Cannabinoid receptor 2 agonist attenuates blood-brain barrier damage in a rat model of intracerebral hemorrhage by activating the Rac1 pathway

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Abstract. Blood-brain barrier (BBB) disruption and consequent edema formation are the most common brain injuries following intracerebral hemorrhage (ICH). Endocannabinoid receptors can alter the permeability of various epithelial barriers and have potential neuroprotective effects. The present study aimed to explore whether the selective cannabinoid receptor 2 (cNR2) agonist, JWH133, can ameliorate BBB integrity and behavioral outcome by activating Ras-related C3 botulinum toxin substrate 1 (Rac1) following ICH. Autologous arterial blood was injected into the basal ganglia of rats to induce ICH. Animals were randomly divided into the following groups: Sham-operated, ICH+vehicle, ICH+JWH133+vehicle, ICH+JWH133+AM630 (a selective cNR2 antagonist), ICH+AM630, ICH+JWH133 +NSc23766 (a Rac1 antagonist) and ICH+NSc23766. JWH133 and AM630 were independently intraperitoneally administrated at 1 h prior to ICH. NSc23766 was intracerebroventricularly (ICV) administered 30 min prior to ICH. A modified Garcia test, corner test, Evans blue extravasation and brain water content analysis were performed at 24 and 72 h following ICH. Western blotting and pull-down assays were performed at 24 h following ICH. The results demonstrated that JWH133 treatment improved neurofunctional deficits, reduced perihematomal brain edema and alleviated BBB damage at 24 and 72 h following ICH. In addition, JWH133 treatment increased the protein expression levels of guanosine-5'-triphosphate-Rac1 and of the adherens junction proteins occludin, zonula occludens-1 and claudin-5. However, these effects were reversed by AM630 and NSc23766 treatment. In conclusion, the present findings revealed that JWH133 treatment attenuated brain injury in a rat model of ICH via activation of the Rac1 signaling pathway, thus preserving BBB integrity.

Introduction

Intracerebral hemorrhage (ICH) is a fatal stroke subtype, associated with high mortality worldwide, and it accounts for 10-15% of all hospitalized stroke patients (1,2). Blood-brain barrier (BBB) damage and angioedema occur in the early stages of injury. BBB breakdown leads to brain injury progression and long-term neurological deficits (3). Destruction of the BBB directly aggravates vasogenic brain edema, and has been identified as an important contributor of secondary brain damage (4). Endothelial cells are interconnected by tight junctions (TJs) and mainly composed of zonula occludens-1 (ZO-1), occludin and claudin proteins (5). ZO-1 anchors the transmembrane protein occludin to the actin cytoskeleton, which confers the capacity of BBB to preclude permeation of blood substances (6). Changes in TJ components result in loss of BBB integrity and BBB decomposition (7). A variety of signal transduction pathways mediate the opening of tight junctions, including Ras-related c3 botulinum toxin substrate 1 (Rac1) (8). Therefore, regulating BBB permeability and reducing the degradation of TJ proteins may allow for a more effective ICH treatment strategy.

The endocannabinoid system has been studied as a potential therapeutic target for neuroprotection (9). There are two major subtypes of cannabinoid receptor, cannabinoid receptor 1 (CNR1) and cannabinoid receptor 2 (CNR2) (10,11). CNR2 is highly expressed in immune cells and mediates inflammatory responses (12). Previous studies have reported that cannabinoid receptors serve an important regulatory role in normal BBB physiology and protect BBB during ischemic stroke (13). Pharmacological data has suggested that CNR2 agonists increase the presence of tight junction proteins in membrane fractions in endothelial resistance and neuroinflammation (14). JWH133 (a specific CNR2 agonist), which exhibits a very high affinity for CNR2 (Ki=3.4 nmol/l) but low affinity for CNR1 (Ki=677 nmol/l), has been reported to attenuate brain edema in rat models of germinal matrix hemorrhage (15), and to improve functional outcomes and reduce brain edema following experimental subarachnoid hemorrhage in rats. In addition, JWH-133 treatment attenuates BBB damage in traumatic brain injury (16). These results...
indicate that CNR2 activation has potential as a practical and therapeutic intervention.

