Effect of monoacylglycerol lipase inhibition on intestinal permeability in chronic stress model

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Research article

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Abstract

Background: The endocannabinoid 2-arachidonoylglycerol (2-AG) is an anti-nociceptive lipid, which is inactivated through cellular uptake and subsequent catabolism by monoacylglycerol lipase (MAGL). The present study aimed to explore the effects of inhibition of MAGL on intestinal permeability.

Methods: We first tested it in differentiated CaCO2 cells after 21 days' culture. The rat model of water avoidance stress (WAS) was established, and rats were divided into four groups according to intervention. Rats received intraperitoneal injection (i.p.) of an MAGL inhibitor (JZL184) alone, JZL184 and the cannabinoid receptor 1 (CB1) antagonist (SR141716A), JZL184 and a cannabinoid receptor 2 (CB2) antagonist (AM630) or vehicle alone (control). We analyzed the fluorescein isothiocyanate-dextran (FD4) permeability and 2-AG level. Expression of MAGL and tight-junction-associated proteins were detected by western blot.

Results: Compared with the control group, MAGL expression was higher and 2-AG levels lower among WAS rats. Intestinal permeability was increased following administration of JZL184 which occurred due to up-regulation of tight-junction-associated proteins Claudin-1, Claudin-2, Claudin-5 and Occludin.

Conclusion: The effects of MAGL inhibition were mediated by CB1, indicating that MAGL may represent a novel target for the treatment of reduced intestinal permeability in the context of chronic stress.

Background

N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are two of the most important endocannabinoids in the human body. It is generally believed that AEA is mainly degraded by fatty acid amine hydrolase (FAAH), and 2-AG is mainly degraded by monoacylglycerol lipase (MAGL). The endocannabinoid system comprises the cannabinoid receptors 1 and 2 (CB1 and CB2, respectively); the receptors for AEA and 2-AG, respectively; and the enzymes involved in synthesis and degradation of endocannabinoids[1]. Studies have confirmed the endocannabinoid system to be closely related to the development of irritable bowel syndrome (IBS), nervous system diseases, cardiovascular disease, pain, inflammation and tumors[2–6]; thus, the components of the endocannabinoid system represent potential therapeutic targets to treat these diseases.

In healthy individuals, the cell junctions between intestinal epithelial cells form a highly selective mechanical barrier between the mucosa and submucosa, allowing small molecules to pass through and preventing large molecules from passing through the epithelial layer and induce an immune response. Tight junctions are multiprotein complexes made up of occludin, claudin-1–5, ZO-1, E-cadherin, etc., which play an important role in the formation and function of the mechanical barrier between epithelial cells. Tight junction proteins participate in the maintenance of the barrier function of the intestinal epithelium by regulating mucosal permeability[7]. It has been reported that the function of the intestinal

epithelial barrier is compromised, possibly increasing intestinal permeability, in a variety of gastrointestinal dysfunction diseases including IBS[8].

Water avoidance stress (WAS) is a simple animal model of psychological stress. Because accumulation of life stress can aggravate symptoms in the majority of patients with IBS-D, repeated WAS is a commonly used animal model of IBS-D[9].In the present study, we administered the MAGL inhibitor JZL184 to rat models of chronic stress to analyze the effects of increased 2-AG on intestinal permeability and elucidate the underlying mechanism.

Methods

Cell culture and treatment

Caco-2 cells(Shanghai Cell Bank, Shanghai, China) were seeded on 24-well 12-mm polyester Transwell filters (Corning, NY, USA) with 0.4μm pore size at a concentration of 2×105 cells per transwell. Cells were grown in DMEM supplemented with 10% FBS, and cultured for 21 days until they formed a differentiated monolayer. For treatment, JZL184 was added to the upper chamber of the transwell cultures.

In vitro measurement of FITC-Dextran permeability

To determine the effect of JZL184, the dextran permeability was measured after JZL184(1 umol/L) treatment for 24 hours. After washing the cells, DMEM was dispensed into each filter in the apical and basolateral chamber, fluorescein isothiocyanate (FITC)-dextran (4 kDa; 3mg/mL) was added to the upper chamber without medium change. Aliquots were withdrawn from the lower chambers after 4 hours, fluorescence values were compared with those of serial dilutions of known FD4 concentrations. Measurements were carried out in triplicate.

Animals

Male Wistar rats (200–230 g) were obtained from the Experimental Animal Center of Shandong University (Jinan, Shandong, China). Animals were housed in an animal facility that was maintained at 22°C with an automatic 12-hour light/dark cycle. All experiments were approved by the Shandong Provincial Hospital Committee on Use and Care of Animals. The researcher was blinded to details of animal treatments.

