Research Article

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Ammonia produces pathological changes in human hepatic stellate cells and is a target for therapy of portal hypertension☆

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Background & Aims: Hepatic stellate cells (HSCs) are vital to hepatocellular function and the liver response to injury. They share a phenotypic homology with astrocytes that are central in the pathogenesis of hepatic encephalopathy, a condition in which hyperammonemia plays a pathogenic role. This study tested the hypothesis that ammonia modulates human HSC activation *in vitro* and *in vivo*, and evaluated whether ammonia lowering, by using L-ornithine phenylacetate (OP), modifies HSC activation *in vivo* and reduces portal pressure in a bile duct ligation (BDL) model.

Methods: Primary human HSCs were isolated and cultured. Proliferation (BrdU), metabolic activity (MTS), morphology (transmission electron, light and immunofluorescence microscopy), HSC activation markers, ability to contract, changes in oxidative status (ROS) and endoplasmic reticulum (ER) were evaluated to identify effects of ammonia challenge (50 μM, 100 μM, 300 μM) over 24–72 h. Changes in plasma ammonia levels, markers of HSC activation, portal pressure and hepatic eNOS activity were quantified in hyperammonemic BDL animals, and after OP treatment.

Results: Pathophysiological ammonia concentrations caused significant and reversible changes in cell proliferation, metabolic activity and activation markers of hHSC *in vitro.* Ammonia also induced significant alterations in cellular morphology, characterised by cytoplasmic vacuolisation, ER enlargement, ROS production, hHSC contraction and changes in pro-inflammatory gene expression together with HSC-related activation markers such as α-SMA, myosin IIa, IIb, and PDGF-Rβ. Treatment with OP significantly reduced plasma ammonia (BDL 199.1 μmol/L ± 43.65 *vs.* BDL + OP 149.27 μmol/L ± 51.1, $p < 0.05$) and portal pressure (BDL 14 ± 0.6 *vs.* BDL + OP 11 ± 0.3 mmHg, $p < 0.01$), which was associated with increased eNOS activity and abrogation of HSC activation markers.

Conclusions: The results show for the first time that ammonia produces deleterious morphological and functional effects on HSCs *in vitro*. Targeting ammonia with the ammonia lowering drug OP reduces portal pressure and deactivates hHSC *in vivo*, highlighting the opportunity for evaluating ammonia lowering as a potential therapy in cirrhotic patients with portal hypertension.

Keywords: Human hepatic stellate cells, (hHSC); Ammonia; Glutamine synthetase, (GS); Ornithine phenylacetate, (OP); Bile duct ligation, (BDL); Endoplasmic reticulum stress, (ER); Oxidative stress; Hepatic encephalopathy, (HE).

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Abbreviations: 3-NTyrosine, 3-Nitrotyrosine; α-SMA, alpha Smooth Muscle Actin; AAs, Amino acid-rich; ATF6, Activating transcription factor 6; BDL, Bile Duct Ligation; BiP, Immunoglobulin heavy chain binding protein; BrdU, 5-Bromo-2-Deoxyuridine; carboxy-DCF, (5(6)-Carboxy-2′,7′-dichlorofluorescein; carboxy-H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; CCL2, Chemokine (C-C motif) ligand 2; CHOP, CCAAT-enhancer-binding protein homologous protein; CM, Complete Medium; Col1A1, Collagen type I alpha 1; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, Enzyme-Linked Immunosorbent Assay; eNOS, Endothelial Nitric Oxide Synthase; ER, Endoplasmic Reticulum ET1, Endothelin 1; GFAP, Glial Fibrillary Acidic Protein; GS, Glutamine Synthetase; HE, Hepatic Encephalopathy; hHSC, Human Hepatic Stellate Cells; HPRT1, Hypoxanthine phosphoribosyltransferase 1; IL1β, Interleukin-1 beta; IL6, Interleukin-6; IL8, Interleukin-8; iNOS, Inducible Nitric Oxide Synthase; LOX, Lysyl oxidase; MAP, Mean arterial pressure; MMP2, Matrix metalloproteinase-2; mRNA, Messenger RNA; MSO, L-Methionine sulfoximine; MTS, (4-sulfophenyl)-2H-tetrazolium, inner salt); NFkB, Nuclear Factor kappa B; NH4Cl, Ammonium Chloride; OP, Ornithine Phenylacetate; p38MAPK, p38 mitogen-activated protein kinases; PDGF-Rβ, Platelet derived growth factor receptor-β; RNA, Ribonucleic Acid; ROS, Reactive Oxygen Species; SEM, Standard Error of the Mean; SFM, Serum Free Medium; SOD2, Superoxide dismutase 2; TGFbeta1, Transforming Growth Factor Beta 1; TIMP-1, Metallopeptidase inhibitor 1; TRITC, Tetramethylrhodamine; XBP1, X-box binding protein 1.

