Ammonia reduction with ornithine phenylacetate restores brain eNOS activity via the DDAH-ADMA pathway in bile duct-ligated cirrhotic rats

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Balasubramanian V, Wright G, Sharma V, Davies NA, Sharifi Y, Habtesion A, Mookerjee RP, Jalan R. Ammonia reduction with ornithine phenylacetate restores brain eNOS activity via the DDAH-ADMA pathway in bile duct-ligated cirrhotic rats. Am J Physiol Gastrointest Liver Physiol 302: G145–G152, 2012. First published September 8, 2011; doi:10.1152/ajpgi.00097.2011.—Ammonia is central in the pathogenesis of hepatic encephalopathy, which is associated with dysfunction of the nitric oxide (NO) signaling pathway. Ornithine phenylacetate (OP) reduces hyperammonemia and brain water in cirrhotic animals. This study aimed to determine whether endothelial NO synthase activity is altered in the brain of cirrhotic animals, whether this is associated with changes in the endogenous inhibitor, asymmetric-dimethylarginine (ADMA) and its regulating enzyme, dimethylarginine-dimethylaminohydrolase (DDAH-1), and whether these abnormalities are restored by ammonia reduction using OP. Sprague-Dawley rats were studied 4-wk after bile duct ligation (BDL) (n = 16) or sham operation (n = 8) and treated with placebo or OP (0.6 g/kg). Arterial ammonia, brain water, TNF-α, plasma, and brain ADMA were measured using standard techniques. NOS activity was measured radiometrically, and protein expression for NOS enzymes, ADMA, DDAH-1, 4-hydroxynonenol (4HNE), and NADPH oxidase (NOX)-1 were measured by Western blotting. BDL significantly increased arterial ammonia (P < 0.0001), brain water (P < 0.05), and brain TNF-α (P < 0.01). These were reduced significantly by OP treatment. The estimated eNOS component of constitutive NOS activity was significantly lower (P < 0.05) in BDL rat, and this was significantly attenuated in OP-treated animals. Brain ADMA levels were significantly higher and brain DDAH-1 significantly lower in BDL compared with sham (P < 0.01) and restored toward normal following treatment with OP. Brain 4HNE and NOX-1 protein expression were significantly increased in BDL rat brain, which were significantly decreased following OP administration. We show a marked abnormality of NO regulation in cirrhotic rat brains, which can be restored by reduction in ammonia concentration using OP.

hepatic encephalopathy; cirrhosis; inflammation; oxidant injury; blood flow

FOR OVER ONE HUNDRED YEARS, ammonia has been thought to be central in the pathogenesis of hepatic encephalopathy, but more recently an important role of inflammation has been hypothesized. Studies in patients with acute liver failure and cirrhosis have shown incontrovertibly that peripheral and brain inflammatory responses are important in modulating the effect of hyperammonemia (15, 38), which in turn is key to the development of HE, promoting astrocyte swelling (33).

More recently, the relationship between hyperammonemia and altered brain nitric oxide (NO) signaling has been described (28). The studies have shown very different results depending on whether the hyperammonemia is acute or chronic. In the acute situation, severe hyperammonemia induces excessive brain glutamate release, which leads to increased stimulation of the N-methyl-D-aspartate receptors resulting in NO release (2). However, chronic hyperammonemia has been shown to reduce cGMP activity, resulting in reduced availability of brain NO and cGMP (7). The use of phosphodiesterase inhibitors has been shown to replete cGMP, resulting in the improvement of memory function of chronic hyperammonemic rats (7). Our prior scanning electron microscopic studies of bile duct-ligated (BDL) cirrhotic rats support the observation that, in chronic liver disease, the microvessels are markedly vasoconstricted, consistent with a NO depleted state in the brain (44). In patients with cirrhosis, Guevara et al. (11) showed evidence of increased cerebral vascular resistance and a decrease in cerebral blood flow.

Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of endothelial nitric oxide synthase (eNOS), formed during proteolysis of methylated proteins (23, 42). The concentration of circulating ADMA is tightly regulated by dimethylarginine diaminohydrolase (DDAH), which catalyses its metabolism (24). Thus decreased activity of DDAH leads to a corresponding increase in circulating levels of ADMA. Elevated plasma concentrations of ADMA are observed in patients with severe acute alcoholic hepatitis (30) and acute liver failure (29). We recently demonstrated that hepatic DDAH protein expression was decreased in a rat model of cirrhosis. This led to increased hepatic levels of ADMA and decreased eNOS activity (1). As hyperammonemia is known to generate inflammation through effects on NF-κB (39) and oxidative stress (35), this may reduce expression of brain DDAH, leading to increased ADMA, resulting in eNOS dysfunction (34).

We have recently shown in cirrhosis models that ornithine and phenylacetate act synergistically to restore ammonia concentration and reduce brain swelling (5). They also attenuate the rise in intracranial pressure in hepatic devascularized pigs with acute liver failure (45). Moreover, our data have shown that reduction in ammonia levels reduces systemic and brain inflammatory responses (37, 44). This study was designed to test the hypothesis that, in cirrhotic BDL rats, reduction in hyperammonemia with ornithine phenylacetate (OP) would reduce brain inflammation and that this would in turn restore eNOS activity by impacting on the brain DDAH-ADMA pathway.
MATERIALS AND METHODS

Animals. All animal experiments were conducted according to the Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. The animals were maintained as per the principle, guidelines, and approval of the ethical committee for animal care of University College London. This study was performed in male Sprague-Dawley rats (Charles River UK, Margate, UK), weighing 220–250 g, obtained from the comparative biological unit at University College London. All rats were housed in this unit and given free access (ad libitum) to standard rodent chow and water, with a light/dark cycle of 12 h:12 h (the dark phase extended from 1900–0700 h), at a temperature of 22–23°C and humidity of ~50%.

Animal models. For BDL, under anesthesia (diazepam 1 mg/kg intravenously, followed by Hypnorn 150 μl/kg intramuscularly; Janssen Pharmaceuticals, Beerse Belgium), all rats underwent BDL to randomized into three groups: sham-operated (control) rats receiving intravenously, followed by Hypnorm 150 μl/kg 0700 h), at a temperature of 22–23°C and humidity of 50%.

Study design. Four weeks after BDL or sham operation, rats were randomized into three groups: sham-operated (control) rats receiving intraperitoneal (i.p.) saline (placebo, n = 30) twice a day for the experimental period of 5 days; BDL disease control rats were administered i.p. saline twice a day for 5 days; a further group of BDL rats received i.p. OCR-002 (OP) 0.3 g/kg twice a day for 5 days. Between weeks 4 and 5 [BDL group; median day 33 (28–35); OP group median day 32 (28–35); P = ns], following anesthesia (2% isofluorane), rats from each study group underwent assessment of mean arterial pressure via isolation and cannulation of the right carotid artery. All rats were then euthanized by exsanguination under anesthesia, and blood was withdrawn from the descending aorta and immediately put into ice-cold heparin/EDTA-containing tubes and centrifuged at 3,000 revolution/min and 4°C for 10 min, and the plasma collected and stored at −80°C until assayed. Brain tissue was also harvested and snap-frozen for storage at −80°C until analyzed.

Assessment of level of consciousness. The conscious levels of rats used in this study were rated using an established neurological scale (26). It was rated as either normal, or loss of the scatter reflex (c.f. Grade 1 encephalopathy) and ataxia (c.f. Grade 2 encephalopathy), together representing precoma stages, or loss of the righting reflex (c.f. Grade 1–4 encephalopathy) representing the coma stage.

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 (25, 40). Tissue water content was then calculated as percentage of water = (1 − dry wt/wet wt) × 100%.

Measurement of plasma biochemistry. All biochemical parameters [alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, total protein, ammonia, bilirubin, and creatinine] were analyzed using 200 μl of respective plasma samples using a Cobas Integra 400 multianalyzer with the appropriate kits (Roche Diagnostics, Burgess Hill, West Sussex, UK).

