Cannabinoid CB$_2$ receptor activation decreases cerebral infarction in a mouse focal ischemia/reperfusion model

Ming Zhang$^1$, Billy R Martin$^2$, Martin W Adler$^3$, Raj K Razdan$^4$, Jack I Jallo$^5$, and Ronald F Tuma$^{1,3}$

$^1$Department of Physiology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA
$^2$Department of Pharmacology and Toxicology, Virginia Commonwealth University School of Medicine, Richmond, Virginia, USA
$^3$Center for Substance Abuse Research, Temple University School of Medicine, Philadelphia, Pennsylvania, USA
$^4$Organix Inc., Woburn, Massachusetts, USA
$^5$Department of Neurosurgery, Temple University Hospital, Philadelphia, Pennsylvania, USA

Abstract

Cannabinoid CB$_2$ Receptor (CB$_2$) activation has been shown to have immunomodulatory properties without psychotropic effects. The hypothesis of this study is that selective CB$_2$ agonist treatment can attenuate cerebral ischemia/reperfusion injury. Selective CB$_2$ agonists (O-3853, O-1966) were administered intravenously 1 h before transient middle cerebral artery occlusion (MCAO) or 10 mins after reperfusion in male mice. Leukocyte/endothelial interactions were evaluated before MCAO, 1 h after MCAO, and 24 h after MCAO via a closed cranial window. Cerebral infarct volume and motor function were determined 24 h after MCAO. Administration of the selective CB$_2$ agonists significantly decreased cerebral infarction (30%) and improved motor function ($P < 0.05$) after 1 h MCAO followed by 23 h reperfusion in mice. Transient ischemia in untreated animals was associated with a significant increase in leukocyte rolling and adhesion on both venules and arterioles ($P < 0.05$), whereas the enhanced rolling and adhesion were attenuated by both selective CB$_2$ agonists administered either at 1 h before or after MCAO ($P < 0.05$). CB$_2$ activation is associated with a reduction in white blood cell rolling and adhesion along cerebral vascular endothelial cells, a reduction in infarct size, and improved motor function after transient focal ischemia.

Keywords
cannabinoid receptors; CB$_2$ agonists; stroke; leukocytes

Introduction

Cannabis, the natural marijuana plant, has been used for its psychotropic and possible medical properties for thousands of years. Cannabinoids, the synthetic analogs of cannabis, have been found recently to have different neuromodulatory properties in both in vivo and in vitro studies (Klein et al., 2001). So far, there are two cloned cannabinoid receptors, designated CB$_1$ and CB$_2$. The CB$_1$ receptor is primarily expressed in the central nervous system (CNS), exhibiting...
a presynaptic location and playing a prominent role in synaptic neurotransmission. Because of the involvement of the CB$_1$ receptor in modulating synaptic transmission, and therefore the potential to attenuate excitotoxic injury after ischemia, the CB$_1$ receptor has been a target for the modulation of injury after ischemia (Muthian et al., 2004; Nagayama et al., 1999; Parmentier-Batteur et al., 2002).

The CB$_2$ receptor is expressed predominantly by cells of the immune system, such as lymphocytes and neutrophils, but is also expressed on resident inflammatory cells within the CNS (Klein et al., 2001; Maresz et al., 2005; Pertwee, 1999). CB$_2$ stimulation has been shown to have immunomodulatory properties, such as decreasing antigen-presenting cell (APC) activity and down regulation of cytokine (interferon-$\gamma$ and tumor necrosis factor-$\alpha$) production during an inflammatory response. Recently, the CB$_2$ receptor has been identified on microglial and dendritic cells, implying a complicated role in CNS inflammatory response (Croxford, 2003; Pertwee, 1999). A number of investigations have shown that CB$_2$ receptor activation has anti-inflammatory therapeutic potential in CNS diseases, such as multiple sclerosis, traumatic brain injury, and Alzheimer’s disease (Grundy et al., 2001; Jackson et al., 2005; Ni et al., 2004). As inflammatory responses after ischemia have been shown to be important contributors to secondary injury, we hypothesized that selective activation of the CB$_2$ receptor might provide protection from reperfusion injury. Although cannabinoids have been tested in models of cerebral ischemia/reperfusion injury and reported to influence stroke outcomes by a variety of mechanisms, none of these studies has focused on the possible role of the CB$_2$ receptor in modulating the inflammatory response during cerebral ischemic and reperfusion injury. To the best of our knowledge we are the first to investigate the role of selective CB$_2$ receptor activation in attenuating ischemia/reperfusion injury.

