (P < 0.05; Fig. 3A). In the OP-treated group, however, glycine levels were reduced significantly compared with the ALF group (P < 0.05). Furthermore, a significant correlation between glycine and ICP (P < 0.001, r² = 0.60) was observed (Fig. 3B).
Phenylacetylglycine was detected in urine in all three groups. OP-treated pigs demonstrated a significant (P < 0.05) increase in the concentration of phenylacetylglycine in the urine compared with ALF pigs (Fig. 4A). Furthermore, the amount (μmol) of phenylacetylglycine excreted was also increased significantly (P < 0.05) in the OP-treated group (Table 1). At T = 0, there was no significant difference in the mean phenylacetylglycine excreted between the ALF and the OP-treated groups (ALF 32.5 ± 10.9 μmol vs. OP 32.2 ± 11.0 μmol).

**Hippuric Acid**
No significant (P = 0.20) difference in the hippuric acid excretion at T = 8 was observed between the OP-treated ALF group and the ALF group (Table 1).

**Glutamate, Serine, and Alanine**
A significant (P < 0.05) decrease in arterial glutamate, from T = 0 to T = 8, was observed in both the ALF group and the OP-treated groups. The decrease in arterial glutamate was less in the OP-treated group, as there was a significantly (P < 0.05) higher concentration of arterial glutamate in the OP-treated ALF group compared with the ALF group at T = 8 h (Table 2). A significant (P < 0.05) intergroup difference for the VA difference between the sham group and ALF group was demonstrated at T = 8 (Table 2). There was no difference between OP-treated and ALF groups at T = 8.

![Figure 2](http://ajpgi.physiology.org/)

**Phenylacetylglycine**
Phenylacetylglycine was detected in urine in all three groups. OP-treated pigs demonstrated a significant (P < 0.05) increase in the concentration of phenylacetylglycine in the urine compared with ALF pigs (Fig. 4A). Furthermore, the amount (μmol) of phenylacetylglycine excreted was also increased significantly (P < 0.05) in the OP-treated group (Table 1). At T = 0, there was no significant difference in the mean phenylacetylglycine excreted between the ALF and the OP-

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**Fig. 2.** A–C: venous-arterial (VA) changes across kidney, portal-drained viscera, and hind leg, respectively. VA concentration changes for glycine across 1 kidney (A), portal-drained viscera (B), and 1 hind leg (C) in sham group, ALF group, and OP-treated ALF group. Positive values = net release; negative values = net uptake; †P < 0.05, ALF group vs. OP-treated group.

**Fig. 3.** A: glycine frontal lobe. Glycine in brain (frontal lobe) at T = 8. *P < 0.05, sham vs. ALF; †P < 0.05, ALF vs. OP. B: correlation arterial glycine and intracranial pressure (ICP). Correlation between arterial glycine and ICP for all time points and all groups (P < 0.001, r² = 0.596).
There was a significant increase in arterial concentration of alanine at $T = 8$ in the OP-treated group compared with the ALF group ($P < 0.05$). There were no significant intergroup differences for VA concentration difference across the kidneys for alanine at $T = 8$ (Table 2).

No significant intergroup differences for arterial concentration of serine or VA difference across the kidney were observed at $T = 8$ (Table 2).

**DISCUSSION**

The results of this study provide strong evidence for the existence of a novel and effective ammonia detoxification pathway involving the conjugation of glycine (an ammonia-generating amino acid) with phenylacetate-producing phenylacetylglutamine in ALF. Previous studies have consistently shown that the administration of ornithine and phenylacetate results in a reduction in ammonia levels and an increase in urinary phenylacetylglutamine (7, 20, 31). However, in each study, the reduction in ammonia did not stoichiometrically correlate with an increase in urinary phenylacetylglutamine. The present study is a secondary analysis from our previous study, which demonstrated an attenuation in the rise of ammonia (and a subsequent attenuation in ICP) in OP-treated ALF (liver-devascularized) pigs. The reduction in blood ammonia could not solely be explained through an increase in phenylacetylglutamine. In the present study, we observed an increase in arterial glycine in the ALF group, which was reduced significantly in the OP-treated ALF animals (Fig. 1A). This reduction in glycine was associated with a highly significant increase in urinary phenylacetylglycine (Fig. 4A) in the OP-treated group, providing a mechanistic explanation for the observed reduction in ammonia. Furthermore, the observation that the quantity of phenylacetylglycine (Table 1), excreted at $T = 8$, was >1,000-fold greater than phenylacetylglutamine (31) confirms that the former is the primary pathway involved in attenuating the rise of ammonia in this model.

We have previously shown the reproducibility of this model, recapturing the cardinal features of ALF, including hyperammonemia and intracranial hypertension (30, 31). We have also previously reported a strong correlation between increased...
arterial ammonia and increased ICP in this model, a finding confirmed in other ALF animal models (20) and patients with ALF (21–23).

This anhepatic model becomes an excellent experimental paradigm for studying the extrahepatic metabolism of ammonia and amino acids in relation to the OP treatment. Several studies suggest that the kidneys are important ammonia-producing organs in liver failure (19), as well as in acute hyperammonemia (8, 33). In this ALF model, we observed that the organ most likely responsible for both the generation of increased glycine and conjugation to phenylacetylglycine was the kidneys. A three-fold increase in the release of glycine from the kidneys into the systemic circulation was observed in the ALF group compared with the sham group (Fig. 2A). This increase was significantly attenuated following OP treatment (Fig. 2A), which was associated with a 15-fold increase in the amount of phenylacetylglycine in urine vs. ALF (Fig. 4A). The conjugation of glycine with phenylacetate is mediated through acyl-CoA:glycine N-acyltransferase (14, 29), using phenylacetyl-CoA as the substrate and glycine as the preferred acyl acceptor (18). Our data strongly suggest that in this anhepatic model of ALF, the kidneys play a major role in the conjugation of glycine. Similar results were not found across the portal-drained viscera or hind leg (at T = 8; Fig. 2, B and C, respectively). Taken together, these results provide compelling evidence that in hepatic, devascularized-induced ALF pigs, the kidneys play a major role in regulating glycine production and the conjugation between glycine and phenylacetate.