Plasma and cortical brain cytokines. A sample (100 μg) of cerebral cortex was homogenized and deproteinized in 300 μl of ice-cold cell lysis buffer solution. After centrifugation at 12,000 g for 10 min at 4°C, and following protein concentration quantification, equilibrated brain protein samples and plasma supernatants (50 μl) were analyzed for TNF-α cytokine levels (pg/ml) by flow cytometry using the Becton Dickinson (BD Biosciences, San Diego, CA) rat inflammation cytometric bead array (CBA) kit, per manufacturer’s instructions. Fluorescence produced by the CBA beads was measured on a FACS Cantor II flow cytometer system (BD Sciences), and the data were analyzed with BD CTA software.

Measurement of nitrite/nitrate. Combined NO2/NO3 levels were determined from the heparinized plasma samples by a modified Greiss test, using previously described methods (10). Nitrite concentrations were measured in the filtrate by chemiluminescence as described previously (12).

Western blot analysis. Proteins were isolated from fresh-frozen brain tissues homogenates, and, after protein concentration determination, extracts containing equal amounts of protein were denatured and separated on 4–12% NuPAGE Bis-Tris gels and blotted on to PVDF membranes (Invitrogen, Paisley, UK). The membranes were then incubated with different primary antibodies including a goat anti-DAH 1 (1:1,000; in-house preparation), mouse anti-ADMA (1: 1,000; Acris Antibodies, Herford, Germany), mouse anti-eNOS, inducible NOS (iNOS), and rabbit anti-neuronal NOS (nNOS) (1:500, 1:10,000 and 1:1,000, respectively; Transduction Laboratories/Pharmingen, San Jose, CA and Cell Signaling Technology, Beverly, MA), mouse anti 4-hydroxynonenol (HNE) monoclonal antibody (1:1,000; Japan Institute for the Control of Aging, Nihken, Japan) and rabbit anti-NADPH oxidase (NOX)-1 (1:1,000; Novus Biologicals, Littleton, CO) with specific protein bands detected using respective horseradish peroxidase-conjugated secondary antibodies. The bands were visualized using an enhanced chemiluminescence detection kit and quantified by densitometry. Loading accuracy was evaluated via membrane rehybridization with antibodies against mouse and rabbit anti-α tubulin (1:1,000; Upstate Biotechnology, Albany, NY).

Tissue NOS activity. The conversion of 14C L-arginine to 14C l-citrulline was used as an index of NOS activity, as previously described (31). The activity was expressed as micromoles of citrulline made per milligram of protein per 30 min. The differences in the measured NOS activities between the calcium present/chelated (EGTA present for iNOS activity determination) assays were interpreted as representing constitutive NOS (cNOS) activity. For nNOS activity, brain homogenate was incubated with the assay mix containing 5 μl (from 1 mM stock) 7-nitroindazole (7-NI), a potent competitive inhibitor of nNOS. Final incubation volume was 1.170 μl. The Ki value calculated for 7-NI in the present study was 4.2 μM. nNOS activity was calculated by subtracting cNOS + 7-NI activity from cNOS-7-NI activity.

Plasma and brain ADMA, and L-arginine measurement. ADMA and L-arginine were measured as previously (30), using fragmenta- tion-specific stable isotope dilution electrospray tandem mass spectrometry. In brief, samples deproteinized with deuterated ADMA and L-arginine were chromatographed (acetonitrile/water, 1:1, with 0.025% formic acid) on a Teicoplanin guard column 10 mm × 2.1 mm ID (Chirobiotic T, ASTEC, Congleton, UK) and analyzed using a SCIEX API4000 (Applied Biosystems, Warrington, UK) in positive ion multiple-reaction monitoring mode. The plasma and brain concentrations were expressed as nmol/l and μmol/mg protein, respectively.

