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Cannabinoid type 2 receptor stimulation attenuates brain edema by reducing cerebral leukocyte infiltration following subarachnoid hemorrhage in rats

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Abstract

Early brain injury (EBI), following subarachnoid hemorrhage (SAH), comprises blood-brain barrier (BBB) disruption and consequent edema formation. Peripheral leukocytes can infiltrate the injured brain, thereby aggravating BBB leakage and neuroinflammation. Thus, anti-inflammatory pharmacotherapies may ameliorate EBI and provide neuroprotection after SAH. Cannabinoid type 2 receptor (CB2R) agonism has been shown to reduce neuroinflammation; however, the precise protective mechanisms remain to be elucidated. This study aimed to evaluate whether the selective CB2R agonist, JWH133 can ameliorate EBI by reducing brain-infiltrated leukocytes after SAH. Adult male Sprague Dawley rats were randomly assigned to the following groups: sham-operated, SAH with vehicle, SAH with JWH133 (1.0mg/kg), or SAH with a co-administration of JWH133 and selective CB2R antagonist SR144528 (3.0 mg/kg). SAH was induced by endovascular perforation and all reagents were administered 1 hour after surgery. Neurological deficits, brain water content, Evans blue dye extravasation, and Western blot assays were evaluated at 24 hours after surgery. JWH133 improved neurological scores and reduced brain water content; however, SR144528 reversed these treatment effects. JWH133 reduced Evans blue dye extravasation after SAH. Furthermore, JWH133 treatment significantly increased TGF- β 1 expression and prevented an SAH-induced increase in E-selectin and myeloperoxidase. Lastly, SAH resulted in a decreased expression of the tight junction protein zonula occludens-1 (ZO-1); however, JWH133 treatment increased the ZO-1 expression. We suggest that CB2R stimulation attenuates neurological outcome and brain edema, by suppressing leukocyte infiltration into the brain through TGF- β 1 up-regulation and E-selectin reduction, resulting in protection of the BBB after SAH.

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Disclosures

None.

Keywords

Cannabinoid type 2 receptor; JWH133; Subarachnoid hemorrhage; Early brain injury; Brain edema

INTRODUCTION

Subarachnoid hemorrhage (SAH) accounts for only 5% of all cerebrovascular accidents but comprises a case fatality rate of approximately 50% [1]. Early brain injury (EBI) following SAH is thought to occur as a consequence of hemorrhage-induced elevation of intracranial pressure (ICP), reduced cerebral blood flow (CBF), global ischemia, oxidative stress, neuroinflammation, activation of inflammatory and cell death pathways, as well as formation of brain edema [2, 3]. The latter has been identified as an accurate predictor of poor outcomes in SAH patients [4]; thus, targeting BBB disruption and edema formation ought to be considered an innovative treatment strategy for this condition.

Recent studies have described the immunosuppressive and anti-inflammatory properties of marijuana-derived cannabinoids [5]. The cannabinoid type 1 (CB1R) and type 2 receptor (CB2R) are well characterized heterotrimeric Gi/o-protein-coupled receptors [6, 7]. While CB1Rs are highly expressed in the brain, causing the psychoactive effects of cannabinoids, CB2R stimulation has been shown to reduce neuroinflammation [8]. In fact, specific CB2R agonists attenuated brain edema in animal models of traumatic brain injury [9], as well as following lipopolysaccharide-induced encephalitis [10]. Furthermore, CB2R agonists impeded leukocyte/endothelial interactions and reduced adhesion molecule expression following ischemic stroke [11–13] and autoimmune uveoretinitis [14]. Although our previous work has provided some evidence for the effectiveness of CB2R agonism in reducing brain edema after experimental SAH [15], it remains unclear whether CB2R stimulation can confer neuroprotection by inhibiting cerebral leukocyte infiltration, thereby preventing BBB disruption and brain edema formation.

We therefore aimed to test the following experimental hypotheses: First, the selective CB2R agonist, JWH133 improves functional outcomes and reduces brain edema after experimental SAH in rats; and second, CB2R stimulation decreases leukocyte recruitment and cerebral infiltration, which is associated with preserved BBB integrity and reduced brain edema formation following SAH.