**Materials and methods**

**Animals**

The cerebral ischemia/reperfusion studies were performed on 8-week-old male C57BL/6 mice (weighing 23 to 27 g; Taconic, NY, USA) and conducted in accordance with the guidelines approved by Institute for Animal Care and Use Committee at Temple University. The behavioral evaluation of the cannabinoid analogs was performed on 8-week-old ICR male mice weighing 23 to 27 g in accordance with the guidelines approved by the Institute for Animal Care and Use Committee at Virginia Commonwealth University.

**In Vitro and In Vivo Evaluation of CB$_2$ Receptor Selectivity**

Both analogs depicted below were assessed for cannabinoid properties by determining their affinities for CB$_1$ and CB$_2$ receptors, functional activity using $^{35}$S-GTPyS binding, and assessment in cannabinoid behavioral assays in mice using methodologies described in detail in recent publications (Martin et al., 2002; Wiley et al., 2002). CB$_1$ and CB$_2$ receptor affinities were determined using $^3$H-CP 55,940 binding to rat brain membranes and to Chinese hamster ovary cells stably expressing the human CB$_2$ receptor, respectively. *In vitro* functional activity was determined in these same preparations using $^{35}$S-GTPyS binding. For *in vivo* behavioral effects, mice were injected intravenously with the drugs prepared in ethanol:emulphor:saline (1:1:18). The mice were evaluated for locomotor activity, analgesia, body temperature, and catalepsy. These behaviors are collectively referred to as the tetrad test and are indicative of CB$_1$ receptor activity.

**Cranial Windows**

On the day of cranial window implantation, the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/mL)—xylazine (20 mg/kg) mixture (1:1) at a dose of 1 mL/kg. The head was shaved and positioned in a stereotactic head holder. A 1 cm
area of skin on the dorsal surface of the skull over the right cortical hemisphere was excised and the periosteum was removed. A 4 mm diameter circular craniotomy was performed using a high-speed drill (Champ-Air Dental Drill Benco Dental) over the right parietal cortex extending from attachment of the temporal muscle to midpoint of sagittal suture in the coronal direction and aligned to the middle of sagittal suture, so that the window contained some terminal branches of the middle cerebral artery. Normal saline was dripped over the cranium to avoid thermal injury of the cortex. The dura was removed and the exposed brain was kept moist with 37°C artificial cerebrospinal fluid solution. A 5 mm diameter coverglass was then placed over the exposed brain, and an airtight seal was produced using Nexaband Quick seal. The coverglass provided adequate mechanical protection from infection or contamination. As shown in Figure 1, a recovery period of 4 days was allowed between implantation of the cranial window and the induction of transient focal ischemia (Ni et al., 2004). A typical closed cranial window is presented in Figure 2.

**Middle Cerebral Artery Occlusion and Reperfusion**

The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/mL)—xylazine (20 mg/kg) mixture (1:1) at a dose of 1 mL/kg. Body temperature was maintained at 37°C±5°C by a heating lamp and heating pad. Middle cerebral artery occlusion (MCAO) was achieved by the intraluminal filament methods (Hata et al., 1998). Briefly, a midline neck incision was made under the operation microscope; the right common carotid artery, external carotid artery (ECA), and internal carotid artery were isolated. The ECA was ligated with 6 to 0 silk suture distal from the internal carotid artery—ECA branch and then cut distal from ligated point. Another 6 to 0 silk suture was tied loosely around ECA close to the origin at the common carotid artery. A blunted 5 to 0 monofilament nylon suture coated with poly-L-lysine (0.1% in deionized water, Sigma Inc., St Louis, MO, USA) (Belayev et al., 1999) was introduced into a small incision on ECA and then advanced into the circle of Willis, and finally to the origin of the middle cerebral artery. The silk suture around the ECA stump was tied tightly to prevent bleeding and secure the nylon suture. The nylon suture was removed after 60 mins occlusion and the ECA was permanently tied. Reperfusion was confirmed when pulsations were again observed in the internal carotid artery.