Removal of glycine by the administration of benzoate is used for ammonia-lowering in patients with urea cycle enzyme deficiency (9). The enzyme responsible for synthesizing hippuric acid has been shown to be present in the pig kidney (26). To rule out the possibility of spontaneous induction of the hippuric acid pathway, we also measured urinary hippuric acid. The data showed no significant difference in the hippuric acid production in OP-treated pigs compared with ALF pigs, suggesting that the preferable route for excretion of glycine was as a phenylacetate conjugate (Table 1).

Glycine acts as an inhibitory neurotransmitter in the brain. Increased concentration of glycine has been shown to result in severe encephalopathy, a condition referred to as hyperglycinemic encephalopathy, occurring in children with an inborn error in the glycine-cleavage system (16). We found a significantly increased glycine level in brain tissue from animals with ALF that was significantly attenuated in OP-treated animals (Fig. 3A). An increase in glycine has been reported in other animal models, as well as in patients with ALF (5, 27), and in the present study, a strong and significant correlation was observed between arterial glycine levels and arterial ammonia levels (31) (Fig. 1B), in addition to ICP levels (31) (Fig. 3B). The reduction in glycine levels in the OP-treated animals (Fig. 3A), along with the reduction in ICP (4), suggests a possible role for glycine in the development of increased ICP in this model of ALF. At present, it is not clear whether its deleterious effects on increased ICP are mediated through ammoniagenesis or whether glycine directly affects the osmolarity of the brain (astrocytes); these pathophysiological pathways merit to be further investigated.

The removal of glycine is believed to stimulate ammonia removal through its incorporation with glutamate in de novo synthesis of glycine to compensate and replenish the glycine stores (3). Glutamate, serine, and alanine are important metabolites involved in glycine metabolism (Fig. 4B) (22). Glycine can be synthesized from serine through removal of one carbon atom by serine hydroxymethyltransferase. Serine is synthesized from 3-phosphoglycerate, an intermediate from glycolysis (Fig. 4B). 3-Phosphoglycerate is oxidized to 3-phosphohydroxypyruvate and undergoes transamination by glutamate as an amino group donor. Glycine can also be synthesized from transamination of glyoxylate using alanine as an amino group donor (Fig. 4B). The transamination enzyme, L-alanine-glyoxylate aminotransferase, is found primarily in the liver but is also expressed in the kidney (24). Additionally, studies have shown increased glycine synthesis from glyoxylate to be important in draining ammonia nitrogen through transamination with alanine in hyperammonemic mice (21).

The activity of the glycine-cleavage complex/glycine synthase is mainly catabolic, producing free ammonia (15, 28). Also, glycine can produce ammonia from the direct oxidase reaction catalyzed by glycine oxidase, leaving ammonia and glyoxylic acid (Fig. 4B). With the conjugation of glycine with phenylacetate, glycine-derived ammonia production will be reduced, preventing a potential ammonia-rebound effect (Fig. 4B).

Glycine has an important position in the general amino acid metabolism as a receiver of amino groups from other amino acids for further metabolism in the urea cycle or for interorgan transport/signaling as part of glutamine and alanine (through transamination) (2). Glutamate is used for glutamine production and plays an important role in hyperammonemia, taking up ammonia as an amide group forming glutamine (12). However, ammonia can also be taken up in the glutamate
dehydrogenase (GDH) reaction using \( \alpha \)-ketoglutarate (Fig. 4B). The \( \alpha \)-ketoglutarate-glutamate transamination (GDH reaction) is reversible, and its direction is set by the concentration of its substrates and products. Glutamine, glycine, and alanine are known inhibitors of glutamine synthetase (11), the enzyme responsible for amidating glutamate to glutamine. The increase in arterial glutamine (31) and alanine (Table 2), seen in this model, following OP treatment, could possibly have an inhibitory effect on glutamine synthetase, therefore stimulating other ammonia-removing pathways, including glycine removal. With the revisitation of the hypothesis, ornithine is given to increase the provision of glutamate in the muscle and stimulate its amidation through glutamine synthetase and lower ammonia (12). In addition, ornithine-derived glutamate can generate glycine (through serine), producing \( \alpha \)-ketoglutarate. In turn, \( \alpha \)-ketoglutarate can form glutamate (removing ammonia), forming a cycle of glutamate-\( \alpha \)-ketoglutarate-glycine production (Fig. 4B). This “ammonia-removal” cycle merits further investigation.

In conclusion, the data presented here provide experimental evidence for a novel ammonia-removing pathway via glycine that is active in the kidneys in pigs with ALF. The data provide an explanation for the mechanism of reduction in ammonia concentration with OP treatment in this porcine ALF model.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the skillful technical assistance and support from our colleagues at the Surgical Research laboratory, UiT The Arctic University of Norway.

GRANTS

Support for this work was provided by the Norwegian Research Council, Helse Nord, and Fonds de recherche en santé du Quebec (FRSQ; Canada).

DISCLOSURES

University College London has patented the use of ornithine and phenylacetate, which has been licensed to Ocera Therapeutics, for the treatment of hyperammonemia and hepatic encephalopathy (R. Jalan). None of the other authors have anything to disclose.

AUTHOR CONTRIBUTIONS


REFERENCES