Statistics. Data are expressed as means ± SE. Three groups were analyzed by ANOVA, and, if the F value was significant, post hoc

Table 1. Effect of OP on arterial ammonia levels, plasma, biochemistry, and brain water in BDL rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (n = 10)</th>
<th>BDL (n = 10)</th>
<th>BDL + OP (n = 10)</th>
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<tr>
<td>Arterial ammonia, μmol/l</td>
<td>62.5 ± 4.0</td>
<td>215.7 ± 29.5</td>
<td>75.6 ± 16.5</td>
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<td>Nitrite, nmol/l</td>
<td>0.6 ± 0.1</td>
<td>1.0 ± 0.4</td>
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<td>ALT, U/l</td>
<td>47.6 ± 4.3</td>
<td>155.0 ± 14.9</td>
<td>102.0 ± 7.9</td>
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<tr>
<td>ALP, U/l</td>
<td>174.5 ± 12.3</td>
<td>564.7 ± 37.7</td>
<td>342.7 ± 20.5</td>
</tr>
<tr>
<td>Total protein, g/l</td>
<td>51.3 ± 2.1</td>
<td>43.1 ± 1.2</td>
<td>52.5 ± 3.6</td>
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<td>Bilirubin, μmol/l</td>
<td>3.2 ± 0.1</td>
<td>250.1 ± 16.1</td>
<td>259.7 ± 6.2</td>
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<tr>
<td>Albumin, g/l</td>
<td>33.9 ± 1.0</td>
<td>20.2 ± 0.8</td>
<td>26.1 ± 1.8</td>
</tr>
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<td>Creatinine, μmol/l</td>
<td>23.1 ± 1.0</td>
<td>40.9 ± 2.4</td>
<td>32.4 ± 1.4</td>
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<tr>
<td>Brain water, %</td>
<td>78.2 ± 0.2</td>
<td>79.6 ± 0.3</td>
<td>78.6 ± 0.1</td>
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</tbody>
</table>

Data are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with sham-operated control rats; 7P < 0.05, 7P < 0.01, and 7P < 0.001 compared with bile duct-ligated (BDL) rats. NS, nonsignificant compared with BDL rats. 7P < 0.01 and 7P < 0.001 compared with sham-operated control rats. OP, ornithine phenylacetate; ALT, alanine aminotransferase; ALP, alkaline phosphatase.
significant increase in plasma ALT, ALP, bilirubin, and creatinine levels observed in sham-operated rats (\(P < 0.001\), Table 1). Administration of OP to BDL rats significantly lowered plasma ALT, ALP, and creatinine (\(P < 0.0001, P < 0.0001\), and \(P < 0.01\), respectively), but plasma albumin levels significantly increased (\(P < 0.01\)). OP treatment in BDL rats had no significant effect on bilirubin and total protein concentrations (Table 1).

Neurobehavioral changes. All rats were still alive at the time of the study following sham operation or BDL. Administration of i.p. saline or OP had no overt effect on conscious levels, with all study rats remaining fully alert throughout the study period.

Arterial ammonia. Compared with sham operation, BDL rats were associated with significantly higher arterial ammonia concentrations (\(P < 0.0001\), Table 1). Treatment with OP in BDL rats resulted in significant lowering of arterial ammonia to levels no different from sham operation (\(P < 0.0001\), compared with control BDL rats).

Brain water measurements. Compared with sham operation, there was a significant increase in frontal cortex brain water content in saline-administered BDL rats (\(P < 0.05\)). This increase in brain water with BDL was ameliorated by OP treatment to levels observed in sham-operated rats (\(P < 0.01\), Table 1).

Plasma and brain cytokine concentrations. Compared with sham operation, plasma and brain TNF-\(\alpha\) concentrations were significantly increased with BDL (\(P < 0.05; P < 0.01\), respectively, Table 2). In BDL rats, treatment with OP was associated with significant lowering of plasma and brain TNF-\(\alpha\) levels (\(P < 0.05; P < 0.01\), respectively), not significantly different to sham-operated rats.

### RESULTS

Following surgery, all animals continued to gain weight. From the final body weight taken before termination, cirrhotic rats were found to be significantly lighter than sham (350 ± 5 g vs. 472 ± 9 g, respectively; \(P < 0.001\)). In BDL rats, OP treatment was associated with significant weight gain compared with cirrhotic controls (399 ± 16 g, \(P < 0.01\)). BDL rats showed significantly lower mean arterial pressure than control rats (85 ± 3 vs. 123 ± 2, \(P < 0.01\)). Mean arterial pressure did not change significantly after treatment with OP.

