Cannabinoid CB_2 Receptor Activation Attenuates Motor and Autonomic Function Deficits in a Mouse Model of Spinal Cord Injury

Darric E. Baty, M.D., Ming Zhang, M.D., Hongbo Li, M.D., Carolyn J. Erb, B.S., Martin W. Adler, Ph.D., Doina Ganea, Ph.D., Christopher M. Loftus, M.D., Jack I. Jallo, M.D., Ph.D., and Ronald F. Tuma, Ph.D.

Traumatic injury to the spinal cord can have devastating consequences. Two major problems for patients with spinal cord injury (SCI) are persistent paralysis and bladder dysfunction.[1] Knowledge of the mechanisms contributing to the pathophysiology of SCI has increased dramatically over the last 2 decades. There is general agreement that inflammatory cell invasion and activation after the initial injury exacerbate damage.[11,33] Neutrophils, macrophages, microglia, and lymphocytes are thought to be involved in the generation of secondary damage and later in the cleaning and preparation of the injury site for wound repair.[7,10] Pro-inflammatory cytokines play a crucial role in the signaling required for recruitment and activation of these cells.[14] Modulation of inflammatory responses after SCI may provide therapeutic opportunities for recovery of function.[15] Although the exploration of neuroprotective strategies to minimize secondary injury has been a primary goal of many investigators, little progress has been made along this line in the clinic. We are hopeful that continued research by many laboratories throughout the world will eventually change this disheartening fact.

The hypothesis investigated in this study is that the administration of a selective cannabinoid agonist offers an effective therapy for treating injury after trauma to the spinal cord, thus improving neurological function. Cannabis, the natural marijuana plant, has been used for its psychotropic and medicinal properties for thousands of years. Cannabinoids, including synthetic analogs of cannabis, have been recently found to have different neuromodulatory properties both *in vivo* and *in vitro*.[8,16,22] 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. 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.[30] CB_2 stimulation has been shown to have immunomodulatory properties such as decreasing antigen presenting cell function and downregulating the production of cytokines like interferon-γ and tumor necrosis factor-α (TNF-α) during an inflammatory response.[21,25] CB_2 receptor activation has also been shown to have immunomodulatory properties without causing psychoactive effects.[24,28] The therapeutic potential of CB_2 receptor activation has been demonstrated in animal models of CNS disorders such as multiple sclerosis, traumatic brain injury, stroke, and Alzheimer's disease.[26,27,34,42] However, to date, there are very few reports regarding the effects of cannabinoids on posttraumatic recovery in SCI.

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MATERIALS AND METHODS

Animals

The SCI studies were carried out using 6- to 8-week-old, female C57BL/6 mice (Taconic, NY) in accordance with the guidelines approved by the Institution for Animal Care and Use Committee at Temple University.

Spinal Cord Injury Model

The animals were anesthetized with an intraperitoneal injection of a ketamine (100 mg/mL)–xylazine (20 mg/kg) mixture (1:1) at a dose of 1 mL/kg. Body temperature was maintained at 37 $\pm$ 0.5°C by a heating lamp and pad. The surgical site was shaved and prepared using a povidone–iodine solution. A dorsal thoracic incision was made followed by laminectomies at T8–T10. The animal was transferred gently to the modified, Adson's forceps holder provided with the Infinite Horizons Impactor device (IH Impactor; PSI Inc., Lexington, KY); the forceps were fitted snugly on the lateral spinal column cranial and caudal to the laminectomy site.[18,20] An impact force of 60 kDynes was used to provide a moderate to severe contusion injury. After impact, the back

muscles were sutured together across the midline, and the skin was closed with surgical clips.

CB_2 Agonist Properties and Injection

The affinity of O-1966 for CB_1 and CB_2 cannabinoid receptors was reported previously to be 5055 ± 984 and 23 ± 2.1 nM, respectively. This was determined using ^{3}H-CP 55,940 binding to rat brain membranes and to Chinese Hamster Ovary cells stably expressing the human CB_1 and CB_2 receptor, respectively.[40] The CB_2 agonist stimulated ^{35}S-GTPγS binding with an EC_{50} of 70 ± 14 nM and an E_{max} of 74 ± 5 (percent of maximal stimulation produced by the full agonist CP 55940). 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.[42]

The CB_2 agonist (O-1966) was dissolved in a pure ethanol:emulphor:saline mixed solution at 1:1:18. The CB_2 agonist (1 mg/kg) or an equal volume of vehicle was administered as an intraperitoneal injection 1 hour before injury and 24 and 48 hours after injury. The investigators were blinded with regard to whether the animal was a member of the vehicle or treatment group during all experimental procedures and measurements.