A laserPro Blood Perfusion Monitor (TSI Inc., Shore-view, MN, USA) was used to monitor regional cerebral blood flow (rCBF) before ischemia, during MCAO, and after reperfusion. A 1-mm-diameter microfiber laser—Doppler probe was attached to the skull 4 mm lateral and 2 mm posterior of bregma. The MCAO was considered adequate if rCBF showed a sharp decrease to 25% of baseline (preischemia) level; otherwise animals were excluded (Tsuchiya et al., 2003).

**Injection of CB₂ Agonists in Middle Cerebral Artery Occlusion and Reperfusion**

The CB₂ agonists (O-1966 and O-3853) were dissolved in a pure ethanol:emulphor:saline mixed solution at 1:1:18. The CB₂ agonists (1 mg/kg) or an equal volume of vehicle were administered as an intravenous injection into the jugular vein 1 h before MCAO or 10 mins after reperfusion. The investigator was masked with regard to whether the animals were members of vehicle or treatment groups during all experimental procedures and measurements.

**Intravital Microscopy**

The animals were anesthetized and immobilized on a plexiglas stage and secured on the microscopic stage. Intravital microscopy was performed with an epi-illuminescence microscope (BHI Water Immersion, Olympus, Japan). A × 20 water-immersion objective (WI 20, 0.4; Olympus, Tokyo, Japan), an image intensifier (Ceniisys Image Intensifier, Dage-MTI, Michigan City, IN, USA), and a monitor (12VM968; Audiotronics, North Hollywood, CA, USA) were used to gain a final total magnification of × 660. Leukocytes were stained *in vivo*.
by a bolus injection of 0.05 mL of a 0.01% solution of the fluorescent dye Rhodamine 6G (Sigma Inc.) into the jugular vein. The light leaving the lamp housing was filtered to allow light with a peak wavelength of 605 nm to be transmitted to tissue. Excitation of fluorescent dyes in the leukocytes caused a shift in the wavelength of the emitted light. Selective filtering allowed visualization of the fluorescent cells on a dark background. A nonintensified black-and-white charge-coupled device camera (CCD72, Dage-MTI) was used for visualization of the microscopic image. The image from the CCD72 camera was then displayed on the monitor, captured, and recorded by a computer-controlled real-time TV Tuner (ATI-TV WONDER, ATI Technologies Inc., Markham, Ontario, Canada) at a video frame rate of 36 frames/sec (Figures 2B—D). The interactions between leukocyte and endothelium were investigated offline. The investigator was masked to drug treatment of the experimental animals when making measurement of leukocyte/endothelial (L/E) interactions.

Measurement of Leukocyte/Endothelial Interactions

Leukocyte/endothelial interactions were evaluated before MCAO, 1 h after MCAO, and 24 h after MCAO. Each vessel was exposed to the light of the microscope for 30 secs at each viewing to minimize phototoxicity (Saetzler et al, 1997). Three venules (with diameter 30 to 50 μm) and three arterioles (with diameter 20 to 40 μm) in each animal were assessed. The number of rolling leukocytes was considered to be the total number of leukocytes moving along the endothelial cells at substantially slower velocity compared with the midstream blood cell velocity. They were counted when they passed an arbitrary line perpendicular to the longitudinal axis of the vessel for a period of 30 secs. Adhering leukocytes were defined as the total number of the leukocytes firmly attached to the micro-vascular endothelium that did not change their location during the entire 30 secs of the observation period. Adhering leukocytes were scored as the number of cells per mm² of the vascular surface area, calculated from the diameter and standardized length (100 μm) of the vessel segment under investigation.