Compared to sham operation, BDL surgery promoted a significant increase in plasma ALT, ALP, bilirubin, and creatinine (\(P < 0.0001; P < 0.05; P < 0.0001; P < 0.0001\), respectively) and significantly decreased albumin and total protein concentrations (\(P < 0.0001; P < 0.01\), respectively) consistent with secondary biliary cirrhosis (Table 1). Administration of OP to BDL rats significantly lowered plasma ALT, ALP, and creatinine (\(P < 0.0001, P < 0.0001\), and \(P < 0.01\), respectively), but plasma albumin levels significantly increased (\(P < 0.01\)). OP treatment in BDL rats had no significant effect on bilirubin and total protein concentrations (Table 1).

### Table 2. Effect of OP on arterial and cerebral TNF-\(\alpha\) in BDL rats

<table>
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<tr>
<th>Parameters</th>
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<th>BDL ((n = 10))</th>
<th>BDL + OP ((n = 10))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TNF-(\alpha), pg/ml</td>
<td>121.9 ± 46.4 822.9 ± 203.1*</td>
<td>270.5 ± 59.4†</td>
<td>33.16 ± 5.0‡</td>
</tr>
<tr>
<td>Brain TNF-(\alpha), pg/ml</td>
<td>45.3 ± 12.9 236.8 ± 79.4†</td>
<td>33.16 ± 5.0‡</td>
<td>33.16 ± 5.0‡</td>
</tr>
</tbody>
</table>

Values are given as means ± SE. *\(P < 0.05\) and †\(P < 0.01\) compared to sham-operated control rats; ‡\(P < 0.05\) and §\(P < 0.01\) compared to BDL rats.

Comparisons were made by the Newman-Kuels multiple-range test; \(P < 0.05\) was taken to be statistically significant. Software used included Microsoft Excel 2007 (Microsoft, Redmond, WA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

### Fig. 1. Cerebral nitric oxide synthase (NOS) protein expression.

A: endothelial NOS (eNOS) expression. Compared with sham-operated controls (\(n = 6\)), there was a significant increase in eNOS protein expression in bile duct-ligated (BDL) rats (\(n = 6\)) (\(\ast\ast\ast P < 0.001\)), which was partly ameliorated by treatment with (0.6 g/kg) administration of ornithine phenylacetate (OP) (\(n = 6\)) over 5 days (\(\ast\ast\ast P < 0.05\)). B: neuronal NOS (nNOS) expression. Compared with sham-operated controls (\(n = 6\)), there was a significant increase in nNOS protein expression in BDL rats (\(n = 6\)) (\(\ast\ast\ast P < 0.01\)), which was significantly decreased following treatment with (0.6 g/kg) OP (\(n = 6\)) over 5 days (\(\ast\ast\ast P < 0.01\)). C: inducible NOS (iNOS) expression. Compared with sham-operated controls (\(n = 6\)), there was a significant increase iNOS protein expression in BDL rats (\(n = 6\)) (\(\ast\ast\ast P < 0.01\)), which was also ameliorated by treatment with (0.6 g/kg) administration of OP (\(n = 6\)) over 5 days (\(\ast\ast\ast P < 0.05\)). ††\(P < 0.01\), compared with sham-operated control rats.
Control rats. nNOS activity. Significantly increased nNOS activity was observed in BDL rat brain (n = 6) compared with sham-operated control rat brain (n = 6) (**P < 0.01). Treatment with (0.6 g/kg) OP (n = 6) over 5 days showed no significant changes in nNOS activity compared with BDL. †P < 0.01, compared with sham-operated control rats. C: iNOS activity. Compared with sham-operated controls (n = 6), BDL rats showed a significant increase in iNOS activity (n = 6) (**P < 0.01), which was decreased by treatment with (0.6 g/kg) administration of OP (n = 6) over 5 days ($$P < 0.01)

Cerebral NOS protein expression. eNOS, nNOS, and iNOS protein expression were significantly increased in BDL rat brains (P < 0.01, P < 0.01, and P < 0.01, respectively, Fig. 1, A–C) compared with sham. In BDL rats, treatment with OP was associated with a significant reduction in eNOS, nNOS, and iNOS protein expression (P < 0.05, P < 0.01, and P < 0.05, respectively).