Locomotive Function Evaluation

Preoperatively, and on postoperative Days 1, 7, and 14, the animals were assessed using the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB) as modified for mice[3,12,19] and the newly developed Basso Mouse Scale for Locomotion (BMS).[4] In these scales, the following are assessed: limb movement (ankle, knee, and hip), paw placement (plantar with support versus plantar without support), stepping (dorsal versus plantar), coordination, paw orientation (parallel versus internally rotated versus externally rotated), trunk stability, and tail position. A score of 17 represents normal function on the BBB and a score of 9 is completely normal on the BMS.

Autonomic Function Evaluation

The animals were tested daily for recovery of bladder function. Urine was manually expressed from the bladder using the Credé maneuver twice each day and then massed. Bladder function recovery was regarded as complete when less than 500 mg of urine was collected during 3 consecutive days.

Real-time Reverse Transcriptase–Polymerase Chain Reaction

CB_2 receptor and TNF-α expression were detected using the SYBR green-based real-time reverse transcriptase–polymerase chain reaction (RT-PCR) technique. Total RNA was isolated from thoracic spinal cord by use of the Ultraspec reagent (Biotecx Laboratories, Houston, TX). cDNA was prepared by reverse transcription. The 20 μL (total volume) PCR mixture consisted of 4 μL of diluted cDNA, 10 μL of SYBR green-containing PCR master mixture (2×), and 150 μM of each primer. The CB_2 and TNF-α primers for real-time RT-PCR were designed using the Primer Express software from Applied Biosystems (Foster City, CA) and were:

CB_2 sense: 5′-TGA ATG AGC AGA CCG ACA GG-3′

CB_2 antisense: 5′-AGA GAT GTT TGC TGG GTG GC-3′

β-actin sense: 5′-TCC ACC ACC ACA GCT GAG AGG-3′

β-actin antisense: 5′-CAG CTT CTC TTT GAT GTC ACG-3′

Real-time RT-PCR was performed using the Stratagene Mx3005P, and the cycling conditions were 95°C for 15 seconds, then 60°C for 1 minute for 40 cycles, followed by a melting point determination or dissociation curve analysis. The expression level of each gene was indicated by the cycle numbers needed for the cDNA to be amplified to a threshold value. The amount of DNA was calculated from the number of cycles by using standard curves, and the results were normalized to the housekeeping gene β-actin from the same sample.

Statistics

Student's *t* test was used to compare the difference in CB_2 mRNA expression and locomotive function between the CB_2-treated group and the vehicle-treated control group. The mRNA expression of TNF-α was analyzed by one-way analysis of variance followed by Bonferroni's test. Data were presented as mean ± standard error of the mean (±SEM). A statistically significant difference was assumed at $P < 0.05$.

RESULTS

CB_2 Expression Was Upregulated after Spinal Cord Injury

There was little CB_2 expression in the spinal cord tissue of sham animals (i.e., those without SCI). CB_2 expression was significantly up-regulated in spinal cord by 24 hours after contusion (*Fig. 23.1*).

Administration of a CB_2 Agonist Improved Locomotive Function after Spinal Cord Injury

Administration of the CB_2 agonist (O-1966) 1 hour before and 24 and 48 hours after injury significantly improved motor function after SCI. In BMS testing, there were significant differences in the motor function scores between the treatment and control groups on postprocedure Days 1 (1.23 ± 0.25 versus 0.30 ± 0.21), 7 (5.36 ± 0.31 versus