Establishment of rat model of water avoidance stress and experimental protocol

The WAS protocol is a well-established model of chronic stress considered to represent moderate psychological stress[10]. Chronic stress was induced as follows: Rats were placed on a plastic platform in the middle of a tank filled with water (25°C) to 1 cm below the height of the platform. The animals were maintained on the platform for 1 hour per day for 10 consecutive days. The selective MAGL inhibitor JZL184 (Cayman Europe, Talin, Estonia) was prepared in a mixture of saline/ethanol/Tween-80 and administered at a dosage of 10 mg/kg by intraperitoneal (i.p.) injection during chronic stress, then twice

daily until sacrifice. The vehicle alone was administered as a control. Antagonists of the CB1 and CB2 receptors; SR141716A and AM630, respectively (Tocris Bioscience, Bristol, UK); were administered once daily at 1 mg/kg in the same vehicle as JZL184. Rats were divided into four groups, with 4 rats in each group, according to the intervention method, denoted the control group, JZL184 group, JZL184 and SR141716A group, and JZL184 and AM630 group.

In vivo measurement of FITC-Dextran permeability

In vivo intestinal permeability was assessed on the first day after completion of the 10 days of WAS. Rats were administered 400 mg/kg of 4 kDa fluorescein isothiocyanate-dextran (FD4) in phosphate-buffered saline (PBS) pH 7.4 by gavage, blood was collected by heart puncture after 4 hours and transferred to ethylenediaminetetraacetic acid-coated tubes. Samples were centrifuged 1,000 *g* for 5 minutes, to separate the serum and stored at −80°C until analysis. To determine FD4 levels, we diluted 25 μL serum in 175 μL of PBS in a 96-well black-wall microplate and fluorescence values were compared with those of serial dilutions of known FD4 concentrations. Measurements were carried out in triplicate.

Tissue collecting

The rats were sacrificed with Carbon dioxide for further use. We dissected out colorectal tissues (approximately 10 cm from the anus) of rats. The dissected colorectal segments were reversed inside-out and washed with cold PBS. The mucosal layers were scraped off and collected in PBS, and the resulting mixture was centrifuged at 1,000 *g* for 5 minutes, then stored at −80°C refrigerator.

Western Blot

The prepared mucosal layers were extracted in radioimmunoprecipitation buffer containing 1 mmol/L phenylmethanesulfonylfluoride, 10 mg/mL leupeptin and 10 mg/mL aprotinin. The extract was centrifuged at 12,000 *g* for 20 minutes. Samples containing equal amounts of protein (20 mg) were separated by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Millipore, USA). Blots were probed with mouse antibodies against occludin (dilution 1:5000; Invitrogen, USA), rabbit antibodies against claudin-1, (dilution 1:1000; Invitrogen, USA), rabbit antibodies against claudin-2 (dilution 1:1000; Invitrogen), mouse antibodies against claudin-5 (dilution 1: 200; Invitrogen) and with rabbit anti-β-actin antibody (1: 5000, Cell Signaling, USA) or mouse anti-β-actin antibody (1:5000, Cell Signaling) as a loading control. Experiments were carried out in triplicate and β-actin protein levels were analyzed as a control for equal protein loading.

2-arachidonolyglycerol quantification

Tissues were homogenized in CHCl3 (5 mL), and deuterated standards (2-arachidonoylglycerol-d5, 200 pmol) were added. Then MeOH (5 mL) and H_2O (2.5 mL) were added and the lipids were extracted by vigorous mixing, and the organic layer was recovered and dried under nitrogen. The resulting lipid fraction

was pre-purified by solid-phase extraction over silica, and 2-AG was eluted using ethyl acetate-acetone (1:1, v/v). The resulting lipid fraction was analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific; Waltham, MA, USA) coupled to an Accela HPLC system (Thermo Fisher Scientific). Analytes were separated using a C-18 Supelguard precolumn and a Supelcosil LC-18 column. The mobile phases A and B were MeOH-H_2O-acetic acid (75:25:0.1, v/v/v) and MeOH-acetic acid (100:0.1, v/v). The gradient used was as follows: 100% A to 100% B in 15 minutes (at 0.5 mL/minute), followed by 10 minutes at 100% B and subsequent re-equilibration at 100% A. Mass spectrometry analysis in positive ion mode was performed with an atmospheric pressure chemical ionization source. Capillary and APCI vaporizer temperatures were 250 and 400°C, respectively. We quantified 2-AG by isotope dilution using the respective deuterated standards. Calibration curves were generated as described, and data were normalized to tissue sample weight.

Statistical analysis

We used SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) for all statistical analysis. Data are expressed as the mean ± standard deviation. The two-tailed Student's *t*-test and one-way analysis of variance were used to evaluate differences between experimental and control groups. Statistical significance was accepted at $P < 0.05$.