MATERIAL AND METHODS

Animals and Treatments

Our experiments were conducted in a controlled laboratory setting, and male Sprague Dawley rats (226 to 330 g; Harlan, Indianapolis, IN) were utilized. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University. SAH animals received the following agents as intraperitoneal (IP) injections 1 hour after surgery: vehicle (0.2 ml of ethanol with 1.8 ml of 0.9% saline), the selective CB2R agonist JWH133 (1.0 mg/kg, Tocris bioscience), or the selective CB2R antagonist SR144528 (3.0 mg/kg, Cayman chemical), which was injected 15

min before JWH133 administration. The dosages of JWH133 and SR144528 were selected based on previous publications [11, 16].

A total of 118 rats were operated; however, 15 animals died within 1 hour after SAH-induction. The surviving animals were assigned to one of the following groups: sham-operated (Sham group: n=20), SAH + vehicle (Vehicle group: n=31), SAH + JWH133 (JWH group: n=33), and SAH + SR144528 + JWH133 (SR+JWH group: n=19). All animals were sacrificed at 24 hours after surgery. The severity of SAH was evaluated in a blinded fashion, as previously described [17]. Briefly, after removing the brain from the skull, a photograph of the base of the brain was taken and divided into six parts. Each part was sub-scored (0 to 3) according to the presence of blood in the subarachnoid space; and a total score was calculated as the sum of all sub-scores. Operated animals received a total score ranging from 0 (no SAH) to 18 (most severe SAH), and SAH rats with a score of 7 or less were excluded from this study.

Surgery

The endovascular perforation model of SAH was performed as previously described [18–20]. Briefly, rats were anesthetized, intubated and kept on artificial ventilation during surgery with 3% isoflurane in 70%/30% medical-air/oxygen. Body temperature was monitored by a rectal probe and normothermia was maintained with a heating lamp. A sharpened 4-0 nylon suture was inserted into the left internal carotid artery and advanced until a resistance was felt (approximately 18 mm from the common carotid bifurcation). The suture was then further advanced to perforate the bifurcation of the anterior and middle cerebral arteries, and withdrawn immediately after artery perforation. During sham operations, the suture was inserted into the left carotid artery; however, no perforation was performed. After removal of the suture the skin incision was sutured, and rats were individually housed in heated cages until recovery. Buprenorphine (0.01 mg/kg) was given for pain management.

Neurological Score

At 24 hours after surgery, immediately before euthanasia, neurological scores were evaluated in a blinded fashion, using a modification of the Garcia scoring system as previously described [19, 21]. This composite sensorimotor assessment evaluates the rodent's spontaneous activity (0–3 points), its reaction to side stroking (1–3 points) and vibrissae touch (1–3 points), as well as limb symmetry (0–3 points), forelimb outstretching (0–3 points), as well as its climbing (0–3 points) and beam walking ability (0–4 points). The latter evaluated the walking distances on a wooden beam for 1 minute. The sum of all sub-tests was calculated to determine neurological function; best test performances were scored with 22, and worst performances were scored with 2 points.

Brain Water Content (Brain Edema)

Brains were collected at 24 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem as previously described [19, 20]. Each part was weighed immediately after removal (wet weight) and after drying in 100 °C for 72 hours

(dry weight). The percentage of brain water content was calculated as $[(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100\%$.

Blood-Brain Barrier Disruption

At 24 hours after surgery, Evans blue dye (2%; 5 ml/kg) was injected into the right femoral vein, over a period of 2 minutes, allowing the dye to circulate for a total of 60 minutes [20]. Under deep isoflurane anesthesia, rats were subjected to transcardial perfusion with phosphate-buffered saline (PBS), and brains were removed and divided into left hemisphere, right hemisphere, cerebellum, and brain stem. Brain specimens were weighed, homogenized in 1 ml of PBS, and centrifuged at 15,000 G for 30 min. Then, 0.6 ml of the resultant supernatant was added to an equal volume of trichloroacetic acid. After overnight incubation at 4°C and centrifugation at 15,000 g at 4°C for 30 min, the supernatant was used for spectrophotometric quantification of extravasated Evans blue dye at 615 nm.

