Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Ornithine phenylacetate targets alterations in the expression and activity of glutamine synthase and glutaminase to reduce ammonia levels in bile duct ligated rats

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Background & Aims: In liver failure, ammonia homeostasis is dependent upon the function of the ammonia metabolising enzymes, glutamine synthetase (GS) and glutaminase (GA) but data about their protein expression and activity are lacking. The aims of this study were to determine the protein expression and activity of GS and GA in individual organs in a rat model of chronic liver disease and to test whether the treatment with the ammonia-lowering agent ornithine phenylacetate (OP) modulates their activities.

Methods: 49 SD rats were studied 35 days after sham-operation or bile duct ligation (BDL). The BDL group received: L-ornithine (0.6 mg/kg/day), Phenylacetate (0.6 mg/kg/day), OP (0.6 mg/kg/day) or placebo (saline) for 5 days prior to sacrifice. Arterial ammonia, amino acids and liver biochemistry were measured. Expressions of GS and GA were determined by Western-blotting and activities by end-point methods in liver, muscle, gut, kidney, lung, and frontal cortex.

Results: In BDL rats, hepatic GS enzyme activity was reduced by more than 80% compared to sham rats. Further, in BDL rats GA activity was reduced in liver but increased in the gut, muscle and frontal cortex compared to sham rats. OP treatment resulted in a reduction in hyperammonemia in BDL rats, associated with increased GS activity in the muscle and reduced gut GA activity.

Conclusions: In a rat model of chronic liver failure, hyperammonemia is associated with inadequate compensation by liver and muscle GS activity and increased gut GA activity. OP reduces plasma ammonia by increasing GS in the muscle and reducing GA activity in the gut providing additional insights into its mechanism of its action. GS and GA may serve as important future therapeutic targets for hyperammonemia in liver failure.

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Introduction

Ammonia is a neurotoxin and its plasma concentration increases with the progression of liver disease [1]. At high plasma levels, ammonia is taken up by the brain, which is thought to be central in the pathogenesis of hepatic encephalopathy (HE). Ammonia can cause astrocytic swelling, neurotransmitter synthesis and release, neuronal stress, and an impairment of mitochondrial function [2]. Moreover, hyperammonemia has been associated with muscle loss in cirrhosis [3]. Therefore, there is a need to develop strategies to reduce ammonia levels in liver disease. Therapies that successfully target ammonia removal are an unmet need.

In liver failure, when the capacity of the liver to synthesise urea is impaired [4], ammonia metabolism is critically controlled by two enzymes; glutamine synthetase (GS) (EC 6.3.1.2), which catalyses the synthesis of glutamine from ammonia and glutamate (removing ammonia from the body pool), and glutaminase (GA) (EC 3.5.1.2), which catalyses the reverse reaction regenerating ammonia from the breakdown of glutamine. Circulating levels of ammonia depend on several organs (liver, muscle, small bowel, and kidney) [5,6]. Glutamine deamidation by intestinal GA has been proposed as one of the main sources of ammonia production in patients with cirrhosis [7]. Also the kidney GA could contribute to the production of the systemic hyperammonemia in liver failure under some circumstances [8]. Moreover, the development of portosystemic shunting and alteration of blood flow is thought to affect circulating ammonia levels in cirrhosis [9,10]. The contribution of each component remains unknown. Gut glutaminase has been suggested as a target for therapy in HE [7,11,12]. GS protein expression is known to be

Keywords: Glutamine synthetase; Glutaminase; Hepatic encephalopathy; Amino acids; Ammonia; Cirrhosis; Chronic liver failure; Ornithine phenylacetate.

Received 11 March 2013; received in revised form 7 October 2013; accepted 8 October 2013; available online 26 October 2013

In this publication the term *ammonia* refers to NH_4^+/NH_3, ammonium (NH_4^+), and ammonia (NH_3) indistinctly.