Results

1. **Inhibition of monoacylglycerol lipase increased expression of tight-junction-associated proteins in vitro**

To test whether tight junction could be disrupted in human cells, differentiated Caco-2 cells were treated with JZL184. We revealed that the expression of claudin-1, claudin-2, claudin-5 and occludin were significantly increased in JZL184 treated group compared with the control group (Figure 1).

2. **Inhibition of monoacylglycerol lipase improved cell permeability in vitro**

In order to show JZL184 treatment's influence on cell permeability, the concentration of FD4 was measured in differentiated Caco-2 cell monolayers. As shown in Figure 2, the concentration of FD4 was lower in the JZL184 treated group than the control group($P < 0.05$).

3. **Expression of monoacylglycerol lipase was increased and 2-arachidonolyglycerol decreased in rats under chronic stress**

Western blot revealed the expression of MAGL to be significantly higher in the WAS group than the control group (Figure 3A). Meanwhile, HPLC-MS analysis showed the level of 2-AG in the intestinal mucosa was

significantly lower in the WAS group compared with the control group (Figure 3B).

4. **Inhibition of monoacylglycerol lipase increased tight-junction-associated protein s and intestinal levels of 2-arachidonolyglycerol in vivo**

To test whether disruption of tight junctions observed in human cells could be reproduced in WAS rats, we treated WAS rats with JZL184. Western blot showed that the expression of claudin-1, claudin-2, claudin-5 and occludin were significantly increased in the intestinal mucosa of the JZL184 group compared with the control group (Figure 4A). Meanwhile, levels of 2-AG were significantly higher in the JZL184 group than the control group (Figure 4B).

5. **Inhibition of monoacylglycerol lipase improved intestinal permeability of WAS rats, which was reversed by CB1 receptor antagonist**

The concentration of FD4 was lower in the JZL184 group than the control group($P < 0.05$). Meanwhile, the concentration of serum FD4 was significantly lower in the JZL184 and SR141716A group than the control group, but there was no significant difference between the JZL184 and AM630 group and the control group ($P > 0.05$) (Figure 5).

6. **Administration of a CB1 antagonist inhibited JZL184-induced upregulation of tight-junction-associated proteins**

As shown in Figure 6, expression levels of claudin-1, claudin-2, claudin-5 and occludin were lower in the JZL184 and SR141716A group than the JZL184 group($P < 0.05$). there was no difference between the JZL184 and JZL184 and AM630 groups ($P > 0.05$).

Discussion

The etiology of IBS has not yet been established. However, recent reports suggest that the endocannabinoid system is involved in the pathophysiology of the condition. It has been reported that patients with IBS-D have lower levels of 2-AG than healthy controls[11]. The administration of MAGL was firstly described by Kupiecki et al in 1966[12]. Dinh et al[13] demonstrated that this enzyme degraded 2-AG, which is the most abundant endocannabinoid in the body. Strong upregulation of 2-AG has been observed in MAGL-knockout mice, implying that most of the endocannabinoid is degraded by hydrolysis[14]. Presently, MAGL is considered a promising therapeutic target for the treatment of a number of diseases including gastrointestinal disorders, cancer and neurodegenerative and inflammatory diseases[15]. The present study identified increased MAGL expression and decreased 2-AG levels in the mucosa of rats in the WAS group compared with the control group. This suggests that MAGL and 2-AG play important roles in the development of IBS-D and may represent therapeutic targets for IBS-D.

A number of MAGL inhibitors exist, including MJN110, KML29, URB602, SAR127303, OMDM169 and ABX1431[16]. In addition, JZL195, SA57, AM6701 and AM4302 are dual inhibitors of fatty acid amine

hydrolase (FAAH)/MAGL[17]. The development of JZL184, a piperidine carbamate which preferentially and irreversibly inhibits MAGL, provided the first pharmacological tool to acutely increase brain levels of 2-AG without altering brain levels of AEA. Thus, JZL184 represents the first selective MAGL inhibitor[18], and there is a growing body of research demonstrating that the inhibition of MAGL reduces nociceptive behavior in animal models of neuropathic pain[19,20]. Sakin et al[21] reported that selective FAAH inhibitors and dual FAAH/MAGL inhibitors are effective for both inflammatory and mechanically evoked visceral pain, while MAGL inhibitors have analgesic effects in inflammatory, but not in distension-induced visceral pain.