Western Blot Analyses

At 24 hours after SAH, rats under deep isoflurane anesthesia were subjected to transcardial PBS perfusion. Next, brains were collected and large vessels on the surface of the brain were identified via a microscope and removed. The brains were then divided as mentioned above, and stored at -80 °C. Protein extraction of the left brain hemispheres was obtained by homogenizing the tissue in RIPA buffer (Santa Cruz Biotechnology) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich), followed by centrifugation at 14,000 g at 4 °C for 20 min. The supernatant was used as whole cell protein extract and stored at -80 °C until usage. Protein concentration was determined by detergent compatible assays (Bio-Rad, DC protein assay).

The supernatants were used for Western blot analyses as previously described [19, 20]. Briefly, equal amounts of protein (40 µg) were loaded onto a SDS-PAGE gel. After electrophoresis and transfer of the samples to a nitrocellulose membrane, the membrane was blocked and incubated with the primary antibody overnight at 4 °C with the following primary antibodies: anti-transforming growth factor (TGF)-β1 (1:200; Santa Cruz Biotechnology), anti-E-selectin (alias CD62E, 1:1000; Abcam), anti-myeloperoxidase (MPO) (1:200; Santa Cruz Biotechnology), and anti-ZO-1 (1:200; Santa Cruz Biotechnology). Following that, the membranes were incubated at room temperature for 1 hour with the appropriate secondary antibodies (1:2000, Santa Cruz Biotechnology). Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Bioscience). Blot bands were quantified by densitometry using Image J software (Image J 1.40g, NIH). β-Actin (1:2000, Santa Cruz Biotechnology) was used as loading control.

Statistics

SAH grades and neurological scores were expressed as median and 25th to 75th percentiles, analyzed by Mann-Whitney U test or Kruskal-Wallis one-way Analysis of Variance (ANOVA) on Ranks followed by Dunn's post hoc analysis. All other results were expressed as mean ± standard deviation and were analyzed by one-way ANOVA followed by Tukey

post hoc analysis. Mortality was analyzed by Fischer exact test. A P value of <0.05 was considered statistically significant.

RESULTS

We performed 118 surgeries, 19 rats were excluded because of low SAH grade, 15 SAH animals died within 1 hour after surgery, and 6 animals died after treatment or intervention (3 animals in the vehicle group, 1 in the JWH group, and 2 in the SR-JWH group). The mortality rates in each group were as follows: Sham group 0.0% (0 of 20 rats), Vehicle group 12.5% (3 of 24 rats), JWH group 4.2% (1 of 24 rats), and SR+JWH group 12.5% (2 of 16 rats). There was no statistical difference in mortality between the Vehicle, JWH and SR+JWH group. After mortality and exclusions, we enrolled 78 rats for outcome evaluations at the 24 hour time point.

Neurological score and brain water content at 24 hours after surgery

For the outcome study, we evaluated Sham, Vehicle, JWH, and SR+JWH animals. There was no significant difference in SAH grade between Vehicle (14.0 [14.0 to 17.0], $n=21$), JWH (16.0 [14.0 to 17.0], $n=23$), and SR+JWH (15.0 [12.0 to 16.0], $n=14$) ($P=NS$, Figure 1A). However, the neurological score was significantly lower in the Vehicle and the SR+JWH group when compared to Sham ($P<0.05$; Figure 1B); however, JWH133 treatment resulted in improved neurological scores ($P<0.05$ compared to Vehicle). This treatment effect was reversed by SR144528 ($P<0.05$, compared to JWH). Brain water content (brain edema) in the left brain hemisphere was significantly higher in the Vehicle group as compared to sham-operated and treated animals ($P<0.01$, Figure 1C). Vehicle animals demonstrated significantly more brain edema in the right hemisphere than rats in the Sham ($P<0.01$) and JWH group ($P<0.05$). The brain water content in the SR+JWH group was also significantly higher in both hemispheres compared to the Sham ($P<0.01$) and the JWH ($P<0.05$) group.