Plasma nitrite levels. Compared with sham-operated rats, plasma nitrite concentrations were significantly increased in BDL rats (P < 0.01, Table 1). Following treatment with OP, there was no significant change in nitrite levels (P = 0.1).

Brain NOS activity. cNOS activity was significantly lower in BDL rat brain compared with sham (P < 0.05, Fig. 2A). Treatment with OP was associated with increased brain cNOS activity to levels no different from sham operation but significantly higher than untreated BDL rats (P < 0.05). By comparison, nNOS activity was markedly elevated in BDL rat brain compared with sham (P < 0.01, Fig. 2B), and this did not change with OP treatment. Similarly, iNOS activity was markedly elevated in BDL rat brains (P < 0.01, Fig. 2C) compared with sham; however, OP treatment was associated with a significant reduction in iNOS activity to levels observed in sham-operated rats and significantly lower than seen with untreated BDL (P < 0.01).

In contrast to the decrease in overall cNOS activity after BDL and its restoration with OP treatment, nNOS activity increased after BDL (P < 0.01; Fig. 2B) and was unchanged by OP treatment. Thus the directionally opposite changes seen in cNOS activity in Fig. 2A are assumed to represent changes in eNOS activity.

Plasma and brain ADMA, L-arginine, and L-arginine/ADMA ratio. Plasma and brain ADMA levels were significantly higher in the BDL animals compared with controls (P < 0.0001 and P < 0.05, respectively, Table 3). In BDL rats, OP treatment was associated with a significant lowering of brain ADMA levels (P < 0.05) toward sham values, albeit there was a minimal reduction in plasma ADMA compared with non-treated BDL (P = 0.2). There were no significant differences in brain L-arginine concentrations between sham-operated and BDL rats with or without OP treatment. However, the calculated L-arginine/ADMA ratios were significantly lower in brain tissue from BDL (P < 0.05), and this was restored towards sham levels following treatment of BDL rats with OP (P < 0.05).

Brain ADMA protein expression. ADMA protein expression was significantly increased in BDL rat brain (P < 0.01) compared with sham. Following treatment with OP to BDL, rats show significant (P < 0.01) decrease of ADMA protein expression (Fig. 3).

Brain DDAH-I protein expression. In BDL rats, there was a significant reduction in DDAH-I expression compared with

Table 3. Effect of OP treatment on plasma/cerebral ADMA, L-arginine and L-arginine/ADMA ratio in BDL rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (n = 6)</th>
<th>BDL (n = 6)</th>
<th>BDL + OP (n = 6)</th>
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</thead>
<tbody>
<tr>
<td>Plasma: ADMA, nmol/l</td>
<td>589.8 ± 45.5</td>
<td>942.7 ± 40.0†</td>
<td>864.3 ± 21.2</td>
</tr>
<tr>
<td>Brain: ADMA, µmol/mg protein</td>
<td>3.1 ± 0.4</td>
<td>4.8 ± 0.4*</td>
<td>3.4 ± 0.2§</td>
</tr>
<tr>
<td>Brain: L-arginine, µmol/mg protein</td>
<td>19.2 ± 1.3</td>
<td>23.9 ± 3.1</td>
<td>21.7 ± 2.2</td>
</tr>
<tr>
<td>Brain: L-arginine/ADMA ratio</td>
<td>6.1 ± 0.4</td>
<td>4.3 ± 0.6*</td>
<td>6.0 ± 0.4.5§</td>
</tr>
</tbody>
</table>

Values are given as means ± SE. *P < 0.05, †P < 0.0001, and ‡P < 0.0001 compared with sham-operated control rats; $P < 0.05 compared with BDL rats. NS, nonsignificant compared with BDL rats. ADMA, asymmetric-dimethylarginine.
sham ($P < 0.01$, Fig. 4), which was ameliorated by treatment with OP ($P < 0.01$).

**Brain 4HNE protein expression.** Compared with sham-operation, there was a significant increase in 4HNE protein expression ($**P < 0.01$), which was ameliorated by treatment with OP ($P < 0.01$) over 5 days ($$$P < 0.001$).

