Research Article

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Hepatic dimethylarginine-dimethylaminohydrolase1 is reduced in cirrhosis and is a target for therapy in portal hypertension

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Background & Aims: Portal hypertension is characterized by reduced hepatic eNOS activity. Asymmetric-dimethylarginine (ADMA), an eNOS inhibitor, is elevated in cirrhosis and correlates with the severity of portal hypertension. Dimethylarginine dimethylaminohydrolase-1 (DDAH-1) is the key enzyme metabolizing hepatic ADMA. This study characterized DDAH-1 in cirrhosis, and explored hepatic DDAH-1 reconstitution through farnesoid X receptor (FXR) agonism and DDAH-1 gene therapy.

Methods: DDAH-1 immunohistochemistry was conducted on human cirrhosis and healthy liver tissue. Subsequently, sham-operated or bile-duct-ligated (BDL) cirrhosis rats were treated with the FXR agonist obeticholic acid (OA, 5 mg/kg) or vehicle for 5 days. Further, animals underwent hydrodynamic injection with DDAH-1-expressing plasmid or saline control, which resulted in the following groups: sham + saline, BDL + saline, BDL + DDAH-1-plasmid. Portal pressure (PP) measurements were performed. Plasma ALT was measured by COBAS INTEGRA, DDAH-1 expression by qPCR and Western blot, eNOS activity by radiometric assay.

Results: Immunohistochemistry and Western-blotting confirmed hepatic DDAH-1 was restricted to hepatocytes, and expression decreased significantly in cirrhosis. In BDL rats, reduced DDAH-1 expression was associated with elevated hepatic ADMA, reduced eNOS activity and high PP. OA treatment significantly increased DDAH-1 expression, reduced hepatic tissue ADMA, and increased liver NO generation. PP was significantly reduced in BDL + OA *vs.* BDL + vehicle (8 ± 1 *vs.* 13.5 ± 0.6 mmHg; $p < 0.01$) with no change in the mean arterial pressure (MAP). Similarly, DDAH-1 hydrodynamic injection significantly increased hepatic DDAH-1 gene and protein expression, and significantly reduced PP in BDL + DDAH-1 *vs.* BDL + saline ($p < 0.01$).

Conclusions: This study demonstrates DDAH-1 is a specific molecular target for portal pressure reduction, through actions on ADMA-mediated regulation of eNOS activity. Our data support translational studies, targeting DDAH-1 in cirrhosis and portal hypertension.

Keywords: Portal hypertension; ADMA; DDAH-1; Nitric oxide.

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Abbreviations: ADMA, asymmetric dimethylarginine; DDAH-1, dimethylarginine dimethylaminohydrolase 1; eNOS, endothelial nitric oxide synthase; FXR, farnesoid X receptor; HVPG, hepatic venous pressure gradient; MAP, mean arterial pressure; NO, nitric oxide; OA, obeticholic acid.

Introduction

Sinusoidal portal hypertension, as a consequence of chronic liver disease, heralds the onset of the most lethal complications of cirrhosis. The pathobiology of portal hypertension is characterized by elevated intrahepatic resistance to flow and increased splanchnic blood flow. Intrahepatic resistance occurs not only because of hepatic fibrosis, but also due to sinusoidal endothelial dysfunction and increased intrahepatic vascular tone [1]. Nitric oxide (NO) is an essential regulator of intrahepatic vascular tone. In cirrhosis, hepatic NO levels are significantly reduced, with associated elevated sinusoidal vascular resistance [2].

Asymmetric dimethylarginine (ADMA) is a competitive endogenous inhibitor of endothelial nitric oxide synthase (eNOS) associated with eNOS dysfunction in decompensated cirrhosis [3] and acute liver failure [4]. Indeed, we recently demonstrated elevated plasma and hepatic ADMA levels in patients with cirrhosis and superimposed alcoholic hepatitis, associated with significantly reduced hepatic eNOS activity, increased portal pressure and increased mortality [5]. ADMA is formed ubiquitously within cells through proteolysis, and acts in a paracrine fashion to inhibit the action of all nitric oxide synthases [6]. Hepatic ADMA levels are predominantly controlled through elimination by the enzyme dimethylarginine dimethylaminohydrolase-1 (DDAH-1), hence hepatic DDAH-1 dysfunction is thought to be a key pathogenic mechanism for ADMA accumulation [7].

The farnesoid X receptor (FXR) is part of a family of nuclear hormone receptors that have an important role in bile, lipid and glucose homeostasiss [8]. In addition, FXR modulates the transcription of many inflammatory and cell-cycle control genes and is particularly abundant in the liver, kidney and intestine. Recently, *DDAH-1* was identified as an FXR target gene from studies in diabetic Zucker rats, where a synthetic FXR agonist was

shown to significantly increase hepatic *DDAH-1* gene expression [9].

The aim of this study was to determine if DDAH-1 expression was altered in cirrhotic rodents, and if so, to investigate the potential therapeutic benefit of augmenting hepatic DDAH-1 with an FXR agonist on hepatic ADMA levels, eNOS activity and portal pressure. Subsequently, to confirm that the portal pressure lowering effects of the FXR agonist were mediated through a DDAH-1 mechanism, a gene therapy approach was used to similarly augment hepatic DDAH-1 levels and confirm a therapeutic lowering of portal pressure.

Materials and methods

Human tissue samples

Human liver samples were obtained with local ethical committee approval and all patients gave written informed consent. Transjugular liver biopsy specimens were obtained from a series of patients with alcoholic cirrhosis and portal hypertension, and fixed in formalin for subsequent histological evaluation. Further control liver samples from patients without liver disease were also obtained for histology.