Infarct Volume Assessment

Animals were euthanized with an overdose of pentobarbital (200 mg/kg intraperitoneal) 24 h after MCAO and then the brains were removed. The brains were chilled in ice for 10 mins to slightly harden the tissue. Five 2 mm coronal sections were cut using a mouse brain matrix (Zivic lab, Pittsburgh, PA, USA). The brain sections were placed in 2% triphenyltetrazolium chloride (Sigma Inc.) dissolved in saline and stained for 20 mins at 37°C in the dark. The brain sections were then fixed in 4% paraformaldehyde at 4°C for 24 h and the anterior and caudal face of each section was scanned by a flatbed color scanner (Microtek Inc., Carson, CA, USA). The resulting images were captured as JPEG files (Figure 2E) and analyzed with NIH image software. The infarct volumes were expressed as mm³ as well as the percentage of the ipsilateral hemisphere.

Neurological Evaluation

The severity of neurological deficits were evaluated 24 h after ischemic insult using a five-point deficit score (0 = normal motor function; 1 = flexion of torso and of contralateral forelimb on lifting of the animal by tail; 2 = circling to the contralateral side but normal posture at rest; 3 = leaning to contralateral side at rest; and 4 = no spontaneous motor activity) (Hata et al, 1998).

Statistical Analysis

Numbers of leukocyte rolling on and adhering to venules or arterioles were analyzed by one-way (times) analysis of variance in control group or two-way (treatments, times) analysis of variance with repeated measurements followed by Bonferroni’s test in treated groups. Bonferroni’s test after one-way analysis of variance was used for analyzing differences in the
average of rCBF, infarct volume, or neurological score. Data were presented as means±s.e.m. A statistically significant difference was assumed at $P < 0.05$.

Results

**In Vitro and In Vivo Characterization of CB$_2$ Cannabinoid Selectivity**

The affinity of O-1966 for CB$_1$ and CB$_2$ cannabinoid receptors was reported previously to be 5055±984 and 23±2.1 nmol/L, respectively (Wiley et al, 2002). We report herein that it stimulated $^{35}$S-GTP$_{\gamma}$S binding with an EC$_{50}$ of 70±14 nmol/L and an E$_{\text{max}}$ of 74±5 (percent of maximal stimulation produced by the full agonist CP 55,940). O-3853 binds to CB$_1$ and CB$_2$ receptors with affinities of 815±127 and 17.3±2.5 nmol/L, respectively. It was also effective in stimulating $^{35}$S-GTP$_{\gamma}$S binding with an EC$_{50}$ of 6.0±2.5 nmol/L and an E$_{\text{max}}$ of 87±5%, whereas its ability for stimulating CB$_1$ $^{35}$S-GTP$_{\gamma}$S was very low (EC$_{50}$ = 1509±148 and E$_{\text{max}}$ of 43%±3%). Intravenous administration of O-1966 to mice failed to produce effects in the tetrad test (the measurements for locomotor activity, analgesia, body temperature, and catalepsy) in doses up to 30 mg/kg, consistent with its very low CB$_1$ receptor affinity. Intravenous administration of O-3853 to mice resulted in weak activity in two of the tetrad measures. It depressed spontaneous activity and blocked tail-flick response with ED$_{50}$ values (confidence limits) of 10.3 (6.2 to 17.0) and 11.4 (8.2 to 16.0) mg/kg. It failed to either alter body temperature or produce catalepsy up to doses of 30 mg/kg. The ED$_{50}$ values of the CB$_1$/CB$_2$ cannabinoid receptor agonist Δ$^9$-tetrahydrocannabinol are approximately 1 to 2 mg/kg in these four measures.

**CB$_2$ Agonists did not Change the rCBF during Middle Cerebral Artery Occlusion**

During MCAO, rCBF decreased to approximately 25% of baseline value. Administration of the CB$_2$ agonists (O-3853 or O-1966) 1 h before occlusion at a dose of 1 mg/kg had no effect on rCBF during the 1 h occlusion period when compared with the vehicle-treated group (Figure 3).