Rac1 is an important and well-studied member of a small-molecular-weight guanosine-5′-triphosphate (GTP) binding protein located inside the cell membrane. It serves an important role in cell proliferation and differentiation (17) and in regulation of cytoskeletal organization in many cell types. Rac1 maintains and stabilizes the barrier function of microvascular endothelial cells (18). Rac1 activation inhibits RhoA, which may preserve BBB integrity and, therefore, reduce the development of vasogenic brain edema after ICH (19). CNR2 concurrently or sequentially affects TJ protein stability via the Rho GTPase pathway (20). CNR2 agonists reduce integrin activation and lamellipodia formation in primary monocytes via inhibition of small GTPases (RhoA and Rac1) and affects cytoskeletal proteins (21). However, whether CNR2 activation can prevent BBB disruption and brain edema by inhibiting Rac1 activation in ICH, remains unknown.

Therefore, the present study investigated the effects of the CNR2 agonist, JWH133, on a rat model of ICH-induced BBB damage, and the role of Rac1 in the neuroprotective process. The present study demonstrated that JWH133, a selective CNR2 agonist, improved neurofunctional deficits, reduced brain edema and alleviated BBB damage following ICH, and these effects were reversed by AM630 (CNR2 antagonist) and NSC23766 (Rac1 antagonist) treatment.

Materials and methods

Chemicals. JWH133 (CNR2 agonist), and AM630 (CNR2 antagonist) were purchased from Toecris Bioscience (Bristol, UK). JWH133 and AM630 were dissolved in dimethyl sulfoxide (DMSO) and then diluted with sterile PBS. NSC23766 (Rac1 inhibitor) was purchased from Abcam (Cambridge, UK) and also dissolved in DMSO. Rac1 Pull-down Activation Assay kit was obtained from Cytoskeleton Inc. (Denver, CO, USA). Rat anti-Rac1 monoclonal antibody (cat. no. PAI-091; 1:1,000) was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-occludin (cat. no. sc-133256; 1:800), anti-ZO-1 (cat. no. sc-33725; 1:800) and anti-claudin-5 (cat. no. sc-374221; 1:800) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). An antibody targeting β-actin (cat. no. 3700; 1:1,000) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat anti-rabbit (cat. no. sc-2004; 1:2,000) and goat anti-mouse secondary antibodies (cat. no. sc-2005; 1:2,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Animals and treatments. A total of 180 male Sprague-Dawley rats (275-300 g) were purchased from the Laboratory Animal Center, Third Military Medical University (Chongqing, China). Rats were housed in a light and temperature controlled environment at 20-22°C. Food and water were available ad libitum. Experimental protocols were approved by the Medical Ethics Committee of the Third Military Medical University.

The experiments were divided into two parts. In experiment I, animals were randomly divided into the following groups (15 rats per group): Sham-operated (Sham group), ICH+vehicle (Vehicle group), ICH+JWH133 (JWH133 group), ICH+JWH133+vehicle (IWH133+vehicle group), ICH+JWH133+AM630 (IWH133+AM630 group) and ICH+AM630 (AM630 group). JWH133 (5.0 mg/kg) and AM630 (1.0 mg/kg) were intraperitoneally (i.p.) injected 1 h prior to ICH. In experiment II, animals were randomly divided into the following groups (15 rats per group): Sham-operated (Sham group), ICH+vehicle (Vehicle group), ICH+JWH133 (JWH133 group), ICH+JWH133+vehicle (JWH133+vehicle group), ICH+JWH133+NSC23766 (JWH133+NSC23766 group) and ICH+NSC23766 (NSC23766 group). JWH133 (5.0 mg/kg) was injected i.p. at 1 h prior to IHC, and NSC23766 (0.1 mg/kg) was administered intracerebroventricularly (ICV) at 30 min prior to ICH. Animals were sacrificed 24 and 72 h following surgery. The control rats received an equal volume of vehicle. The physiological parameters of rats were measured at 24 and 72 h following the ICH procedure. The experimental design is presented in Fig. 1.

Rat ICH model. The rat ICH model was established as previously described (22). Briefly, animals were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and placed on a stereotaxic apparatus. A 1-mm cranial burr hole was drilled, and a needle was inserted into the right basal ganglia under stereotactic guidance (coordinates: 0.2-mm anterior, 3.5-mm lateral to the midline and 5.5-mm ventral), and 100 µl of autologous arterial blood extracted from the femoral artery was slowly infused at a rate of 5 µl/min using a microinfusion pump. After the injection was complete, the needle was left in place for 20 min before withdrawal. Control animals were injected with 100 µl of normal saline. Bone wax was used to close the burr hole, and the skin incision was closed with sutures. In order to avoid infection, all procedures were performed under aseptic conditions.