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Abbreviations: HE, Hepatic encephalopathy; ALF, Acute liver failure; CLF, Chronic liver failure; Gln, Glutamine; Glu, Glutamate; GS, Glutamine synthetase; OP, O-rnithinephenylacetate; GA, Phosphate-activated glutaminase; BDL, bile duct ligation; O, Ornithine; P, Phenylacetate; PAGN, phenylacetylglutamine (PAGN).

reduced in the peri-central hepatocytes in cirrhosis [13,14] and the stimulation of the skeletal muscle GS isoform plays a pivotal role in ammonia detoxification [15,16]. These findings suggest that approaches to target GA (by inhibition) or GS (by stimulation) may provide targets for ammonia detoxification as a valid therapeutic strategy for HE.

Ornithine phenylacetate (OP) is a novel treatment for hyperammonemia and hepatic encephalopathy in liver failure [17,18]. Studies have demonstrated that the administration of OP decreases plasma ammonia levels in animal models of liver disease and patients with cirrhosis [17,19–22]. The mechanism of action of OP is thought to occur in two main steps. Firstly, ornithine is delivered to the liver and muscle and converted to glutamate, which favors the substrate availability for the synthesis of glutamine. Then, glutamine binds covalently with phenylacetate to produce a non-reabsorbable substance, phenylacetylglutamine (PAGN), which can be excreted in urine. The increase in urinary excretion of PAGN could not fully account for the decrease in arterial ammonia described in a previous study [20] suggesting that OP may work through additional mechanisms, such as increasing glutamine production in the liver [23]. In addition, it may well modulate ammonia levels by impacting on the function of the enzymes, GS and GA.

This study aimed to investigate the impact of chronic liver failure on the protein expression and activity of GS and GA in the main organs involved in ammonia homeostasis (liver, muscle, small intestine, kidney, lung, and frontal cortex) in a 5 week bile-duct ligation (BDL) rat model and the effect of OP, ornithine (O) alone and phenylacetate (P) alone.

Materials and methods

Animals

All animal experiments were conducted in accordance with the UK Animals in Scientific Procedures Act 1986 and with approval from the local ethics committee Fifty-eight male Sprague-Dawley rats, body weight (260 ± 5.1) g were used.

Experimental design

All animals underwent sham operation (n = 15) or BDL (n = 43) to induce cholestatic fibrosis and portal hypertension under anaesthesia as described previously [24]. Nine animals were excluded because of inadequate ligation or death. Thirty days after BDL (or sham-operation) rats were randomized into four groups. In Group 1 (n = 15) sham-operated (control) rats were administered saline intraperitoneally (I.P.) (placebo) twice a day for the experimental period of 5 days. In Group 2 (n = 18) BDL rats were administered saline I.P. twice a day for 5 days and served as disease controls. In Group 3 (n = 15) BDL rats received OP, I.P. (OCR-002; Ocera Pharmaceuticals, US) 0.3 g/kg twice a day for 5 days. In Group 4 (n = 5) BDL rats received L-ornithine (O) I.P. 0.3 g/kg twice a day for 5 days. In Group 5 (n = 5) BDL rats received phenylacetate (P) I.P. 0.3 g/kg twice a day for 5 days. We used the groups 4 and 5 for checking effects of O and P separately to determine whether either component of OP was responsible for any observed change. The investigators were blinded to the treatment administered to the animals, which was performed by the animal technicians.

The experiments were performed in 3 successive batches and the data presented combines the results from these experiments. We performed a first study (Study 1) and two supplemental studies (Study 2a and 2b) as a confirmation of the findings in the enzymatic activities. In Study 1, we measured biochemistry and amino acids, protein expression for GS and GA and enzyme activities and in the other 2 studies we repeated the enzymatic activities and biochemistry. Tables 2 and 3 shows the averages and standard deviations for the pooled data.