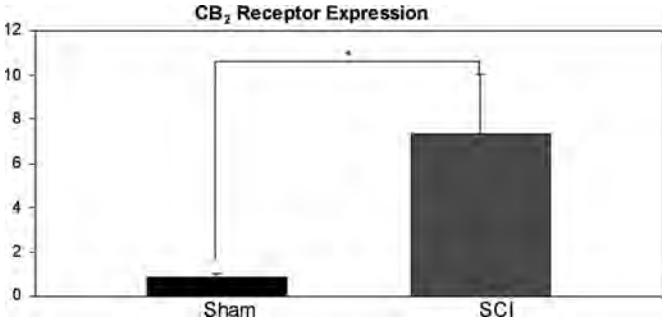

FIGURE 23.1. CB_2 receptor expression in the spinal cord was tested in sham animals (i.e., those without SCI) and in animals with SCI 24 hours after injury. There was little CB_2 expression in the spinal cord of sham animals, but CB_2 expression was significantly up-regulated in the spinal cord 24 hours after injury. N = 3 in each group; data expressed as mean ± SEM; $^*P < 0.001$.

4.00 ± 0.70), and 14 (6.45 ± 0.34 versus 5.25 ± 0.37), whereas with BBB testing, the animals treated with CB_2 agonist demonstrated significant improvement over control animals only on postprocedure Day 14 (12.8 ± 0.3 versus 10.4 ± 0.8) (*Fig. 23.2A–B*).

Administration of a CB_2 Agonist Improved Autonomic Function after Spinal Cord Injury

Administration of the CB_2 agonist O-1966 1 hour before and 24 and 48 hours after injury significantly improved the percentage of animals regaining spontaneous bladder function. More animals in the CB_2 agonist treatment group recovered bladder function compared with the vehicle-treated control group at each evaluation period, and this was statistically significant by the 14th day postinjury (*Fig. 23.3*).

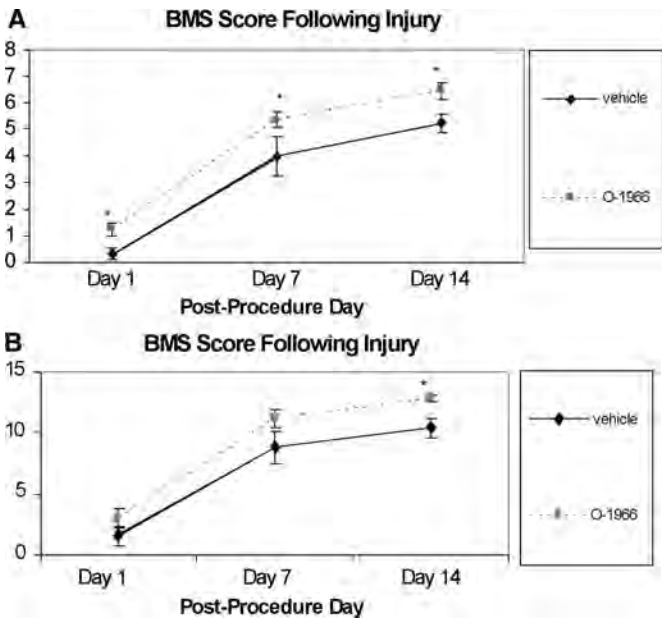

FIGURE 23.2. The CB_2 agonist (O-1966) was administered (1 mg/kg) 1 hour before injury and 24 and 48 hours after injury. The control group received an equal volume of vehicle. Motor function was improved significantly at all evaluation times after injury by BMS (*A*) and at day 14 by BBB (*B*). N = 10 to 12 in each group; data expressed as mean ± SEM; $^*P < 0.05$.

CB_2 Agonist Decreased Tumor Necrosis Factor-α Expression after Spinal Cord Injury

The CB_2 agonist (O-1966) or vehicle was administered 1 hour before SCI. Thoracic spinal cord TNF-α was tested 24 hours after injury by real-time RT-PCR. Spinal cord tissues from normal animals were also tested by real-time RT-PCR to acquire normal values (sham animals). TNF-α expression was significantly increased 24 hours after SCI in vehicle-treated animals as compared with sham animals. This increase in expression was significantly attenuated by the administration of CB_2 agonist (*Fig. 23.4*).

DISCUSSION

The major finding of this study was that treatment with a selective CB_2 agonist significantly improved recovery in both locomotive and autonomic functions in a mouse model of SCI. Animals treated with the CB_2 agonist showed improved hind limb motor function and earlier recovery of bladder function compared with vehicle-treated control animals. In addition, these improvements were associated with a decreased production of the pro-inflammatory cytokine TNF-α in the injured spinal cord. These results support the hypothesis that activation of the CB_2 receptor promotes improvement of function after SCI through inhibition of inflammatory responses.