Western blot analysis. Rats were sacrificed at 24 and 72 h post-ICH, and brain tissues surrounding the hemorrhagic region were isolated. Total protein was isolated from brain tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology, Beijing, China). The protein concentrations were determined by bicinchoninic acid (BcA) assay. Equal amounts of protein (40 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred into PVDF membranes (EMD Millipore, Billerica, MA, USA). The membrane was blocked in 5% non-fat milk at room temperature for 1 h and incubated overnight at 4°C with the following primary antibodies: Anti-occludin, anti-ZO-1, anti-claudin-5 and β-actin. Then the membranes were incubated with secondary antibody for 1 h at room temperature. (1:2,000; Santa Cruz Biotechnology, Inc.) and immunoblots were visualized using an ECL Plus chemiluminescence kit (Amersham; GE Healthcare, Chicago, IL, USA). Blot bands were semi-quantitatively analyzed using ImageJ (version 4.0; National Institutes of Health, Bethesda, MD, USA).

Functional behavioral test. The sensorimotor Garcia (23) and Corner test (24) were conducted in a blinded fashion, and used to assess neurofunctional deficits in rats at 24 and 72 h post-ICH. The Garcia Test was modified to examine spontaneous activity, axial sensation, vibrissae touch and limb symmetry, as well as the animal's ability to turn laterally, outstretch forelimbs and climb. The worst performance...
was awarded 0 points, and the best performance, 3 points, for each subtest. The sum point score was calculated to determine neurological function (maximum score, 21).

The corner test was conducted as described by Li et al. (24). The rat was placed between two boards, each with dimension of 30x20x1 cm³. The edges of the two boards were connected at an angle of 30°. A small opening along the joint between the two boards encouraged entry into the corner. The rat was placed between the two angled boards facing the corner and halfway to the corner. When the rat entered far into the corner, both sides of the vibrissae were stimulated together. Rearing forwards and upwards was observed, followed by turning to face the open end. The turns in each direction were recorded for each test. A total of 10 trials were performed for each rat, with a break of ≥30 sec between trials. The % of right turns was calculated. Only turns involving full rearing along either board were included.

Analysis of the brain water content. The brain water content was examined in rats at 24 and 72 h post-ICH, according to a previous study (25). Animals were anesthetized with pentobarbital (40 mg/kg; i.p.), sacrificed and the brains were surgically removed. The cerebrum was divided into 5 parts: ipsilateral cortex (Ipsi-cX) and contralateral cortex (Cont-cX), ipsilateral basal ganglia (Ipsi-BG) and contralateral basal ganglia (Cont-BS), and cerebellum (cerebel). Each part was immediately weighed using an analytical microbalance (APX-60; Denver Instrument, Bohemia, NY, USA) to measure the wet weight, and then they were dried at 100˚C for 24 h to measure the dry weight. The brain water content was calculated using the formula: Brain water content (%) = (wet weight–dry weight)/wet weight x 100.

Evans blue staining. Evans blue staining was performed at 24 and 72 h post-ICH to evaluate blood-brain barrier permeability, as previously described by Ma et al. (26). The rats were anesthetized and received femoral vein injection of 2% Evans blue solution (5 ml/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), allowing the dye to circulate for 3 h prior to transcranial perfusion with PBS pH 7.4. Following transcranial perfusion, the brains were surgically removed, divided into right and left brain hemispheres and stored at -80˚C until use. Brain specimens were homogenized in PBS, sonicated and centrifuged at 12,000 x g at 4˚C for 30 min. The supernatant was collected and an equal amount of trichloroacetic acid (50%) was added. The supernatant was incubated overnight at 4˚C and centrifuged at 15,000 x g at 4˚C. The absorbance of the sample was measured at 620 nm using a spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Inc.) and quantified according to a standard curve.

Rac1-GTPase pull-down assay. Rac1 activation assays were performed as described previously (27). In brief, brain tissues surrounding the hemorrhagic region underwent homogenization in RIPA buffer (Santa Cruz Biotechnology, Inc.) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; Merck KGaA) at 4˚C. The samples were incubated in a 4˚C water bath for 1.5 h. Samples were centrifuged for 10 min at 10,000 x g at 4˚C and the supernatants were collected. The protein concentration was determined using a BcA kit. A portion of each supernatant was added to Pak-GST-conjugated beads (10 µl) for detection of total Rac1 GTPases. The solution was continuously shaken for 2 h at 4˚C, and centrifuged for 1 min at 3,000 x g at 4˚C. The samples were washed three times with lysis buffer by centrifugation at 4,500 rpm at 4˚C for 5 min. Proteins bound to the beads were rinsed with distilled water and the Rac1-GTPase activity was determined by western blotting analysis.