**Effects of CB$_2$ Agonists on Cerebral Infarction**

Administration of CB$_2$ agonists (O-3853 or O-1966) at either 1 h before MCAO (preischemic treatment) or 10 mins after reperfusion (postreperfusion treatment) significantly reduced the cerebral infarction compared with the vehicle-treated group. Infarct volumes were similar in preischemic treated controls (99.2±6.9 mm$^3$, 34%±2.3%) and in postreperfusion treated controls (99.8±4.6 mm$^3$, 38%±1.6%). Administration of O-3853 before ischemia reduced infarct size to 68.2±5.0 mm$^3$ and 24%±2.4%. Furthermore, administration of O-3853 after reperfusion reduced infarct size to 71.9±6.1 mm$^3$ and 28%±1.3%. Likewise, O-1966 reduced infarct size to 65.6±4.0 mm$^3$ and 25%±2.5%, and 71.3±5.5 mm$^3$ and 27%±2% when administered either before ischemia or after reperfusion, respectively (Figure 4).

**Effects of CB$_2$ Agonists on Neurological Function**

Administration of the CB$_2$ agonists (O-3853 or O-1966) at either 1 h before MCAO (pretreatment) or during reperfusion significantly improved the motor function at 24 h after ischemia (Figure 5). Motor function score in animals receiving vehicle before MCAO was 3.25±0.20, which was reduced to 2.04±0.18 with O-3853 pretreatment and 1.69±0.30 with O-1966 pretreatment.

**MCAO Enhanced Leukocyte/Endothelial Interactions**

Leukocyte rolling and adhesion on both venules and arterioles were significantly enhanced during 1 h MCAO followed by 23 h reperfusion. Leukocyte rolling on venules increased from 3±0.2 (before MCAO) to 6.9±0.8 (1 h after MCAO) and 8.9±0.8 (24 h after MCAO); leukocyte...
adhesion on venules increased from 18±8.5 (before MCAO) to 134.7±30.9 (1 h after MCAO) and 163±25 (24 h after MCAO); leukocyte rolling on arterioles increased from 0.2±0.2 (before MCAO) to 1.7±0.6 (24 h after MCAO); leukocyte adhesion on arterioles increased from 0 (before MCAO) to 196±54.7 (1 h after MCAO) and 160±21.9 (24 h after MCAO) (Figure 6).

**Effects of CB$_2$ Agonists on Leukocyte/Endothelial Interactions during Cerebral Ischemia/Reperfusion Injury**

Treatment with either of the CB$_2$ agonists 1 h before MCAO attenuated leukocyte/endothelial interactions during cerebral ischemia/reperfusion injury. Both CB$_2$ agonists significantly decreased leukocyte rolling and adhesion on venules 1 h after ischemia and at 23 h of reperfusion. Leukocyte adhesion to arterioles was also attenuated by both agonists during both measurement periods after ischemia. Although both agents reduced leukocyte rolling along arterioles 24 h after MCAO, there was no difference in leukocyte rolling as a result of treatment 1 h after MCAO (Figure 7).

**Discussion**

The major finding of this study was that the selective CB$_2$ cannabinoid receptor agonists provide the significant protection for the brain from cerebral ischemia/reperfusion injury in a mouse MCAO/R model. Animals treated with the CB$_2$ agonists had smaller infarct volumes and improved motor function 24 h after the ischemic episode. These results confirm the hypothesis that activation of the CB$_2$ receptor is neuroprotective after ischemia/reperfusion injury. Both analogs have excellent affinity for CB$_2$ receptors and very low affinity for CB$_1$ receptors. They were also effective in activating the CB$_2$ receptor, but not CB$_1$ receptors, as evidenced by their effects on $^{35}$S-GTP$_7$S binding. Consistent with their binding profiles, O-1966 failed to produce CB$_1$ receptor-mediated behavioral effects in mice even up to very high doses and O-3853 produced some modest behavioral effects only at doses that far exceeded those used in the MCAO/R studies.