**Brain NOX-1 protein expression.** NOX-1 protein expression was significantly increased in BDL-induced cirrhotic rat brain compared with sham ($P < 0.05$, Fig. 5B). OP treatment over 5 days to BDL rat shows significant attenuation of NOX-1 protein expression ($P < 0.01$) compared with BDL alone.

**DISCUSSION**

The results of this study confirm the previous observation that hyperammonemia is associated with abnormalities in brain NO metabolism and extend these observations by providing novel insight into the associated mechanisms and the role of ammonia. Brain eNOS activity was shown to be reduced, whereas its protein expression was increased in the cirrhotic rats, associated with an increase in the brain concentrations of its inhibitor, ADMA. This in turn was coupled with a reduction in the protein expression of the ADMA metabolizing enzyme, DDAH (Fig. 4). Lowering of arterial ammonia, which has also been shown to be associated with a reduction in brain ammonia (17) following administration of OP, resulted in a significant reduction in brain cytokines and restoration of eNOS activity and DDAH protein expression. These data support the hypothesis that hyperammonemia in cirrhotic rats induces major defects in brain NO metabolism, and a reduction in its severity with OP restores brain eNOS activity.

NO plays an important role in regulating blood flow and preventing vascular and endothelial dysfunction. Considerable research has been focused on the cerebral circulation in acute and chronic liver diseases. Earlier studies in cirrhosis have suggested that cerebral blood flow is decreased in patients with advanced cirrhosis and that autoregulation is impaired (6, 20, 21). More recently, evidence of increased cerebral vascular resistance correlating with the severity of liver disease has been described (11). Using SPET scanning, we previously showed that induction of hyperammonemia in cirrhotic patients was associated with reduced cerebral perfusion (16), linking ammonia as a possible contributor of cerebral hypoperfusion in cirrhosis. In electron microscopy studies of brains of BDL rats, we observed markedly vasoconstricted cerebral blood vessels together with a maintained blood-brain barrier in keeping with the clinical data, suggesting that BDL rats are a clinically relevant model of cirrhosis (44).

Several lines of evidence suggest that NO availability in the brain of hyperammonemic animals may be altered. In acute hyperammonemia, NO availability was shown to be increased both in animal models and also in isolated astrocyte cultures exposed to ammonia (28, 32). However, chronic hyperammonemia has been shown to be associated with a reduction in the availability of brain NO, possibly through a reduction in the activity of guanylate cyclase (4, 14, 27). In chronically hyperammonemic animals, treatment with the phosphodiesterase inhibitor, sildenafil, or the cyclooxygenase inhibitor, ibuprofen, was shown to improve NO availability and the learning ability of rats. (3, 8). More recently, hyperammonemia has

![Fig. 3. Cerebral asymmetric-dimethylarginine (ADMA) protein expression.](http://ajpgi.physiology.org/)

Compared with sham-operated control ($n = 6$), BDL rats ($n = 6$) showed significant increase of ADMA protein expression ($**P < 0.01$), which was ameliorated by treatment with (0.6 g/kg) OP ($n = 6$) over 5 days ($$$P < 0.001$).

![Fig. 4. Cerebral dimethylarginine-dimethylaminohydrolase (DDAH)-1 protein expression.](http://ajpgi.physiology.org/)

A significant decrease in DDAH-1 protein expression was noted in BDL rats ($n = 6$), compared with sham-operated controls ($n = 6$) ($**P < 0.01$), which was also reversed by treatment with (0.6 g/kg) OP ($n = 6$) over 5 days ($$$P < 0.01$).
been shown to result in the activation of microglia and neuroinflammation (17, 18, 36). The results of our study extend these previous observations and suggest that additional mechanisms such as reduced eNOS activity (but increased eNOS protein expression) due to an increase in ADMA may contribute to reduced brain NO availability. The results also provide strong evidence that reduction in hyperammonemia with OP restores brain eNOS activity associated with a reduction of brain ADMA.