Research Article

Introduction

Hepatic stellate cells (HSCs) orchestrate many important functions in the liver and their activation and consequent dysfunction is associated with many complications including hepatic fibrosis, portal hypertension and liver cancer [1–3]. Astrocytes are cells that are centrally involved in the pathogenesis of hepatic encephalopathy (HE), an important complication of cirrhosis [4,5]. Hyperammonemia is a feature of liver failure and over the past 120 years many studies have demonstrated incontrovertibly its central role in the pathogenesis of HE [6,7]. The mechanism of ammonia-induced HE is hypothesised to be through induction of astrocytic osmotic stress swelling induced by glutamine derived from ammonia detoxification by the action of glutamine synthase [4,5]. More recently, it has been shown that ammonia itself and/or the resulting of cell swelling can initiate a cascade of pathological events such as oxidative stress, resulting in RNA oxidation and nitrosative stress, leading to nitration of critical proteins [8]. Many studies have indicated that human HSCs, like astrocytes, share similar markers when activated indicating that they belong to a similar mesodermal origin [9–12]. Both cell types are characterised by a large cytokine repertoire including NFkB-induced pro-inflammatory cytokines [13–16].

Previous work showed that rat HSCs express glutamine synthetase (GS) leading to our hypothesis that excess ammonia may therefore produce deleterious effects on the activity and function of primary human HSC as it does in astrocytes [17]. Following acute or chronic liver injury, HSCs undergo phenotypic transformation from "quiescent" (non-proliferating and non-contractile) to "activated" (promitogenic, profibrogenic, and pro-inflammatory myofibroblasts-like) cells. Moreover, during the process of activation, HSCs become highly contractile and develop the necessary machinery to contract or relax in response to a number of vasoactive substances/stimuli [18–25]. Several lines of investigation indicate that activation of HSCs and their ability to contract represent crucial contributory mechanisms associated with the development of portal hypertension [21,22,26], the severity of which has been shown to result in variceal bleeding and a hepatic venous pressure gradient of greater than 10 mmHg is strongly predictive of future decompensation [27].

The aim of the present study, was to test the hypothesis that ammonia further activates human HSCs (hHSCs) *in vitro* and has consequences on hHSC biology. We then tested whether this activation of hHSCs occurred *in vivo* by induction of hyperammonemia in a bile duct ligated animal model (BDL). Finally, we tested whether reduction of ammonia by administration of an ammonia lowering drug L-ornithine phenylacetate (OP) (OCR-002) [28,29] in BDL rats was associated with a negative modulation of HSC activation and in the reduction of portal pressure.

Materials and methods

Please refer to Supplementary data section for more detailed descriptions.