Statistical analysis. The data was analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation. One-way analysis of variance was used to compare the differences among the multiple groups.
groups of data, followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**JWH133 treatment reduces neurofunctional deficit and brain edema at 24 and 72 h following ICH.** A modified method of autologous blood injection was used to establish the ICH model, as described by Yang et al (22). Neurofunctional deficits and brain water content were evaluated at 24 and 72 h following surgery. As illustrated in Fig. 2A and B, rats performed significantly lower scores during the Garcia and Corner tests at 24 and 72 h following surgery. In addition, the results demonstrated increased brain water content in the ipsilateral basal ganglia of the brain in the vehicle group, compared...
JWH133 protects against ICH-induced BBB destruction. Evans blue staining was used to detect BBB integrity at 24 and 72 h following ICH. The results demonstrated that Evans blue leakage was significantly increased at 24 and 72 h post-ICH in the vehicle group compared with the sham group (Fig. 3). JWH133 treatment significantly reduced Evans blue leakage at 24 and 72 h post-ICH compared with the vehicle group (Fig. 3). However, the effect of JWH133 on decreasing the Evans blue leakage was reversed by AM630 treatment (Fig. 3).

JWH133 administration upregulates GTP-Rac1 protein expression following ICH. To elucidate the potential mechanism of JWH133 on the CNR2/Rac1/TJ signaling pathway following ICH, a pull-down method was used to analyze the effect of JWH133 on the expression of GTP-Rac1 protein at 24 h post-ICH. As illustrated in Fig. 4, the GTP-Rac1 protein levels were decreased in the ipsilateral basal ganglia at 24 h post-ICH compared with the sham group. However, the GTP-Rac1 protein expression levels were higher in the JWH133-treated group and JWH133+vehicle group, compared with the vehicle group (Fig. 4). The combination treatment of JWH133 with AM630 reversed the GTP-Rac1 protein upregulation, compared with the JWH133 group (Fig. 4).

JWH133 administration prevents ICH-induced TJ-related protein attenuation. The expression levels of the TJ proteins, ZO-1, claudin-5 and occludin, were measured by western blotting at 24 h post-ICH. As illustrated in Fig. 5, the results demonstrated that the protein expression levels of ZO-1, claudin-5 and occludin in the ipsilateral basal ganglia of rats were significantly decreased at 24 h post-ICH compared with the sham group. JWH133 administration significantly upregulated the protein expression levels of ZO-1, claudin-5 and occludin (Fig. 5). The combination treatment of JWH133 with AM630 reversed ZO-1, claudin-5 and occludin protein levels compared with the JWH133 group (Fig. 5).

Rac1 inhibition reverses the neuroprotective effect of JWH133. In order to confirm that the neuroprotection effect of JWH133 was mediated by Rac1, the Rac1 inhibitor, NSc23766, was injected in the rats at 0.5 h prior to ICH. As illustrated in Fig. 6A, NSC23766 administration significantly decreased the JWH133-induced upregulation of GTP-Rac1 protein expression. Next, neurofunctional deficits, brain water content, and Evans Blue permeability were detected at 24 and 72 h post-ICH. NSC23766 administration reversed the protective effect of JWH133 treatment, as demonstrated by significantly lower scores in the Garcia and Corner test (Fig. 6B and C), as well as significantly increased brain water content in the ipsilateral basal ganglia of the brain (Fig. 6D and E). In addition, the combination treatment of JWH133 with NSC23766 increased Evans Blue leakage compared with the JWH133 group (Fig. 6F).

Rac1 inhibition reverses the effect of JWH133 on TJ-related protein expression post-ICH. The results demonstrated that JWH133 treatment increased ZO-1, claudin-5 and occludin protein expression levels at 24 h post-ICH, compared with the vehicle group (Fig. 7). However, NSC23766 administration, alone or in combination with JWH133, efficiently decreased...
ZO-1, claudin-5 and occludin protein expression compared with the JWH133 group (Fig. 7).