Evans blue assay

Evans blue dye extravasation assays were used for the evaluation of BBB leakage in Sham, Vehicle, and JWH animals (Figure 2). The amount of extravasated Evans blue dye in the left hemisphere was significantly higher in the Vehicle group as compared to the Sham ($P<0.01$) and the JWH ($P<0.01$) group. Furthermore, significantly more extravasated dye was found in the right hemisphere of Vehicle animals compared to Sham animals ($P<0.01$). Vehicle animals demonstrated significantly higher levels of extravasated dye in the cerebellum and the brain stem ($P<0.05$ compared to sham). JWH133 treatment resulted in significantly reduced dye extravasation into the left hemisphere ($P<0.01$) and the cerebellum ($P<0.05$) as compared to the Vehicle group.

Western blot analysis

Western Blot analyses were conducted for the quantification of TGF- β 1, E-selectin, MPO, and ZO-1 in left brain hemispheres of Sham, Vehicle, JWH, and SR+JWH animals at 24 hours after surgery. SAH-induction resulted in a lower TGF- β 1 expression ($P<0.01$ compared to Sham; Figure 3A); however, JWH133 treatment increased the TGF- β 1

expression as compared to Vehicle animals ($P<0.01$). The E-selectin expression was significantly higher in the Vehicle group as compared to Sham ($P<0.01$). JWH133 treatment reduced the level of E-selectin significantly ($P<0.05$, compared to Vehicle; Figure 3B). MPO expression was found significantly higher in the Vehicle group ($P<0.01$, compared to Sham; Figure 4A); however, JWH133 treatment significantly reduced MPO expression in the left brain hemisphere ($P<0.01$, compared to Vehicle). Combining the treatment with CB2R antagonist SR144528 (SR+JWH) tended to reverse this treatment effect ($P=NS$). The ZO-1 expression was found significantly lower in the Vehicle group as compared to sham-operated ($P<0.01$) and treated animals ($P<0.01$; Figure 4B). ZO-1 levels in the JWH group were significantly lower than in the Sham ($P<0.01$) and significantly higher than in the SR+JWH group ($P<0.05$). Lastly ZO-1 levels in the SR+JWH group were significantly lower than in the Sham group ($P<0.01$).

DISCUSSION

This current study showed that CB2R agonism via JWH133 ameliorated neurological deficits and brain edema after experimental SAH in rats. These treatment effects were associated with TGF- β 1 up-regulation and reduction of E-selectin, leading to reduced brain infiltration of leukocytes, specifically MPO expressing granulocytes, which ultimately preserved BBB integrity following SAH.

Mortality rates and neurological deficits are important clinical and experimental measurements used to evaluate the outcome following SAH. While mortality rates were similar in all SAH groups, JWH133 treatment improved neurological deficits at 24 hours after surgery. This treatment effect was reversed by combining JWH133 with the selective CB2R antagonist SR144528. Brain edema formation is an important element of EBI and has been described as an accurate predictor and independent risk factor for mortality and poor outcome in SAH patients [4]. Our results demonstrated that JWH133 treatment effectively reduced the water content of the ipsilateral and contralateral brain hemispheres at 24 hours after surgery; however, this treatment-induced reduction in brain edema was reversed by SR144528. It has been demonstrated that brain edema peaks at 24 hours after SAH induction in rats [22]; thus, we chose the same time point for these evaluations.

Evans blue dye binds to serum albumin [23] and since the latter cannot readily cross the BBB under normal physiologic conditions, spectrophotometric determination of extravasated Evans blue dye is a reliable way to estimate the extent of BBB permeability in animal models of central nervous disease. Our data showed that SAH induction resulted in increased parenchymal levels of Evans blue dye; however, JWH133 treatment restored BBB function in the left brain hemisphere as well as in the cerebellum.

Triggered inflammatory responses from immune-endothelial cell interactions can damage the BBB [24]; therefore, anti-inflammatory pharmacotherapies may maintain barrier function, thereby reducing EBI following SAH. Activated leukocytes, especially granulocytes, have been shown to aggravate ischemic brain damage [25].