Previous studies have investigated the potential neuroprotective properties of cannabinoids after ischemia. However, these studies have focused on activation of the CB$_1$ receptor rather than activation of the CB$_2$ receptor. Both CB$_1$ and CB$_2$ receptors are found in the brain. CB$_1$ is predominately expressed in the CNS and peripheral neurons and CB$_1$ stimulation is important in neurotransmission and CNS homeostasis (Grundy, 2002; Pertwee, 1999; Rodriguez de Fonseca et al., 2005). The ability of the CB$_1$ receptor, which is thought to inhibit presynaptic transmission has been postulated to protect neurons from excitotoxic injury after ischemia. Consistent with this hypothesis, the CB$_1$/CB$_2$ agonist Δ$^9$-tetrahydrocannabinol was shown to reduce cerebral infarction in a mouse model of MCAO by CB$_1$ receptor induced hypothermia (Hayakawa et al., 2004). Cannabidiol, the nonpsychoactive constituent of cannabis, also reduced cerebral infarction but this is thought to be due to increasing rCBF during ischemia via the serotonergic 5-hydroxytryptamine$_{1A}$ receptor (Mishima et al., 2005). WIN55212-2, which stimulates both the CB$_1$ and CB$_2$ receptor, with greater affinity for the CB$_2$ receptors, has been shown to be neuroprotective in both global and focal models of ischemia. Based on the use of WIN55212-2 in combination with a CB$_1$ antagonist, these effects were interpreted to be the result of CB$_1$ receptor activation (Nagayama et al., 1999). Moreover, an investigation utilizing CB$_1$ knockout mice showed an increase in infarct size compared with wild-type animals (Parmentier-Batteur et al., 2002). However, in a separate investigation utilizing the CB$_1$ receptor antagonist SR141716, blockade of this receptor was found to reduce infarct volume in a mouse MCAO model (Muthian et al., 2004). Owing to the conflicting nature of these results, the potential of the CB$_1$ receptor as a therapeutic target for neuroprotection after ischemia/reperfusion injury remains an open question.
The hypothesis investigated in the current study was that activation of the CB₂ receptor might provide protection from cerebral ischemia/reperfusion injury through a completely separate mechanism. This hypothesis was based on a number of studies that have showed a modulation of inflammation by CB₂ agonists in models of multiple sclerosis, traumatic brain injury, and Alzheimer’s disease (Jackson et al., 2005; Ni et al., 2004; Schwenkreis and Tegenthoff, 2003; Walter and Stella, 2004). CB₂ is mainly located on immune cells and its stimulation has been shown to modulate immune cells activities and inflammatory responses. CB₂ is a G_i protein-coupled receptor and its activation triggers a series of signal transduction pathways, which eventually lead to either up- or down regulation of gene transcription. In most cases, the genes involved are coded for proinflammatory cytokines (Klein et al., 2001). Inhibition of cytokines such as tumor necrosis factor-α and interleukin-6 by CB₂ activation had been shown in both in vivo and in vitro studies. In addition, iNOS transcription and NO production in macrophage can be largely inhibited by CB₂ activation (Berdyshev, 2000). Both pro-inflammatory cytokines and NO are neurotoxic, leading to neuronal death during stroke. CB₂ stimulation is also able to inhibit antigen-presenting cell activity, decrease antibody production from B lymphocytes, and down regulate inflammatory cytokine production (Klein and Gabral, 2006). All these investigations indicate that activation of the CB₂ receptor may provide a potent mechanism for interference with inflammatory response.

Inflammation has been shown to be an important contributor to the damage to the brain after ischemia/reperfusion injury (Danton and Dietrich, 2003; Iadecola and Alexander, 2001). The contribution of white blood cell invasion from the circulating blood to this process has been shown in early studies that relied on depletion of white blood cells, and later studies that used adhesion molecule blockers (Connolly et al., 1996; Sughrue et al., 2004). Within minutes of ischemia, cerebral vascular endothelium is activated and leukocytes begin to roll on inflamed endothelial cells. Once activated, leukocytes begin to firmly adhere to endothelial cells. This process is mediated by series of interactions between cell adhesion molecules expressed on both leukocytes and endothelial cells. Cell adhesion molecules, such as intracellular adhesion molecule-1, can be highly induced during the early stage of ischemia by pro-inflammatory cytokines such as tumor necrosis factor-α (Kataoka et al., 2004; Kishimoto and Rothlein, 1994). After cerebral ischemia, neutrophils are the first leukocytes to infiltrate at the site of inflammation and monocytes are subsequently recruited. Leukocytes activation and migration have been implicated as primary contributors to ischemia/reperfusion injury. In addition to their role in physical obstruction of capillaries, they participate in inflammatory responses and cause brain tissue damage by various mechanisms. Proinflammatory cytokines (tumor necrosis factor-α and interleukin-1β), secreted by leukocytes, not only activate vascular endothelial cells and amplify inflammatory response but also directly induce neuronal injury (Wood, 2003). Matrix metalloproteases (MMPs), secreted by macrophages, can degrade the constituents of basal lamina and thus contribute to blood—brain barrier disruption and cerebral edema during stroke (Maier et al., 2004). All these studies highlight the involvement of white blood cells in exacerbating ischemic injury, and the protection offered by interfering with the ability of white blood cells to adhere to endothelial cells and undergo diapedesis into the brain (Connolly et al., 1996; Heinel et al., 1994; Kanemoto et al., 2002; Vasthare et al., 1990; Weaver et al., 2002; White et al., 2000).