Changes in mucosal permeability may occur as a result of mucosal damage, and contribute to the development of inflammatory bowel disease or functional gastrointestinal disorders such as IBS, functional dyspepsia and gastro-esophageal reflux disease. Several studies have demonstrated increased intestinal permeability in patients with IBS[22–25]. The underlying mechanisms include alterations in the expression, localization or function of tight junction proteins, as well as changes in the microbiota, presence of active inflammation and/or presence of pro-inflammatory cytokines [26]. Studies involving animal models of IBS, such as the maternal separation[27] and chronic stress models[28], have revealed that both the paracellular and transcellular pathways are involved in barrier dysfunction. Nozu et al[29] reported that pioglitazone blocks visceral allodynia and increases colonic permeability in animal models of IBS. Zhou et al[30] conducted a randomized, double-blind, placebo-controlled, 8-week-long trial to assess the efficacy and safety of oral glutamine therapy for patients who developed IBS-D with increased intestinal permeability following enteric infection. They found that intestinal hyperpermeability was normalized in the glutamine but not the control group.

To date, there have been no studies on the effects of MAGL inhibitors on intestinal permeability. The present study focused on the effect of JZL184 on intestinal permeability, and found that JZL184 improves this parameter. Tight junction proteins are known to participate in the maintenance of intestinal epithelial barrier function by regulating intestine mucosal permeability in vitro and in vivo. The present study identified that JZL184 caused the levels of claudin-1, claudin-2, claudin-5 and occludin in the intestinal mucosa to increase significantly and the levels of 2-AG level in the intestinal mucosa of WAS rats to increase. This suggests that increased levels of 2-AG may directly affect intestinal permeability.

Among the endocannabinoid receptors, CB1 and CB2 are the most well-known. The CB1 receptor is mainly found in the central and peripheral nervous systems, as well as various peripheral tissues including the heart and blood vessels. In contrast, CB2 is mainly expressed in the immune system and hematopoietic cells, and in the central nervous system and the heart. N-arachidonoylethanolamine binds to CB1 receptors of the central nervous system and, to a lesser extent, to CB2 receptors in the peripheral nervous system, while 2-AG binds to CB1 and CB2 receptors with similar binding affinities[1,31]. Long et al[32] reported that MAGL-knockout mice have dramatically increased levels of 2-AG in brain tissues, and the effects of JZL184 were found to be dependent on CB1 receptor activation, being absent in CB1-receptor-deficient mice but not in CB2-receptor-deficient mice. Kerr et al[33] demonstrated that JZL184

attenuates lipopolysaccharide-induced increases in interleukin -1β, IL-6, tumor necrosis factor α and IL-10, but does not affect expression of the inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells in the rat frontal cortex. The CB1 antagonist AM251 was found to attenuate JZL184-induced decreases in IL-1β expression in the frontal cortical. Interestingly, administration of the highly selective MAGL inhibitor KML29 produces analgesia without cannabimimetic side effects. Chronic administration, however, leads to desensitization of CB1 receptors, which is also observed for other MGL inhibitors[34]. Furthermore, genetic mouse models of MAGL deficiency do not experience analgesia due to the constantly increased levels of 2-AG and subsequent desensitization of CB1 receptors[35]. Alhouayek et al[36] reported that MAGL inhibition increases 2-AG levels in a mouse model of colitis, leading to the reduction of macroscopic and histological colon alterations. Co-administration of JZL184 and selective CB1 or CB2 antagonists completely abolished the protective effect on colitis, demonstrating the involvement of both cannabinoid receptors in the effect.

We found the serum concentration of FD4 to reflect intestinal permeability. The concentrations of serum FD4 in the JZL184 and the JZL184 and SR141716A groups were lower than in the control group, but there was no significant difference between the JZL184 and JZL184 and AM630 groups. The expression of claudin-1, claudin-2, claudin-5 and occludin was higher among rats in the JZL184 group than the control group. Compared with JZL184 treatment, the expression of these four proteins was lower following JZL184 and SR141716A treatment, but similar to JZL184 and AM630 combined treatment. Our results of the administration of selective CB1 and CB2 antagonists clearly affect that the level of 2-AG in rat models of WAS is mediated by CB1. This is consistent with previous reports above.

In conclusion, the present study aimed to investigate the changes in intestinal permeability due to the effects of locally produced 2-AG by blocking degradation of this compound. Moreover, 2-AG acts on CB1, which has been shown to reduce intestinal permeability. Thus, administration of 2-AG represents a promising therapeutic approach for the treatment of IBS-D.

Abbreviations

Monoacylglycerol lipase (MAGL)

Water avoidance stress (WAS)

Intraperitoneal injection (i.p.)

Cannabinoid receptor 1 (CB1)

Cannabinoid receptor 2 (CB2)

Fluorescein isothiocyanate-dextran (FD)

N-arachidonoylethanolamine (AEA)

2-arachidonoylglycerol (2-AG)

Fatty acid amine hydrolase (FAAH)

Irritable bowel syndrome (IBS)

Phosphate-buffered saline (PBS)

High-performance liquid chromatography-mass spectrometry (HPLC-MS)

Declarations

Ethics approval and consent to participate: The study was approved by Shandong Provincial Hospital ethics committee.