TGF- β 1 signaling is an important regulator of endothelial cell differentiation, vascular network formation, and maintenance of vessel wall integrity [24]. Cortical and brain-stem TGF- β 1 expressions were increased in SAH rats that received high-dose simvastatin treatment, which was associated with improved neurological function compared to the control group [26]. Furthermore, neutralization of TGF- β 1 increased the number of leukocytes adhering to the endothelial cell surface [27]. Although TGF- β 1 demonstrated pro-inflammatory effects in some experimental settings, its anti-inflammatory effects have been established in previous literature. Indeed, Gamble *et al.* reported that TGF- β 1 suppresses neutrophil recruitment via decrease in the expression of endothelial E-selectin [28], and Melrose *et al.* reported that induction of E-selectin is inhibited by pretreatment of endothelial cells with TGF- β 1 [29].

Three major steps, rolling, adhesion, and trans-endothelial migration, are involved in leukocyte extravasation into the injured organs [30]. The interactions between leukocytes and endothelial cells, are mediated by several groups of cell adhesion molecules, including selectins, integrins, as well as the immunoglobulin superfamily [25]. E-selectin, expressed by endothelial cells, is responsible for the grouping of neutrophils from the axial blood stream to the vessel wall [31]. Accordingly, inhibition of E-selectin reduced the adhesion of MPO-expressing polymorphonuclear neutrophils to the endothelium [32]. Infiltrating neutrophils can damage brain tissue directly by generating reactive oxygen species and by secretion of proinflammatory mediators [33].

BBB dysfunction following SAH may initiate and/or contribute to a “vicious cycle” of the disease process by promoting the influx of blood-borne cells and substances into the brain parenchyma, thus amplifying inflammation, leading to further edema formation and neuronal damage [2, 34]. Endothelial cells are interconnected by tight junctions, mostly consisting of occludin, claudin, and zonula occludens (ZO) proteins [35]. ZO-1 anchors occludin, a transmembrane protein, to the actin cytoskeleton [36]. Recent studies demonstrated that CB2R activation plays an important role in preventing brain edema and neuroinflammation. Ramirez *et al.* showed that JWH133 attenuated BBB dysfunction by decreasing immune-endothelial cell interaction, thus preserving tight junction proteins in the rodent brain following induction of LPS-induced encephalitis [10]. Zhang *et al.* showed that CB2R activation was associated with a significant reduction of leukocyte adhering along cerebral endothelial cells, a reduction in infarct size, and better motor function following transient middle cerebral artery occlusion in mice [12]. Moreover, CB2R activation reduced the number of neutrophils in the ischemic brain, indicated by decreased MPO levels [11]. Furthermore, in a rodent model of autoimmune uveoretinitis, JWH133 treatment resulted in reduced leukocyte trafficking into the retina by reducing cellular adhesion molecules [14].

Hemorrhage-induced EBI and delayed cerebral vasospasm are believed to be responsible for the poor clinical outcome of SAH patients. Pathological contraction of vascular smooth muscle cells, resulting in cerebral vasospasm, occur around the third day after symptom onset and may last for several weeks after SAH [37]. This study focused on EBI rather than on delayed cerebral vasospasm following SAH. Therefore, we removed the large vessels from the brain before conducting Western blot analyses. We evaluated TGF- β 1, E-selectin, MPO, and ZO-1 expressions within the left (ipsilateral) brain hemisphere. JWH133

attenuated leukocyte migration into the brain, indicated by reduced MPO and increased ZO-1 expressions. This BBB-protective effect may have resulted from increased TGF- β 1 production, as a consequence of CB2R stimulation, thus reducing E-selectin expressions following SAH. The barrier-protective effect of JWH133 was reversed by SR144528, a selective CB2R antagonist, supporting the hypothesis that CB2R stimulation was responsible for the observed amelioration of BBB disruption and brain edema following experimental SAH.