In the current investigation, the closed cranial window technique was used to evaluate the effect of selective CB₂ agonists on endothelial/leukocyte interactions. Before ischemia, baseline leukocyte rolling and adhesion on venules was very low and there were almost no leukocyte/endothelial interactions on arterioles because of high shear stress. One hour after ischemia, there was a significant increase in leukocytes rolling on venules. The lack of increase in leukocyte rolling in arterioles during the first hour of reperfusion was probably the result of an increase in shear rate resulting from the ischemia-induced reactive hyperemia. The increased leukocyte/endothelial interactions that resulted from ischemia/reperfusion injury were
dramatically diminished by both CB\textsubscript{2} agonists. As leukocyte rolling and adhesion on endothelial cells are critical steps for their full activation and extravasation into brain tissue to participate in the inflammatory response (Heiné et al., 1994), it is possible that CB\textsubscript{2} activation exerts at least part of its neuroprotective effects via modulation of white cell contributions to inflammatory reactions during ischemia/reperfusion injury. In a previous study using selective CB\textsubscript{1} and CB\textsubscript{2} antagonists, we found that WIN55212-2 exerted its neuroprotective effects in a mouse EAE model via CB\textsubscript{2} not CB\textsubscript{1} activation, and that this neuroprotective effect was also associated with an attenuation of leukocyte/endothelial cell interactions (Ni et al., 2004). Although it is likely that the attenuation of leukocyte rolling and adhesion after stroke is directly caused by CB\textsubscript{2} receptor activation on these cells, it is also possible that the attenuation of rolling and adhesion is not a direct contributory mechanism but rather a reflection of a decrease in damage due to CB\textsubscript{2} receptor activation in other cells such as microglia. In addition to modulating inflammatory responses through inhibition of leukocyte/endothelial adhesion, a number of laboratories have also reported that the CB\textsubscript{2} receptors also exist on microglial cells and that CB\textsubscript{2} receptors were highly upregulated by inflammatory stimulation in microglial cells (Maresz et al., 2005; Nunez et al., 2004). Activated microglia play an active role in cerebral ischemia/reperfusion injury, through phagocytic activity, inflammatory cytokine production, and the release of destructive proteolytic enzymes as well as neurotoxin secretion (Mabuchi et al., 2000; Schilling et al., 2005). It is therefore possible that the beneficial effects of CB\textsubscript{2} agonist treatment in stroke may result in part from inhibition of microglial activation.

Another possible mechanism through which CB\textsubscript{2} receptor agonists could exert a protective effect is by modulating cerebral blood flow through alterations in cerebral vascular resistance. However, rCBF during ischemia was not changed by the agonists in the model used in this investigation. Therefore, it seems unlikely that CB\textsubscript{2} activation exerts its protective effects by influencing cerebral vascular resistance during ischemia. As transient MCAO should result in maximal vasodilation early in the reperfusion period, it is also unlikely that vasodilation caused by the CB\textsubscript{2} agonists during this time period is a contributing factor.

**Acknowledgements**

This project is funded, in part, by a grant from the Pennsylvania Department of Health, a contract from BTG (London), and grants DA P30 13429, DA 03672, and DA 05488 from the National Institute on Drug Abuse.