Consent for publication: Written informed consent for publication was obtained.

Availability of data and material: We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes.

Competing interests: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Authors' contributions：**ZSL** proposed the study. **ZSL, WJ, ZXH, YCM** performed the research and wrote the first draft. ZSL and **WJ** collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. **ZSL** is the guarantor.

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Figures

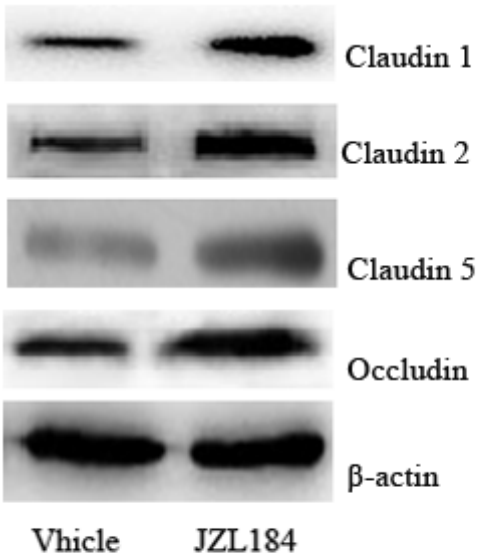

Figure 1

JZL184 intervention increased tight junction associated proteins expression in vitro