The synthesis of NO by eNOS is regulated by its competitive inhibitor ADMA, which has been shown to exist in high concentrations and distributed throughout the brain and to constrict cerebral vessels under resting conditions (8, 41). More recently, it has been shown that infusion of ADMA to normal healthy volunteers increases vascular tone and decreases cerebral perfusion (19). Faraci et al. (9) found that 50% of rat brain NOS activity was inhibited by infusion of ADMA even at low or physiological ADMA concentrations. Our observations show that hyperammonemia in BDL animals is associated with significantly increased ADMA concentration compared with sham controls. Following treatment with OP, which reduced arterial and brain ammonia levels, blood and brain ADMA levels were lowered significantly. The increase in ADMA in the untreated BDL animals was associated with reduced brain eNOS expression through high inhibitory levels of ADMA. OP treatment significantly reduces both hyperammonemia and inflammation in the brain and redresses the imbalance in the brain NO-ADMA-DDAH pathway.

Fig. 5. Cerebral oxidative stress markers. A: cerebral 4-hydroxynonenal (4-HNE) protein expression was significantly increased in BDL (n = 6)-induced cirrhotic rat brain compared with sham (n = 6) (***(P < 0.0001). Treatment with (0.6 g/kg) OP (n = 6) over 5 days to BDL rats shows significant decrease of 4-HNE protein expression (SP < 0.05). +++P < 0.0001, compared with sham-operated control rats. B: compared with sham-operated control (n = 6), there was a significant increase of cerebral NADPH oxidase (NOX)-1 protein expression in BDL rats (n = 6) (***P < 0.05), which was also ameliorated by (0.6 g/kg) OP (n = 6) over 5 days (SSP < 0.01).

Fig. 6. Schematic representation of a working hypothesis for how OP treatment may improve hepatic encephalopathy (HE) in a rat model of cirrhosis. Hyperammonemia and inflammation contribute to HE in cirrhosis associated with a reduction in DDAH-1. A reduced level of this enzyme, required for the metabolism of brain ADMA, leads to decreased brain eNOS activity through high inhibitory levels of ADMA. OP treatment significantly reduces both hyperammonemia and inflammation in the brain and redresses the imbalance in the brain NO-ADMA-DDAH pathway.
NOX-1 protein expressions, which were reduced significantly in the OP-treated animals. Additionally, our data show evidence of increased activity and expression of brain iNOS in the BDL animals, which we and others have previously shown is associated with increased brain nitrotyrosine (13, 43, 44). iNOS protein expression and activity were significantly reduced in the OP-treated group, indicating that reduction in hyperammonemia may attenuate the severity of oxidative stress and potential scavenging of brain NO.

As DDAH activity and protein expression can be markedly reduced during inflammation and or oxidative stress (34), it is interesting to hypothesize that one of the mechanisms that may be involved in the restoration of DDAH protein expression, and therefore ADMA levels in the OP-treated group, may be through a reduction in inflammation. Similar reduction in DDAH protein expression has been described together with increased hepatic ADMA levels in the livers of cirrhotic patients with superimposed alcoholic hepatitis compared with cirrhotic patients in whom there was no hepatic inflammation (30). As brain swelling can initiate transcription of cytokines, it is also possible that reduction in brain water following treatment with OP led to a reduction in brain inflammation. It is likely that the reduction in brain inflammation and brain water is consequent on reduction in ammonia concentration as ammonia has been shown to produce neuroinflammation (38). This may in turn have contributed to improved DDAH expression. Although there was no significant difference in the concentration of l-arginine in the BDL animals compared with the treated group, the ADMA/l-arginine ratio was significantly greater in the untreated group, which was reduced toward sham levels in the animals treated with OP.

In conclusion, the results of this study provide compelling evidence that hyperammonemia contributes to eNOS dysfunction through induction of inflammation and alterations in DDAH protein expression leading to an increase in ADMA. Reduction in ammonia with OP reduces neuroinflammation and restores eNOS activity.

DISCLOSURES
OCR-002 (Ornithine phenylacetate) was donated for this study by Ocera Therapeutics (SD, USA). UCL has licensed its invention ornithine phenylacetate in hepatic encephalopathy to Ocera, and Prof. Jalan is the named inventor on the patents.

REFERENCES