**References**


Muthian S, Rademacher DJ, Roelke CT, Gross GJ, Hillard CJ. Anandamide content is increased and CB1 cannabinoid receptor blockade is protective during transient, focal cerebral ischemia. Neuroscience 2004;129:743–50. [PubMed: 15541895]


Figure 1.
Experimental flow charts for preischemic treatment test (A) and postreperfusion treatment test (B).
Figure 2.
Typical closed cranial window in a mouse. Scale bar = 500 μm (A). Representative video images of capturing leukocytes labeled with Rhodamine 6G in the ischemic area. Video images were taken before MCAO (B), one hour after MCAO (C), and 24 h after MCAO (D) in the same venule. Arrows indicate Rhodamine 6G-labeled leukocytes flowing through venules. Scale bar = 40 μm. Representative brain slices after 1 h MCAO followed by 23 h reperfusion. Slices were stained with 2% triphenyltetrazolium chloride. The unstained areas (white) represent the infarct lesion corresponding to the middle cerebral artery distribution territory (E).
Figure 3.
Pretreatment with either CB₂ agonist (O-3853 or O-1966) had no effect on rCBF in mice subjected to MCAO compared with vehicle-treated control group. rCBF decreased to 25% of baseline level within the first 1 min and was maintained below 25% of baseline value throughout MCAO. Values represent percentage of baseline value at each time spots. (Data were expressed as mean±s.e.m., n = 8–13 in each group.)
Figure 4. Effects of CB₂ agonists on cerebral infarction. Administration of either CB₂ agonist (O-3853 or O-1966) at either 1 h before MCAO or 10 mins after reperfusion significantly reduced the cerebral infarction compared with the vehicle-treated group. There was no difference between the untreated group and the vehicle-treated group. (A) Infarct volume (mm³); (B) percentage of infarction to ipsilateral hemisphere. (Data were expressed as mean±s.e.m., n = 8 to 13 in each group, *P < 0.05 versus vehicle-treated group.)
Figure 5.
Effects of CB\textsubscript{2} agonists on neurological function in mice subjected to 1 h MCAO and 23 h reperfusion. Administration of either CB\textsubscript{2} agonist (O-3853 or O-1966) at either 1 h before MCAO or 10 mins after reperfusion significantly improved the motor function (0 represents normal motor function, 4 represents no spontaneous motor function) compared with the vehicle-treated control group. No difference was found between untreated and vehicle-treated groups. (Data were represented as mean±s.e.m., \(n\) = 8 to 13 in each group, *\(P < 0.05\) versus vehicle-treated group.)
Figure 6.
Middle cerebral artery occlusion enhanced leukocyte rolling and adhesion after reperfusion. Rolling and adhesion were significantly enhanced ($P < 0.05$) on both venules and arterioles, with the exception of rolling on arterioles after 1 h MCAO. (Data were represented as mean ±s.e.m., $n = 6$ in each group, *$P < 0.05$ versus before MCAO.)
Administration of either CB₂ agonist (O-3853 or O-1966) 1 h before MCAO attenuated leukocyte/endothelial interactions. (A) The CB₂ agonists decreased the number of leukocytes rolling on venules at 1 and 24 h after MCAO. (B) The CB₂ agonists decreased the number of leukocyte adhering to venules 1 and 24 h after MCAO. (C) The CB₂ agonists had no effect on leukocyte rolling on arterioles 1 h after MCAO compared with the vehicle-treated group. Both agonists decreased the number of leukocytes rolling on arterioles at 24 h after MCAO. (D) The CB₂ agonist (O-1966) had no effect on leukocyte adhesion to arterioles before MCAO and 1 h after MCAO compared with the vehicle-treated group, whereas O-3853 decreased the number of leukocyte adhesion on arterioles at 1 h after MCAO. Both agonists decreased leukocyte adhesion to arterioles at 24 h after MCAO. There was no difference of baseline of leukocyte rolling and adhesion on venules and arterioles among the groups. (Data were represented as mean±s.e.m., n = 6 in each group, *P < 0.05 versus vehicle.)