The rats were sacrificed by exsanguination, blood was withdrawn from the descending aorta and immediately put into ice cold lithium heparin or EDTA tubes, centrifuged at 3120*g* at 4 °C, and the plasma collected and stored at −80 °C until assayed.

Reagents

All chemicals used for protein and amino acid assays were obtained from Sigma (UK), Human Serum Albumin (HSA) from Promega (UK). All reagents for HPLC-MS were high quality grade for MS.

Biochemical measurements

Plasma biochemistry and arterial ammonia

Plasma biochemistry and arterial ammonia were analysed using a COBAS Integra multiple analyser (Roche Diagnostics, UK) as previously described [24] in all animals studied.

Amino acid determination in arterial plasma

25 µl of plasma was spiked with 100 µl methanol 0.1% of formic acid, shaken for 2 min and centrifuged for 10 min at 4 °C. The measurements were performed by high performance liquid chromatography followed by mass spectrometer in positive mode (HPLC-MS; Dionex uHPLC system with a Ultimate 3000 RS autosampler and BD Biphenyl-1.9 µm, 50 × 2.1 mm column switcher with accompanying Thermo Scientific Orbitrap XL system and with Electrospray source) as described [25].

Tissue homogenization

Snap frozen (−80 °C) and stored 300 mg tissue samples from liver, neck skeletal muscle, small intestine (first portion of the small gut), kidney, lung samples and 100 mg of cortical brain (from the frontal cortex), were homogenized as described previously [22]. To ensure all cell and mitochondrial membrane rupture, release of free glutaminase from the mitochondria and glutamine synthetase from the cytosol, three freeze-thaw cycles were performed and 0.1% dodecyl maltoside was added to the homogenised samples in lysis buffer solution (50 mM Tris, 1 mM EDTA, pH 7.4) before centrifugation at 12,000*g* for 60 min at 4 °C. The supernatants were collected for Western blotting and activity assays. Protein concentration was measured by the Biuret method as we have done before [26].

Western blot analysis

Western-blot was performed following NuPAGE® pre-cast gel system (Invitrogen Ltd, UK) protocol. All membranes were stained with ponceau red after being transferred as a loading gel control test. L-Type GA antibody was used in liver and frontal cortex, and K-Type in gut, muscle, lung, kidney, and frontal cortex. Specific protein bands were detected (around 42 kDa for GS and 60 kDa for GA, in some tissues several bands in GA around 56–58 kDa were detected, these were analysed as a single band). GS and L-Type GA antibodies were obtained from Abfrontier (UK), and Santa Cruz Biotechnology, (USA) respectively. For K-Type GA, the antibodies were custom made in rabbits described previously [11]. Secondary goat polyclonal antibody to rabbit IgG-HRP conjugated (Hycult biotechnology, Netherlands) was used. Alpha-tubulin (α-tubulin; Santa Cruz Biotechnology, Inc. USA) was measured as a loading protein control to establish accurate differences in total protein expression between sample tissues and required a secondary goat polyclonal antibody to mouse IgG-HRP conjugated (Hycult biotechnology, Netherlands) for detection. All primary antibodies were used at a dilution of 1:1000 and secondary at 1:5000. Protein bands were visualized using Amersham ECL™ advance western blotting detection reagents and Hyperfilm™ (GE Healthcare, UK). Densitometry measurements were made using Image-J software (freeware; rsbweb.nih.gov/ij).

Enzyme activities

GS and GA activities were measured by colorimetric end-point methods in liver, muscle, small gut, kidney, lung, and brain (frontal cortex) as described previously [11,27] in all animals. GS activity was measured by Minet method [28] and GA activity was measured as we described before [11]. Specific activities of enzymes were expressed in international units (IU) per milligram of total homogenate of protein. One IU was defined as micromole of substrate transformed per minute.

Statistical analysis

The HPLC-MS analysis was performed by Chromeleon, Xcalibur and Microsoft Excel software. Results were compared using exact Mann-Whitney test (significance p value <0.05, two-tailed).

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