Research Article

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

The relationship between endotoxemia and hepatic endocannabinoids in cirrhotic rats with portal hypertension

Han-Chieh Lin[1,5,*,†], Ying-Ying Yang[2,3,5,†], Tung-Hu Tsai[4,5], Chih-Ming Huang[4,5], Yi-Tsau Huang[4,5], Fa-Yauh Lee[2,5], Tze-Tze Liu[6], Shou-Dong Lee[1,5]

[1]*Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, Taiwan;* [2]*Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, Taiwan;* [3]*Institute of Public Health, National Yang-Ming University School of Medicine, Taiwan;* [4]*Institute of Traditional Medicine, National Yang-Ming University School of Medicine, Taiwan;* [5]*Department of Medicine, National Yang-Ming University School of Medicine, Taiwan;* [6]*Genome Research Center, National Yang-Ming University School of Medicine, Taipei, Taiwan*

Background & Aims: Cirrhosis is characterized by endotoxemia and increased intrahepatic resistance, which is caused by hepatic fibrosis and endothelial dysfunction, as well as the activated endocannabinoids system, including cannabinoid (CB_1 and CB_2) receptors. Besides accelerating hepatic fibrogenesis, endotoxins induce the release of circulating endocannabinoids and portal hypertension in cirrhosis. This study examines how suppression of endotoxemia by antibiotics affects intrahepatic resistance and the hepatic endocannabinoid system in bile-duct-ligated (BDL) rats.

Methods: Measurements were performed that included: mean arterial pressure, cardiac index (CI), systemic vascular resistance, superior mesenteric arterial blood flow and resistance, PVP, plasma endotoxin and hepatic tumor necrosis factor-α (TNFα), anandamide and 2-arachidonylglycerol, hepatic expression of cannabinoid receptors, endothelial nitric oxide synthase (eNOS), phospho-eNOS, Akt, phospho-Akt and thromboxane synthase (TXS), matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), hepatic fibrosis, and leukocyte infiltration. Hepatic endothelial dysfunction was evaluated in BDL rats receiving vehicle (BDL-V) or 2-weeks of ciprofloxacin (BDL-cipro).

Results: Plasma endotoxin and hepatic TNFα, anandamide and 2-arachidonylglycerol, expression of TXS, MMP-2, TIMP-2, hepatic fibrosis and infiltration of hepatic leukocytes, CI, PVP and intrahepatic resistance were significantly lower in BDL-cipro than in BDL-V rats. Conversely, systemic vascular resistance, eNOS and Akt phosphorylation were significantly higher in BDL-cipro than in BDL-V rats. Improvement of hepatic endothelial dysfunction was associated with lower expression of hepatic CB_1 and a higher expression of hepatic CB_2 in BDL-cipro rats.

Conclusions: In cirrhotic rats, ciprofloxacin suppressed endotoxemia and the hepatic endocannabinoid system thus ameliorating hyperdynamic circulation and decreased intrahepatic resistance by preventing hepatic fibrogenesis and endothelial dysfunction.

Keywords: Antibiotics; Biliary cirrhosis; Portal hypertension.

Received 20 March 2010; received in revised form 26 August 2010; accepted 21 September 2010; available online 12 November 2010

* Corresponding author. Address: Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 11217, Taiwan. Fax: +886 2 28739318.

E-mail address: hclin@vghtpe.gov.tw (H.-C. Lin).

† These authors contributed equally to this paper.

Abbreviations: BDL, bile-duct-ligated; CB receptor, Cannabinoid receptor; CI, cardiac index; eons, endothelial nitric oxide synthase; TXS, thromboxane synthase; TIMP-2, tissue inhibitor of metalloproteinase-2; TNF-, tumor necrosis factor-; MMP-2, matrix metalloproteinase-2.

Introduction

In hepatic microcirculation, fibrosis, endothelial dysfunction, and increased local production of various vasoconstrictors such as thromboxanes (TX) increase intrahepatic resistance in cirrhosis [1–3].