In vitro studies in human HSC

Primary hHSCs were isolated from wedge surgical sections of liver normal liver tissue, obtained from patients undergoing surgery in the Royal Free Hospital after giving informed consent (EC01.14-RF). Cells were isolated according to Mederacke *et al.* [30], with the relative modifications for human liver [31]. Briefly, 10 g of total human liver tissue was digested with 0.01% collagenase, 0.05% pronase and 0.001% DNase I without performing perfusion. The homogenate was filtered through a 100 μm cell strainer and the flow-through was centrifuged at 50 g for 2 min at 4 °C. After washing the supernatant, gradient centrifugation was performed at 1400 g for 17 min at 4 °C using an 11.5% Optiprep gradient. Finally, the interface was collected and washed. Purity of hHSCs was established by detection of CD140b (PDGFRβ), CD29 (Integrin beta 1) and cytoglobin (CYGB).

The obtained HSCs were cultured in RPMI supplemented with 20% foetal bovine serum (FBS), GLUTAMAX, 1x nonessential amino acids, 1.0 mM sodium pyruvate, 1x antibiotic-antimycotic (all Life Technologies), referred to as complete HSC medium hereinafter. Experiments described in this study were performed on hHSCs of at least three independent cell preparations between passage 3 and 8.

Treatment

Cells were seeded (density $26 \times 10^3/cm^2$) under basic, serum-rich conditions (CM complete medium) for 24 h, followed by serum deprivation for another 24 h (SFM). It is well established that ammonia leads to the production of glutamine when GS is present and conversely, glutamine can lead to the generation of ammonia, not allowing the effects of exogenous ammonia to be studied. Thus, exogenous glutamine was removed from the culture medium to avoid a confounding element in the experimental protocol. Specific treatment with NH_4Cl treatments were replaced daily for the duration of the experiment as described in the Supplementary information.

Animal models

Animals

All animal experiments were conducted according to the Home Office guidelines under the UK Animals in Scientific Procedures Act 1986 with 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.

In one experimental model, rats were administered a high protein/ammoniagenic diet (AAs) for 5 days [13,32]. Furthermore, all rats underwent BDL to induce cirrhosis or a sham operation as described previously [33].

Study design

(i) In this experimental protocol, animals underwent BDL surgery and were given 4 weeks to develop liver injury. During the 4th week, BDL animals were randomized into 3 groups: the first group included BDL rats receiving an amino acid-rich (AAs) diet in addition to injection of intraperitoneal (i.p.) saline solution (n = 4); the second group received the AAs diet and was treated with an i.p. injection of the ammonia lowering agent OP 0.3 g/kg twice a day for 5 days (n = 4); the third group consisted of BDL rats receiving saline solution i.p. (n = 4). In addition to the BDL animals, a further group of sham-operated rats received saline solution (i.p.) (n = 4). Animals were sacrificed on the 5th day of treatment.

(ii) In a second experimental protocol, the effect of the ammonia lowering agent OP on ammonia-induced portal hypertension was investigated. Four weeks after BDL or sham operation, rats were randomized into three groups: sham-operated rats receiving saline (i.p.) (n = 18) twice a day for the experimental period of 5 days; BDL rats (n = 20) were administered i.p. saline twice a day for 5 days; a further group of BDL rats (n = 11) received i.p. injection of OP 0.3 g/kg twice a day for 5 days. Between weeks 4 and 5, following anesthesia (2% isofluorane), rats from each group underwent assessment of mean arterial pressure (MAP) via isolation and cannulation of the right carotid artery. In addition, portal pressure was measured by direct cannulation of the main portal vein. All measurements were transduced to a Powerlab (4SP) linked to Chart v5.0.1 software. The mean of three readings taken one minute apart was recorded. Liver tissue was harvested and snap-frozen for storage at 80 °C until analysed.

Statistical analysis

Results are expressed as mean values ± SEM and compared using one-way analysis of variance followed by Dunnet's or Tukey's multiple comparison post hoc tests, where appropriate. p values $\leqslant 0.05$ were considered significant.

In vivo experimental data were analysed by t tests and Mann-Whitney U test as appropriate; $p < 0.05$ was considered statistically significant. Results are presented as mean values ± SEM using GraphPad Prism software (GraphPad, La Jolla, CA).