Research Article

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Long-term cannabinoid type 2 receptor agonist therapy decreases bacterial translocation in rats with cirrhosis and ascites

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Background & Aims: Intestinal hyperpermeability, impaired peritoneal macrophages (PMs) phagocytosis, and bacterial translocation (BT), resulting in increased systemic and local infection/inflammation such as spontaneous bacterial peritonitis (SBP) together with increased tumor necrosis factor-α (TNFα) levels, are all implicated in the pathogenesis of cirrhosis-related complications. Manipulation of the cannabinoid receptors (CB_1R and CB_2R), which are expressed on the gut mucosa and PMs, has been reported to modulate intestinal inflammation and systemic inflammatory cytokine release. Our study aims to explore the effects of chronic CB_1R/CB_2R agonist/antagonist treatments on relevant abnormalities in cirrhotic ascitic rats.

Methods: Vehicle, archidonyl-2-chloroethylamide (ACEA, CB_1R agonist), JWH133 (CB_2R agonist), and AM630 (CB_2R antagonist) were given to thioacetamide (TAA) and common bile duct ligation (BDL) cirrhotic rats with ascites for two weeks and various measurement were performed.

Results: Compared to sham rats, CB_2Rs were downregulated in cirrhotic rat intestines and PMs. The two-week JWH133 treatment significantly decreased systemic/intestinal oxidative stress, TNFα and inflammatory mediators, infection, intestinal mucosal damage and hyperpermeability; the JWH133 treatment also decreased bacterial overgrowth/adhesion, BT and SBP, upregulated intestinal tight junctions and downregulated the PM TNFα receptor/NFκBp65 protein expression in cirrhotic rats. Acute and chronic JWH133 treatment corrected the TNFα-induced suppression of phagocytosis of cirrhotic rat PMs, which then could be reversed by concomitant AM630 treatment.

Conclusions: Our study suggests that CB_2R agonists have the potential to treat BT and various relevant abnormalities through inhibition of systemic/intestinal oxidative stress, inflammatory cytokines and TNFα release in cirrhosis. Overall, the chronic CB_2R agonist treatment affects multiple approach mechanisms, and its direct effect on the hyperdynamic circulation is only minor.

Introduction

In cirrhosis, intestinal hyperpermeability-related bacterial translocation (BT) may result in spontaneous bacterial peritonitis (SBP) if local bactericidal mechanisms are insufficient [1–4]. Endotoxemia, increased intestinal/systemic oxidative stress, an inflammatory state, and circulating tumor necrosis factor-alpha (TNFα), have been related to the impaired function of peritoneal macrophages (PMs), increased bacterial infection susceptibility, and hemodynamic disarrangements that carry high mortality in cirrhosis [2,4,5].

Selective decontamination using poorly absorbable antibiotics is highly effective in the prevention of SBP [6]. However, this antibiotic prophylaxis strategy is associated with the development of resistant strains of bacteria [6,7]. Alternative pathogenic

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Abbreviations: BT, bacterial translocation; SBP, spontaneous bacterial peritonitis; TNFα, tumor necrosis factor-α; PMs, peritoneal macrophages; EC, endocannabinoids; CBR, cannabinoid receptors.

manipulation strategies, including TNFα monoclonal antibodies, antioxidants, and pentoxifylline, have been developed for the prevention of SBP and BT [8–11]. However, agents that are able to simultaneously modulate oxidative stress- and endotoxemia-related intestinal hyperpermeability and various related disarrangements in cirrhotic ascitic rats are still limited.

The endocannabinoids (EC) and their CB_1 and CB_2 receptors are highly upregulated and are involved in the pathogenesis of various complications of cirrhosis [12]. CB_1R and CB_2R agonist have been reported to reduce the severity of experimental colitis [13]. Additionally, CB_2R agonists are able to inhibit TNFα-induced cytokine release by human colon epithelial cells [14]. Thus, it seems likely that CBR agonist/antagonists might be potential agents for the simultaneous treatment of intestinal hyperpermeability and the endotoxemia-associated abnormalities of cirrhosis. In this study, we explored the possible effects and mechanisms of chronic modulation of CBR agonists/antagonist on the above mentioned disarrangements in cirrhotic ascitic rats.

Materials and methods

Animals

The study approval was granted by the Institutional Ethics Review Committee of the University of Yang-Ming, Taiwan. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Yang-Ming, and the National Research Council's Guide for the humane care and use of laboratory animals. 16-weeks TAA- and 6-weeks BDL-cirrhotic rats with stable ascites were randomly assigned to receive oral gavage for 2-weeks of one of the following treatments: archidonyl-2-chloroethylamide (ACEA, CB_1R agonist, 3 mg/kg/d dissolved in normal saline [NS] with Tween 80), JWH133 (CB_2R agonist, 1 mg/kg/d dissolved in NS with Tween 80), or vehicle (V) (NS 1 ml/kg/d containing Tween 80). This created nine experimental groups of rats, namely sham-V, TAA-V, BDL-V (n = 9); sham-ACEA, sham-JWH133, TAA-ACEA, TAA-JWH133, BDL-ACEA, and BDL-JWH133 rats (n = 14). Additional experiments were carried out to explore intestinal permeability and phagocytosis of peritoneal monocyte-derived macrophages (PMs) collected from (1) AM630 (CB_2R antagonist, 3 mg/kg/d); (2) JWH133 plus AM630–treated TAA and BDL rats. Moreover, the effects of acute incubation of ACEA, JWH133, AM630 and JWH133 + AM630 on the phagocytic ability of PMs were evaluated in cirrhotic rats.