The final histological lesion is usually far greater in size than the initial physical damage caused by contusion of the spinal cord. Cascades of injury-induced, destructive events that occur after the initial insult are defined as secondary injury. The synthesis of inflammatory cytokines and a coordinated infiltration of the peripheral leukocytes to the damaged site are important contributors.[7,11] This posttraumatic inflammation may further reduce functional recovery through promotion of neuronal and oligodendrocytic necrosis or apoptosis as well as through development of scar tissue.

Attenuation of inflammation after SCI has been shown to improve clinical outcome.[10] White blood cell invasion has been regarded as a primary contributor to the secondary damage during inflammation. The expression of endothelial cell adhesion molecules such as intercellular adhesion molecule-1, which interact with circulating white blood cells, is an initial step in this process. Neutrophils are the first inflammatory cells to arrive at the site of injury.[37] These cells contribute to the secondary injury by releasing neutrophil proteases, reactive oxygen species, and cytokines. Cytokines such as TNF-α not only activate other inflammatory cells like microglia within the SCI site, but may also damage neurons

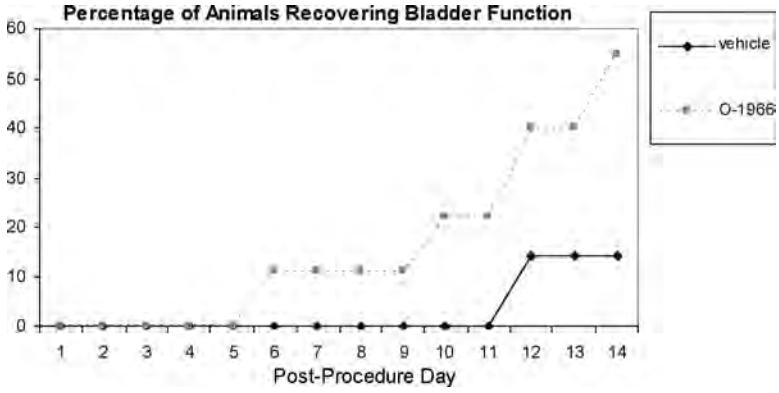

FIGURE 23.3. The CB_2 agonist O-1966 was administered (1 mg/kg) 1 hour before injury and 24 and 48 hours after injury with controls receiving an equal volume of vehicle. A significantly larger fraction of the CB_2-treated animals demonstrated bladder function recovery compared with the vehicle-treated group. At the end of observation (Day 14), 55% of animals showed bladder function recovery in the CB_2-treated group compared with 14% of the animals in the control group. N = 10 in each group.

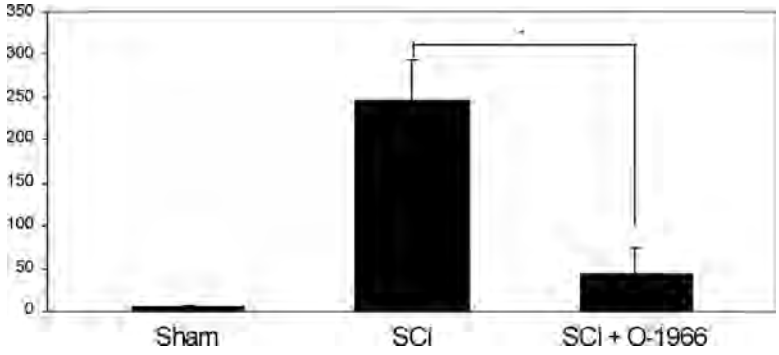

FIGURE 23.4. Sham animals (i.e., those without injury) were used to determine normal, baseline TNF-α expression in the spinal cord (sham group). Vehicle (SCI group) or O-1966, our CB_2 agonist (SCI + O-1966 group), was given at 1 mg/kg 1 hour before injury. Spinal cord TNF-α was measured by real-time RT-PCR 24 hours after injury. N = 3 in each group; data expressed as mean ± SEM; $^*P < 0.01$.