Increased circulating bacterial endotoxins in patients with chronic liver disease may stimulate the release of tumor necrosis factor-α (TNFα) and accelerate liver fibrosis [4–6]. Additionally, endotoxins increase intrahepatic resistance and portal pressure in cirrhosis through the release of hepatic TXA_2 and endotoxemia is believed to trigger variceal bleeding [1,2,7,8]. Ciprofloxacin has the advantage over the majority of fluoroquinolones because it is well tolerated with low hepatotoxicity [9]. Ciprofloxacin had been used to prevent bacterial infection in patients with cirrhosis after upper gastrointestinal bleeding [9]. However, the effects of ciprofloxacin on hepatic microcirculation in cirrhosis are still uncertain.

Endocannabinoids are lipid mediators that are produced by Kupffer and endothelial cells in response to endotoxemia [10–12]. Endocannabinoids act as mediators in the brain (CB_1) and peripheral (CB_2) tissues through the stimulation of CB receptors [9]. In hepatic microcirculation, the activated endocannabinoids system released TXA_2, which then led to increased intrahepatic resistance of cirrhotic rats [1,2,13]. Additionally, both CB_1 receptor antagonism and CB_2 receptor agonism were shown to have anti-fibrotic effects in cirrhotic livers [11,14].

The interactions among endotoxemia, hepatic endothelial dysfunction, and an activated endocannabinoid system, and systemic, splanchnic, and hepatic circulation in cirrhosis are still unclear. This study aimed to examine the effects of ciprofloxacin-induced intestinal decontamination on the

interactions among the above factors in bile duct ligated-cirrhotic rats.

Materials and methods

Animals/protocols

Cirrhosis was induced in adult male Sprague–Dawley rats (250–350 g) by bile-duct-ligation (BDL) [2]. This study was approved by the Animal Experiment Committee of Yang-Ming University and conducted according to the "Guides for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, USA.

Two weeks of ciprofloxacin treatment (10, 20, 50 mg/kg) were given twice a day by oral gavages to BDL rats in a dose-finding study ($n = 5$ for each dose). Interestingly, the most potent portal hypotensive effect was presented at 20 mg/kg of ciprofloxacin. Therefore, BDL and Sham rats were divided into two groups ($n = 7$ in each group) to receive vehicle (BDL-V/Sham-V rats) or 20 mg/kg ciprofloxacin (BDL-cipro/Sham-cipro rats) starting 2-weeks after BDL to mimic the clinical situation.

Experiment 1: hemodynamics, plasma TNF-α and endotoxin levels

The first set of animals was used in this experiment. Mean arterial pressure (MAP), cardiac index (CI), systemic vascular resistance, superior mesenteric arterial blood flow and resistance, and portal venous pressure (PVP) were measured [15,16]. Plasma TNF-α and endotoxin levels were quantified by ELISA kits.

Experiment 2: histopathologic examination and fibrosis quantification

Liver sections from *Experiment 1* were used in this experiment. In addition to hematoxylin–eosin stains, paraffin-embedded livers were used to stain for leukocyte common antigen (LCA) expression, a specific marker for leukocytes. Then, an LCA stained area in 10-field of high power (20×) field was calculated for five slides for each animal. For analysis of the relative fibrotic area, a sirius red stained area in 10-field of high power (20×) field was calculated for five slides for each animal. Finally, the data from the LCA and sirius red stained areas were analyzed using the Image ProPlus analysis system (IPP, version 6.1, Media cybernetics, Silver spring, MD, USA).

Experiment 3: expression of various hepatic proteins and mRNAs

Liver samples from *Experiment 1* were used to measure protein expression of eNOS, Ser^{1177}-phospho-eNOS, Akt, Ser^{473}-phospho-Akt, thromboxanes synthase (TXS), cannabinoid-1 and cannabinoid-2 receptor and matrix metalloproteinases-2 (MMP-2), and tissue inhibitors of metalloproteinase-2 (TIMP-2) (markers of the hepatic deposition of extracellular matrix). GAPDH was used as an internal control for all protein expression. Additionally, *mRNA* levels of the CB_1 and CB_2 receptors were also measured with primers [**CB_1**: 5-GGAGAACATCCAGTGTGGGG-3 (sense) and 5-CATTGGGGCTGTCTTTACGG-3 (antisense); **CB_2**: 5-CCTGTTGAAGATCGGCAGCG-3 (sense) and 5-GGTAGGAGATCAACGCCGAG-3 (antisense)]. Finally, the β-actin gene [5-GTGGGGCGCCCCAGGCACCA-3 (sense) and 5-CTCCTTAATGTCACGCACGATT-3 (antisense)] was used as an internal control for *mRNA* expression.