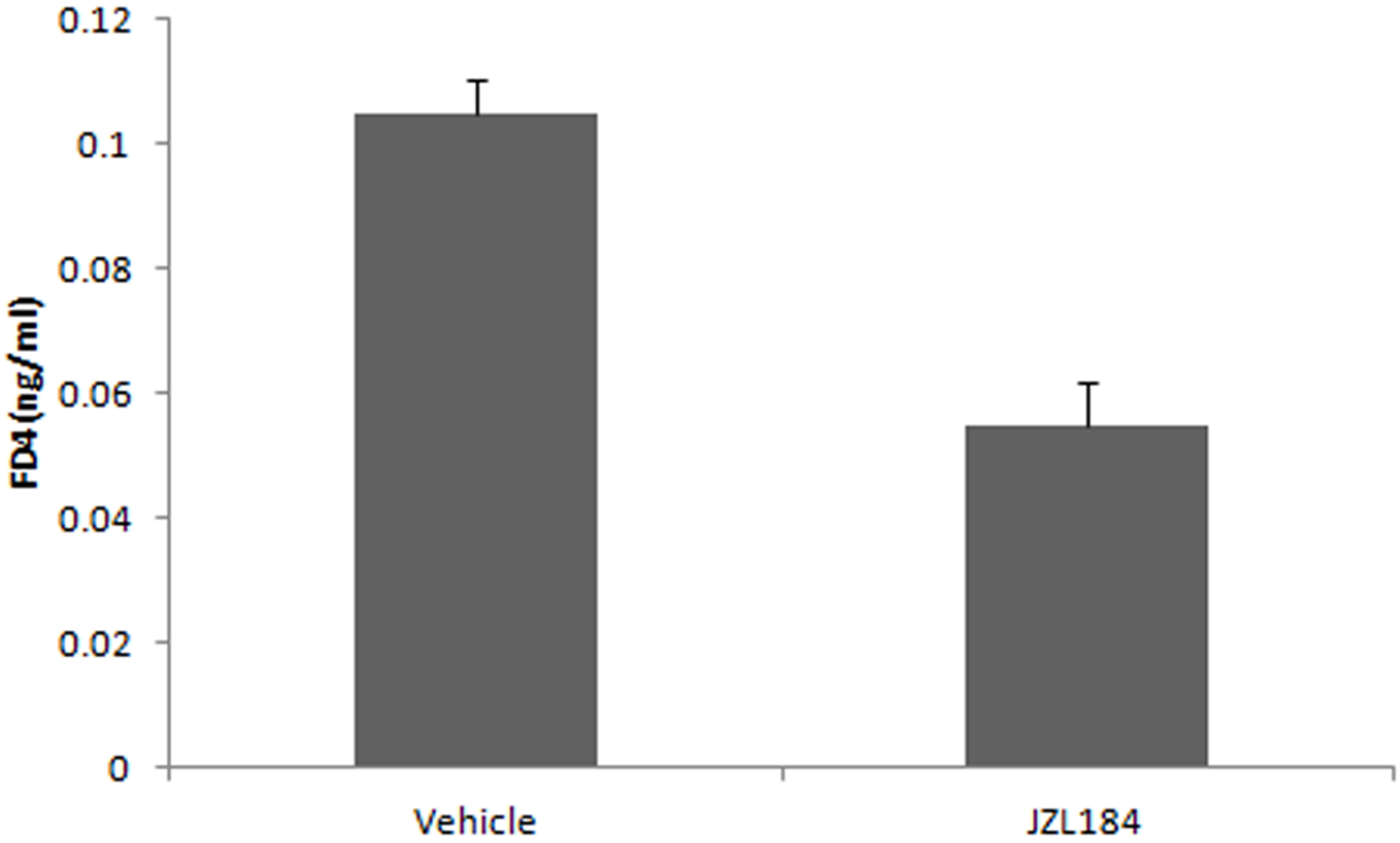

Figure 2

JZL184 treatment improved cell permeability in vitro

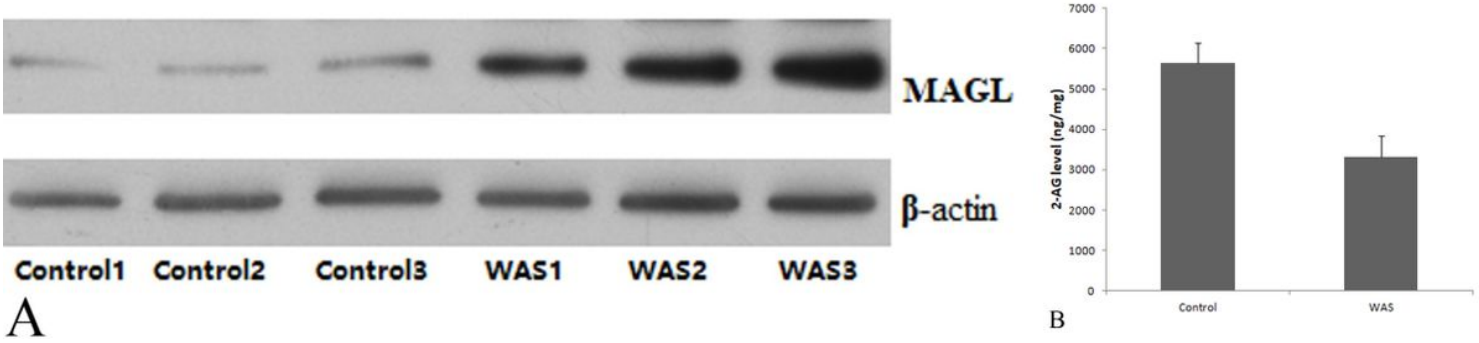

Figure 3

A. MAGL expression increased in chronic stress group, compared with control group. B. 2-AG level decreased in chronic stress group, compared with control group.