Histological evaluation

Human liver sections were embedded, cut and stained with haematoxylin and eosin (H&E) using standard techniques, and immunostaining with anti-DDAH-1 antibodies (Abcam, UK) was performed as previously described [10].

Animals and bile duct ligation model

All animal experiments were conducted in accordance with the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley rats (Charles-Rivers), weighing 220 ± 25 g, were housed in a temperature and humidity controlled environment. Animals had access to food and water *ad libitum*, with a light/dark cycle of 12 h. The bile duct ligation (BDL) model of cirrhosis was used in this study, since this replicates advanced cirrhosis, demonstrating portal hypertension and extra-hepatic organ failure [11]. BDL and sham surgery was performed under induction of anaesthesia with 5% isoflurane and maintenance with 2% isoflurane, as previously described [11]. To confirm findings in the BDL model, hepatic DDAH-1 was also characterised in the carbon tetrachloride (CCl_4) model previously described in Sprague-Dawley rats [12].

Study design

(i) Three weeks after BDL and sham surgery, rats were orally administered the FXR agonist obeticholic acid (OA) (Intercept Pharmaceuticals, USA) at 5 mg/kg in vehicle (corn oil) for 5 days, or vehicle alone. Three groups were studied: (a) Sham + vehicle, (b) BDL + vehicle, (c) BDL + OA. At the end of the study, at week 4, all rats underwent haemodynamic measurements and were then sacrificed.

(ii) A further group of Sprague Dawley rats underwent hydrodynamic injection of plasmid DNA, through modification of the method described by Maruyama *et al.* [13], and extensively reviewed by Sudan and Liu [14], leading to efficient temporary hepatic transgene expression in rodents. Rapid, high-volume, high-pressure injection is thought to lead to transgene expression by causing retrograde flow in the inferior vena cava and hepatic vein, leading to transient permeation of cell membranes and subsequent uptake and expression of plasmid DNA. Endotoxin-free plasmid DNA (1 mg) was prepared using the Qiagen Endo-Free Plasmid Giga Kit, dissolved in 15 ml of 0.9% sterile saline solution and warmed to 37 °C. Following cannulation of the right jugular vein, hydrodynamic injection of the saline-DNA solution, or of saline alone, was performed over a period of 15 s. The *DDAH-1* expressing plasmid was pCMV-Sport6_HsDDAH-1 (Source Bioscience, UK). Three groups were studied: (a) sham + saline injection, (b) BDL + saline injection, (c) BDL + DDAH-1 expressing plasmid. Similarly, haemodynamic measurements were performed up to 5 days following plasmid injection, at 4 weeks post BDL surgery.

Haemodynamic measurements

Portal pressure and systemic haemodynamics were measured 4 weeks following surgery. Briefly, measurements were made under anaesthesia. Portal pressure (PP) was assessed by direct cannulation of the main portal vein, and mean arterial pressure (MAP) was assessed by cannulation of the right carotid artery. All measurements were transduced to a Powerlab (4SP) linked to a computer with Chart v5.0.1 software. The mean of three readings taken one minute apart was recorded.

Plasma biochemistry

Plasma samples were analysed for ALT, albumin and bilirubin (Cobas Integra 400, Roche-diagnostics, Burgess Hill, West Sussex, UK).

Rat liver cell isolation

Hepatocytes and non-parenchymal cells were isolated from Sprague Dawley rats by collagenase perfusion at the Yale Cell Isolation Core Facility (New Haven, Connecticut, USA.) as described [15]. These cells were lysed in a lysis buffer immediately after the isolation and stored until used for Western blot analysis.

Western blot

Proteins were isolated from snap frozen liver tissue using standard techniques. Equal amounts of protein extract were denatured and separated on 4–12% NuPAGE Bis-Tris Gels and transferred on to PVDF membranes (Lifetech, UK), which were then probed with anti-DDAH-1 (Abcam), anti-DDAH-2 (Abcam) and anti-tubulin (Millipore) monoclonal antibodies by standard techniques. The bands were visualized using an enhanced ECL detection kit (Amersham, UK) and quantified by densitometry.

Quantitative PCR

RNA was extracted from whole liver tissue following homogenization, using Trizol (Invitrogen), and was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative PCR (qPCR) was performed using TaqMan (Life Technologies) FAM labelled probes to *DDAH-1* (transcripts NM_012137.3 and NM_001134445.1) and to the housekeeping control gene peptidylprolyl isomerase A (*PPIA*) (transcript NM_017101.1). Quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Life Technologies) according to manufacturer's instructions. Samples were processed in triplicate, and analysed by the $2^{-[delta][delta]Ct}$ method.

Tissue NOS activity

A previously determined method of ^{14}C L-arginine conversion to ^{14}C L-citrulline was used to measure eNOS activity, with slight modification [16]. (See Supplementary Materials and methods).

Statistics

Data were analysed using GraphPad Prism v5.0a (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± SEM. Two-tailed unpaired *t* tests were used to define differences between means of normally distributed data of equal variance. For data that was not normally distributed, a Mann-Whitney test was used; $p < 0.05$ was considered statistically significant.

Results

DDAH-1 is expressed in hepatocytes and levels are decreased in cirrhosis

The precise cellular localisation of DDAH-1 has not been previously determined, in part due to insufficient specificity of commercially available antibodies for rodent DDAH-1. Therefore, to answer this question, we performed immunohistochemistry on