and oligodendrocytes directly.[23,32,41] Neutrophil proteases are capable of damaging endothelial cells and contribute to increased vascular permeability. The concept that neutrophils contribute to microvascular damage after injury to the spinal cord is supported by investigations demonstrating that the administration of a neutrophil elastase inhibitor reduces post-traumatic hemorrhage.[36,38] Previous studies have also shown that inhibition of TNF-α activity eliminates vascular damage after SCI.[29,35] In our study, we found that TNF-α expression was significantly increased after SCI and that this increase was attenuated by CB_2 agonist administration. In an earlier study using a mouse model of cerebral ischemic injury, we demonstrated that CB_2 activation reduced infarct size through inhibition of leukocyte/endothelial interactions.[42] Therefore, it is likely that the improved motor function resulting from CB_2 agonist treatment observed in this study is also related to diminished neutrophil invasion into the injured tissue.

Selective CB_2 agonists have the potential to attenuate many of the inflammatory responses associated with secondary injury to the spinal cord. CB_2 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 code for pro-inflammatory cytokines.[22] Inhibition of cytokines such as TNF-α and interleukin-6 by CB_2 activation has been verified both *in vivo* and *in vitro*. In addition, inducible nitric oxide synthase transcription and nitric oxide production in macrophages can be largely inhibited by CB_2 activation.[6] Both proinflammatory cytokines and nitric oxide are neurotoxic, leading to secondary neuronal death and axonal destruction after SCI.[2,7] CB_2 stimulation is also able to inhibit antigen presenting cell activity, decrease antibody production from B-lymphocytes, and down-regulate inflammatory cytokine production.[21] Prior work in our laboratory demonstrated that the administration of WIN55212-2, a CB_1 and CB_2 agonist, attenuated clinical symptoms in a mouse model of multiple sclerosis through the CB_2 receptor.[27] More recently, we have found that selective stimulation of the CB_2 receptor protected the brain during ischemic–reperfusion injury, a process associated with decreased leukocyte infiltration.[42] All of these investigations indicate that activation of the CB_2 receptor may provide neuroprotection during CNS injuries through interference with inflammatory responses.

Multiple studies have established the neuroprotective properties of cannabinoids in various CNS disorders like stroke, multiple sclerosis, naturopathic pain, and Alzheimer's disease.[8,9,17,26] CB_1 and CB_2 receptors are both found in the CNS; CB_1 is predominantly expressed in the CNS and peripheral neurons. CB_1 stimulation is important in neurotransmission and CNS homeostasis and is thought to inhibit presynaptic transmission. Some evidence has indicated that

the CB_1 receptor may protect neurons from excitotoxic damage after CNS injuries.[13,30] However, the psychotropic effects caused by CB_1 activation potentially limit its clinical applicability. On the other hand, CB_2 is principally located on immune cells, and its stimulation has been shown to modulate immune cell activities and the inflammatory responses without causing psychoactive effects.[30,39] The CB_2 agonist used in this investigation, O-1966, has excellent affinity for CB_2 receptors and very low affinity for CB_1 receptors, as evidenced by its effects on ^{35}S-GTPγS binding. Consistent with its binding profile, O-1966 produced only very mild behavioral effects at doses that far exceeded those used in this investigation.[42]

In addition to improving motor function, CB_2 activation improved recovery of spontaneous bladder function after SCI. The destruction of neurons carrying autonomic and somatic motor fibers from the spinal cord to bladder muscles leads to the uncontrolled bladder. In addition to the initial harm to nerve fibers, the increased intraspinal pressure caused by posttraumatic edema also damages nerve fibers and worsens bladder dysfunction.[5,31] Although the exact mechanisms underlying the improvement of bladder function after CB_2 agonist treatment are perhaps still unclear, it is possible that the attenuated inflammation mediated by CB_2 activation ameliorates postinjury hemorrhage and edema, thereby relieving pressure on nerve fibers, which carry information to bladder muscles, allowing faster and more complete recovery.

In conclusion, in this study, we found that selective stimulation of the CB_2 receptor improves both locomotive and autonomic function in a mouse model of SCI. Furthermore, we provide evidence that this improvement is mediated by the neuroprotective attenuation of inflammatory responses after the initial injury, including inhibition of inflammatory cytokine production.

Disclosure

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