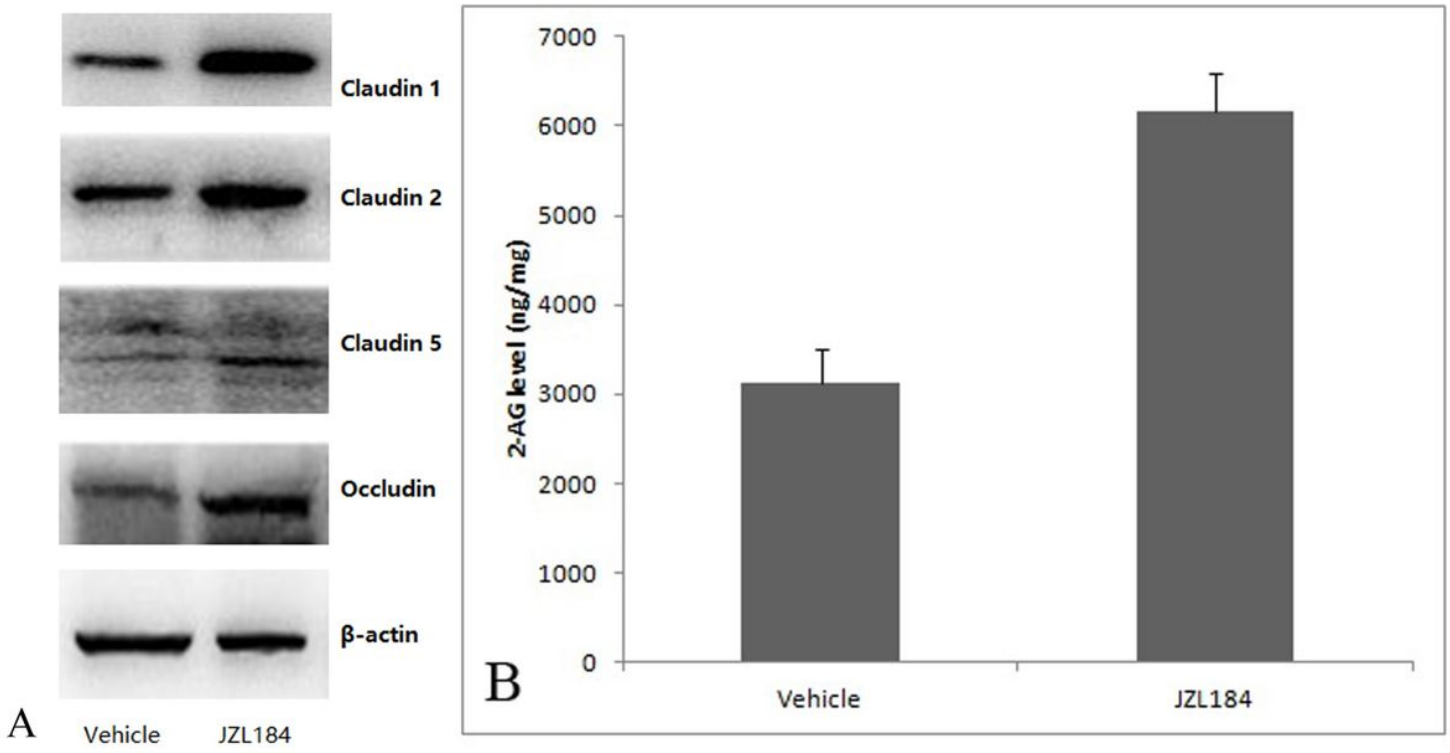

Figure 4

A. JZL184 intervention increased intestinal 2-AG level B. JZL184 intervention increased tight junction associated proteins expression in vivo