Experiment 4: measurement of hepatic endocannabinoids

Liver samples from *Experiment 1* were used to measure hepatic endocannabinoid levels. Liver tissues were homogenized in 0.5 ml of an ice-cold solution of methanol/Tris buffer (50 mM, pH 8.0), 1:1, containing 7 ng of d4-anandamide, synthesized as previously described [17]. The amount of anandamide and 2-arachidonoylglycerol (fmol/mg) in the samples was determined by using inverse linear regression of standard curves.

Experiment 5.1: Hepatic endothelial dysfunction

The second set of animals was used in this experiment. Basal portal perfusion pressure (PPP), intrahepatic resistance, and NO_x and thromboxane B_2 (TXB_2) production were measured from the liver perfusion system as previously described [13]. Then, hepatic endothelial dysfunction was evaluated by ACh (acetylcholine, 10^{-7} to 10^{-5} M) after pre-constriction with methoxamine (MTX, 10^{-4} to 10^{-2} M) [18]. The ACh-response was calculated by the percent change in vasorelaxation from baseline. Additionally, MTX and ACh-related TXB_2/NO_x production was calculated according to the formula in Table 1.

Experiment 5.2: acute effects of lipopolysaccharide (LPS) pre-infusion in response to ACh (hepatic endothelial dysfunction) and hepatic endocannabinoid levels

Preliminary studies were performed to find the peak hepatic TNFα level and the worst hepatic endothelial dysfunction at 1–3 h after infusion of *Escherichia coli* LPS (serotype 0111: B4) in BDL-V and BDL-cipro rat livers [19]. We found that the peak hepatic TNFα levels associated with the worst hepatic endothelial dysfunction were observed at 3-h after LPS in BDL-cipro rat livers. After blood sampling for endotoxin and TNFα, at 3-h after LPS infusion in BDL-V, BDL-cipro, and sham-V rat livers receiving LPS [LPS(+)BDL-V, LPS(+)BDL-cipro and LPS(+)sham-V], perfused livers were included to assess the effect of ciprofloxacin-related suppression of pre-existing endotoxemia on acute endotoxemia-induced hepatic endothelial dysfunction, and MTX and ACh-related TXB_2/NO_x production (Fig. 1A, B–1 and C–1). Additionally, hepatic endocannabinoid (anandamide and 2-arachidonoylglycerol) levels were also measured at 3-h after acute pre-infusion of *Escherichia coli* LPS (serotype 0111: B4) to clarify the interaction between the endocannabinoids system and hepatic endothelial dysfunction.

Experiment 5.3: roles of CB_2 receptor agonist JWH133 in hepatic microcirculation

The fifth set of animals was used in this experiment. Concentration–response curves to JWH133 (10^{-8} to 10^{-4}M) were evaluated in MTX-preconstricted perfused livers. JWH133-related changes in PPP and TXB_2/NO_x productions were calculated as in Table 1.

Table 1. Formula to calculate thromboxane B_2 (TXB_2) and total nitric oxide (NO_x) production in liver perfusates.

	Concentration of TXB_2 /NOx			
Time points collected from liver perfusates	at baseline (before methoxamine, MTX)	after MTX incubation	after cumulative doses of Acetylcholine (ACh)	after cumulative doses of JWH133
Symbol	[A]	[B]	[C]	[D]
MTX+ACh-related TXB_2 and NOx production = [C] — [A]			MTX-related TXB_2 and NOx production = [B] — [A]	
ACh-related TXB_2 and NOx production = [C] — [B]			MTX+JWM133-related TXB_2 and NOx production = [D] — [A]	
JWH133-related TXB_2 and NOx production = [D] — [C]			Percentage of JWM133-related/baseline TXB_2 and NOx production = { [D] — [C]} / [A]	