This study has several limitations. First, we did not show which cell types were primarily stimulated by JWH133 to produce TGF- β 1. In the mammalian brain, CB2Rs are expressed on neurons, activated astrocytes, as well as in microglial and endothelial cells. Microglial cells may be one of the key players in the progression of neuroinflammation after SAH; and CB2R agonism has been shown to reduce microglial cell activation after experimental permanent middle cerebral artery occlusion as well as in an experimental model of traumatic brain injury [38, 39]. Second, CB2Rs have been shown to modulate acute, chronic, as well as post-surgical pain [6]. Although we used buprenorphine to reduce post-surgical pain in all animals, JWH133 injection might have further affected the perception of pain, interfering with activity levels and subsequent neurological performances of JWH133-treated SAH rats. Third, we did not evaluate whether multiple administrations and/or different dosages of JWH133 would have been more effective, since the terminal elimination half-life of this drug in rats is unknown. Further pharmacokinetic/-dynamic studies are needed to improve the translatability of this treatment. Fourth, our results indicate that CB2R agonism ameliorates SAH-induced BBB disruption and consequent edema formation; however, other pathophysiological mechanisms take place during EBI, and the effects of JWH133 on ICP, CBF, microcirculatory dysfunction, oxidative stress, and cell death pathways have yet to be established.

In conclusion, we suggest that CB2R activation by JWH133 may constitute a potential treatment to attenuate EBI following SAH. However, this theory necessitates further research, including *in vivo* magnetic resonance imaging to visualize BBB functions over a time course. Furthermore, the exact anti-inflammatory and BBB-protective mechanisms need to be elucidated.

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Highlights

This study targets antiinflammation strategy for early brain injury after SAH.

The mechanisms of cannabinoid type 2 receptor activation to reduce neuroinflammation studied.

Both selective agonist and antagonist of cannabinoid type 2 receptor are used.

Neurological deficits, brain edema, and blood-brain barrier evaluated.

Activation of cannabinoid receptor increased TGF-beta1 and reduced E-selectin, ZO-1.

Fig. 1.

(A) Grading score of subarachnoid hemorrhage (SAH), demonstrating equivalent SAH severity between Vehicle (n=21), JWH (n=23), and SR+JWH (n=14) groups. (B) Effect of JWH133 and SR144528 combined with JWH133 on neurological scores. Modified Garcia score was used as a neurological score in Sham (n=20), Vehicle (n=21), JWH (n=23), and SR+JWH (n=14). (C) Effect of JWH133, and SR144528 with JWH133 on brain water content at 24 hrs after surgery. Values are mean ± SD. * P < 0.05 compared to Sham group,

†P <0.05 compared to Vehicle group, ‡P <0.05 compared to JWH group, §P <0.01 compared to Sham group, # P <0.01 compared to Vehicle group.

Fig. 2. Evans blue extravasation after SAH. Evans blue detected in Vehicle group showed significantly higher brain-blood barrier permeability in the left (ipsilateral) hemisphere and cerebellum as compared with in the JWH group. Values are mean $\pm$ SD. * $P < 0.01$ compared to Sham group, † $P < 0.01$ compared to Vehicle group, ‡ $P < 0.05$ compared to Sham group, § $P < 0.05$ compared to Vehicle group.

Fig. 3.

Representative Western blots and quantitative analysis after SAH. TGF-β1 (A) and E-selectin (B) in the ipsilateral hemisphere were evaluated 24 hours after SAH. Expression levels of each protein are expressed as a ratio of the Actin levels. N=6 rats per in Sham, Vehicle, JWH, and SR+JWH group. Values are mean ± SD. * P < 0.01 compared to Sham group, †P < 0.01 compared to Vehicle group, ‡P < 0.05 compared to Vehicle group.

Fig. 4.

Representative Western blots and quantitative analysis of MPO (A) and ZO-1 (B) expressions following SAH. N=6 rats per each group. Values are mean $\pm$ SD. * $P < 0.01$ compared to Sham group, † $P < 0.01$ compared to Vehicle group, ‡ $P < 0.05$ compared to Vehicle group, § $P < 0.05$ compared to Sham group, # $P < 0.01$ compared to JWH group.