Hemodynamic measurements and detection of various systemic/intestinal biomarkers

The hemodynamic measurements were performed under ketamine anaesthesia (100 mg/kg, intramuscular). The femoral veins and arteries/portal veins were cannulated with PE-50 tubing to monitor the mean arterial pressure (MAP), heart rate (HR) and portal venous pressure (PVP) as well as to allow blood withdrawal. Cardiac output (CO) was measured using the transit-time ultrasound-dilution method. The cardiac index (CI) was then calculated using the following formula, CI = CO/body weight. Systemic vascular resistance (SVR) was calculated according to the following formula, SVR = MAP (mmHg) × 80/CI. Liver function (total bilirubin, AST and ALT) and plasma levels of TNFα, interleukine-1 (IL-1), IL-6, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 were measured.

The antioxidant glutathione (GSH), glutathione peroxidase (GPx), total superoxide dismutase (SOD) and MnSOD, oxidative stress markers thiobarbituric acid-reacting substances (TBARS), malondialdehyde (MDA), nitrotyrosine and protein carbonyls, as well as TNFα, cannabinoid receptor 1/2 (CB_1R/CB_2R), TNF receptor 1/2 (TNF-R1/2), NFκBp65, and tight junction markers occludin, claudin-1, and zonula occludens-1 (ZO-1)-protein/mRNA expressions were measured using commercially available ELISA kits and appropriate antibodies/primers (Supplementary Table 1).

Fibrosis quantification

The hepatic hydroxyproline content and Sirius red staining for hepatic collagen deposition were measured as described in the Supplementary materials and methods.

Histopathologic examination and immunohistochemical (ICH) quantification of duodenal ZO-1 expression

The degree of mucosal damage of the terminal ileum lumen was assessed using a semi-quantitative grading system [15]. Additionally, the IHC ZO-1 staining index of duodenal tissues was calculated as the product of the staining intensity score (0, 1, 2, and 3) and the proportion of positive cells (0, 1, 2, and 3).

Intestinal permeability and bacterial overgrowth/adhesion study

Intestinal permeability was measured by FITC-dextran (FD-4) and Evan Blue (EB)-based intestinal permeability methods as previously described [16,17].

Subsequently, cecum and cecal luminal contents were collected under sterile conditions for the evaluation of bacterial overgrowth. Additionally, *in vivo* assessment of bacterial adherence was carried out on a HepG2 cell monolayer by counting the number of *E. coli* harvested from rat intestine that adhere on 100 HepG2 cells [10].

Diagnosis of bacteraemia, spontaneous bacterial peritonitis (SBP), bacterial translocation (BT) and systemic infections

Using aerobic/anaerobic culture plates, sterile blood, liver, lung, spleen, and pleural fluid (if available) samples were collected for evaluation of bacteremia and systemic infections, which were defined as any positive culture from any above biological samples. BT was defined as a positive culture result for the sterile mesenteric lymph nodes (MLNs), drained from the terminal ileum, cecum and ascending colon. Additionally, TNFα levels were also measured in MLNs homogenate, whereas ascitic fluid samples were collected for the diagnosis of SBP.

Assessment of the phagocytic ability of rat peritoneal monocyte-derived macrophages after acute and chronic CB_1R/CB_2R agonist treatment

Briefly, rats were anesthetized and injected intraperitoneal with 50 ml (sham rats) or 25 ml (cirrhotic rats with ascites) of sterile Dulbecco's PBS for further isolation of primary peritoneal monocyte-derived macrophages (PMs) as previously described [4].

The phagocytosis of PMs, which were collected from rats receiving chronic CB_1R/CB_2R agonist treatment, were measured by three methods, namely the Vybrant phagocytosis assay, fluorescent confocal microscopy and flow cytometry dot plots [4]. All experiments were carried out in five replicates. Results were calculated by the following formula, where AFI stands for average fluorescence intensity: Phagocytic index = (AFI of experiment−AFI of background)/(AFI of positive control−AFI of background) × 100% or number of PMs containing engulfed cells (FITC-positive PMs)/total number of counted PMs × 100%.

Additionally, the acute effect of the CB_1R agonist ACEA, the CB_2R agonist JWH133, the CB_2R antagonist AM630, and the combination of JWH133 and AM630 together on the TNFα-modulated phagocytic ability of PMs for FITC-labeled *E. coli* from sham-V rats were evaluated separately. Finally, primary PMs collected from sham-V rats were lysed for various protein and mRNA (CB_1R/CB_2R, TNF-R1/2, NFkBp65) measurements.

Chemicals and reagents

JWH133, ACEA, AM630 were purchased from Tocris Cookson (Ellisville, MO, USA). TNFα, IL-1, IL-6, MCP-1, and MIP-2 ELISA kits were purchased from R&D Systems (Inc., Minneapolis, USA). Ficoll-Paque was purchased from Amersham Biosciences (Piscataway, NJ, USA). Antibodies for CB_1R/CB_2R, occludin, claudin-1, ZO-1were purchased from Abcam (Cambridge, MA, USA), antibodies for TNF-R1/2, nuclear NFκBp65 were purchased from Santa Cruz Biotechnology (CA, USA), and antibodies for nitrotyrosine, MnSOD were from Cayman Chemical (Ann Harbor, MI, USA), respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Detailed Materials and methods are provided as Supplementary data.

Results

Chronic CBR agonist treatments attenuate hyperdynamic circulation and systemic inflammatory cytokines release

Significant hepatic inflammation/fibrosis, hyperdynamic circulation (high CI and low SVR) and high portal venous pressure