**Discussion**

In the present study, autologous blood was infused into the caudate nucleus of rats to establish a ICH model, similar to models that are widely used to study the mechanisms of brain injury. This rat model was then used to investigate the effects of CNR2 activation in ICH. The results demonstrated that JWH133 administration attenuated neurofunctional deficits and brain edema in rats at 24 and 72 h following experimental ICH. In addition, JWH133 treatment decreased BBB permeability at 24 and 72 h following experimental ICH. These results suggested that cannabinoid receptor agonists may serve a protective role following ICH. However, whether CNR2 activation can prevent BBB disruption and brain edema by inhibiting Rac1 activation in ICH has not been clarified.

In addition, in the present study, the expression levels of GTP-Rac1 and the tight junction proteins ZO-1, occludin and claudin-5 were detected at 24 h following treatment with JWH133 alone or in combination with AM630 or NSc23766. The results demonstrated that JWH133 treatment increased the expression levels of ZO-1, occludin and claudin proteins, and induced activation of Rac1 (GTP-Rac1) at 24 h following ICH. Combination treatment with the CNR2 antagonist, AM630, or the Rac1 inhibitor, NSc23766, reversed all the treatment effects of JWH133. These results suggest that the neuroprotection was
mediated by CNR2-induced activation of the Rac1 pathway. However, the expression of GTP-Rac1 and the tight junction proteins at 72 h post-treatment of JWH133 alone or combination of AM630 or NSc23766 require further study.

Increased BBB permeability and consequent vasogenic edema formation occurred following ICH. BBB disruption results from ICH by influx of blood-borne cells and substances into the brain parenchyma. This leads to early brain edema and sensorimotor dysfunction (28,29) and ultimately results in high morbidity and mortality rates (30). It has been previously reported that CNR2 activation has an important role in preventing brain edema and neuroinflammation. CNR2 agonists diminish inflammation-induced activation of brain endothelial cells and BBB disruption in conjunction with increased TJ protein expression (31). A previous study reported that JWH133 treatment increased ZO-1 expression and attenuated neurological outcome and brain edema, protecting the BBB following subarachnoid hemorrhage (14). In addition, JWH133 attenuated BBB dysfunction and preserved TJ protein expression in the rodent brain following LPS-induced encephalitis (32). The present findings are consistent with previous reports in which CNR2 activation by JWH133 significantly reduced BBB permeability and increased ZO-1, occludin and claudin expression.

Rac1 is a small GTPase that belongs to the Ras superfamily. Rac1 has been previously reported to maintain and stabilize barrier functions of microvascular endothelial cells (18). CNR2 agonists attenuate the activation of ras homolog family member A (RhoA) and Rac1 during migration (33). CNR2 agonists reduce integrin activation and lamellipodia formation in primary monocytes via inhibition of small GTPases (RhoA and Rac1) and effects on cytoskeletal proteins (34). JWH133 also activates the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways. A previous study has reported that JWH133 induced a greater and sustained activation of ERK1/2 kinase in lipopolysaccharide (LPS)/interferon (IFN)-γ-induced interleukin (IL)-12p40 release from murine macrophages (35). JWH-133 treatment activates ERK1/2 and signal transducer and activator of transcription (STAT)-3 in a mouse model of ischemia/reperfusion (36). JWH-133 induced PI3K/AKT activity in osteoclast precursors and osteoblasts of breast cancer-induced osteolysis (37). JWH133 increased oligodendrocyte progenitor cell proliferation by activating PI3K/AKT (38). In the present study, it was demonstrated that administration of JWH133 increased the GTP-Rac-1/total-Rac-1 protein expression ratio, and the barrier protective effect of JWH133 was reversed by NSc23766 (Rac1 inhibitor). These findings suggest that Rac1 participates in the improvement of BBB destruction and brain edema by JWH133 in experimental ICH. In the present study, only one selective CNR2 agonist, JWH133, was selected to investigate the effects on a rat model of ICH. In conclusion, the present results demonstrated that the CNR2 agonist JWH133 attenuated neurofunctional deficits and brain edema, and improved the BBB dysfunction induced by ICH, through the Rac1 pathway. Therefore, it is likely that targeting CNR2 may be a clinically feasible approach in the future to protect against ICH.

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Authors' contributions
ZW wrote the manuscript and interpreted the data. YL and SC analyzed the data and revised the manuscript. RL searched the literature and collected the data. GC designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Experimental protocols involving animals were approved by the Medical Ethical committee of the Third Military Medical University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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