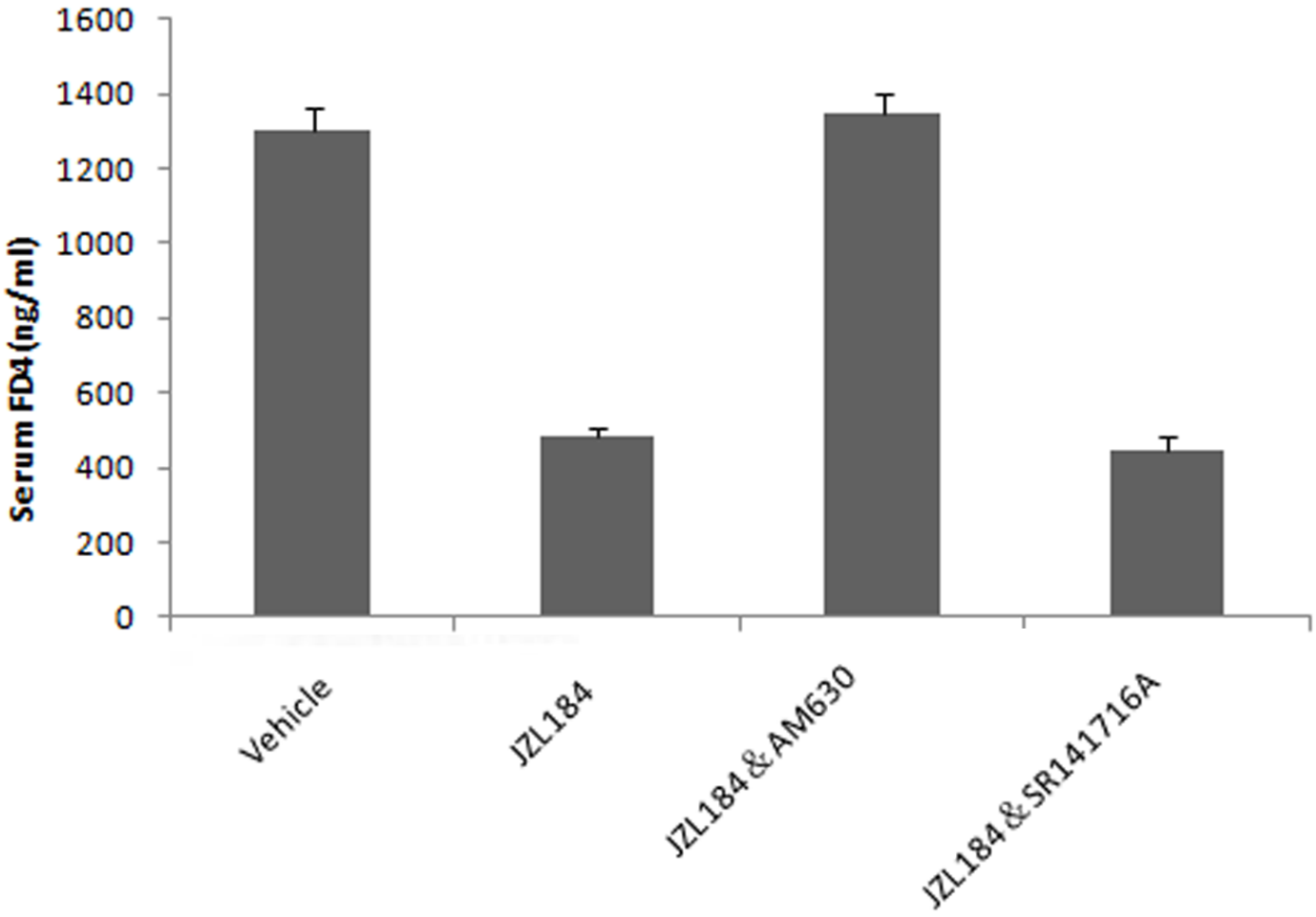

Figure 5

JZL184 improved intestinal permeability of WAS rats, which can be reversed by CB2 receptor antagonist

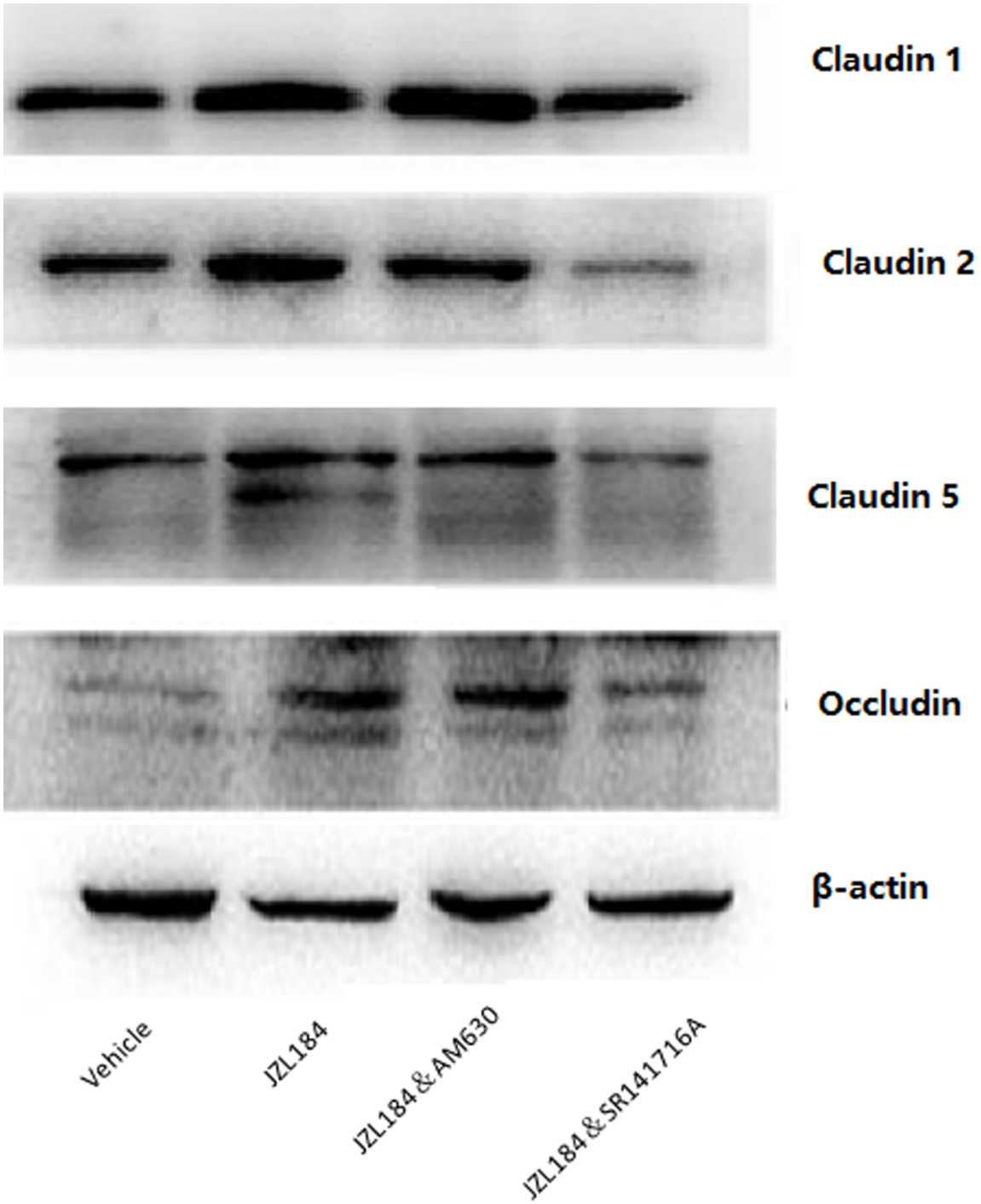

Figure 6

CB2 antagonist reversed JZL184 induced upregulation of tight junction associated proteins

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklist2014.docx