Activation of cannabinoid receptor 2 inhibits experimental cystitis

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Wang Z, Wang P, Bjorling DE. Activation of cannabinoid receptor 2 inhibits experimental cystitis. Am J Physiol Regul Integr Comp Physiol 304: R846–R853, 2013. First published March 20, 2013; doi:10.1152/ajpregu.00585.2012.—Cannabinoids have been shown to exert analgesic and anti-inflammatory effects, and the effects of cannabinoids are mediated primarily by cannabinoid receptors 1 and 2 (CB1 and CB2). Both CB1 and CB2 are present in bladders of various species, including human, monkey, and rodents, and it appears that CB2 is highly expressed in urothelial cells. We investigated whether treatment with the CB2 agonist GP1a alters severity of experimental cystitis induced by acrolein and referred mechanical hyperalgesia associated with cystitis. We also investigated whether the mitogen-activated protein kinases (MAPK), ERK1/2, p38, and JNK are involved in the functions of CB2. We found that treatment with the selective CB2 agonist GP1a (1–10 mg/kg, ip) inhibited the severity of bladder inflammation 3 h after intravesical instillation of acrolein in a dose-dependent manner, and inhibition reached significance at a dose of 10 mg/kg (P < 0.05). Treatment with GP1a (10 mg/kg) inhibited referred mechanical hyperalgesia associated with cystitis (P < 0.05). The inhibitory effects of the CB2 agonist were prevented by the selective CB2 antagonist AM630 (10 mg/kg, sc). We further demonstrated the inhibitory effects of CB2 appear to be at least partly mediated by reducing bladder inflammation-induced activation of ERK1/2 MAPK pathway. The results of the current study indicate that CB2 is a potential therapeutic target for treatment of bladder inflammation and pain in patients.

Cannabinoid receptor 2; GP1a; cystitis; hyperalgesia; mice

PAINFUL BLADDER DISORDERS, such as interstitial cystitis/painful bladder syndrome (IC/PBS), are relatively common. IC/PBS is a chronic inflammatory disorder characterized by increased frequency, urgency, and bladder pain, and it is estimated to affect 2.7% to 6.5% of American women (1, 5, 20). The etiology and pathogenesis of IC/PBS remain unknown. It is highly probable that the etiology of IC/PBS is a multifactorial condition, and no treatment or combination of treatments has been found to be consistently effective in alleviating symptoms in IC/PBS patients. (1, 5, 20). Cannabinoids have been shown to have analgesic and anti-inflammatory effects, and the effects of cannabinoids are mediated primarily by cannabinoid receptors 1 and 2 (CB1 and CB2), both coupled to inhibitory G proteins (2, 12, 17, 19). CB1 is predominantly located in the central nervous system (CNS), whereas CB2 is primarily present in peripheral tissues (2, 12, 29). Therefore, selective CB2 agonists may exert actions without inducing undesirable CNS effects related to activation of CB1, including hypoactivity, hypothermia, and catalepsy (2, 12, 29, 46, 58).

Both CB1 and CB2 are present in bladders of various species, including humans, monkeys, and rodents (26, 32, 49, 69–70), and it appears that CB2 is highly expressed in urothelial cells (26, 32, 49, 69). Intravesical administration of a selective CB1 agonist inhibited sensitization of bladder afferent nerves induced by bladder inflammation (73). In vitro electrically evoked contraction of bladder strips was inhibited by treatment with CB1 or CB2 agonists (70). Treatment with a selective CB2 agonist increased micturition intervals and volumes in normal rats (27), and improved bladder function of rats after partial urethral obstruction (28). Fatty acid amide hydrolase (FAAH), an enzyme that specifically degrades anandamide, an endogenous cannabinoids (or endocannabinoid), is present in urothelium (50, 65). Inhibition of FAAH activity also increased micturition intervals and volumes in rats (65), and the effects of FAAH inhibition were prevented by a selective CB2 antagonist. These studies support a role of cannabinoid receptors, particularly CB2, in regulating bladder functions under physiological and pathophysiological conditions.

Cyclophosphamide (CYP) is an antineoplastic alkylating agent commonly used to treat cancer patients (15), and an undesirable clinical side effect of CYP is hemorrhagic cystitis. CYP is metabolized by the liver to acrolein that is accumulated in urine, and acrolein is primarily responsible for CYP-induced cystitis (13, 15). CYP/acrolein-induced cystitis in rodents is commonly used as an experimental model to study mechanisms underlying cystitis and associated visceral pain (7, 8, 25). Recently, a highly specific CB2 agonist, N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-di-hydro-6-methylindenol[1,2-c]pyrazole-3-carboxamide (GP1a) has been described (22, 53). Systemic treatment with GP1a decreased serum IL-6 concentrations, reduced neutrophil recruitment in lung tissues, and increased mean survival time in a mouse model of sepsis induced by cecal ligation and puncture (68). In the present study, we investigated whether treatment with the CB2 agonist GP1a alters severity of experimental cystitis induced by acrolein and referred hyperalgesia associated with cystitis. We also investigated whether the mitogen-activated protein kinases (MAPK), ERK1/2, p38 and JNK are involved in the functions of CB2.

MATERIALS AND METHODS

Animals. Female C57BL/6NH mice (10–12 wk old) were obtained from Harlan (Indianapolis, IN). Experiments were conducted in accordance with National Institutes of Health Guidelines, and all protocols were reviewed and approved by the Animal Care and Use Committee of the University of Wisconsin.

Histological evaluation of cystitis. Mice were anesthetized with avertin (250 mg/kg, Sigma-Aldrich, St. Louis, MO), injected intraperitoneally (ip), and cystitis was induced by intravesical instillation of acrolein (1 mM, 150 μl total volume; Ultra Scientific, Kingstown, RI) via a urethral catheter (PE 10, i.d. 0.28 mm, o.d. 0.61 mm; Becton Dickinson, Sparks, MD). The dose and volume of acrolein were chosen on the basis of results of preliminary experiments to induce...
cystitis of moderate severity. Control mice received an equivalent volume of intravesical saline (0.9%) instead of acrolein.

Three hours after instillation of acrolein or saline, mice were deeply anesthetized with pentobarbital (50 mg/kg, ip) and perfused with saline through a canula inserted into the left ventricle. Bladders were removed and weighed, and bladder weight (mg) was normalized to body weight (g). Bladders were divided into two parts; the caudal part including the neck region was fixed in 4% paraformaldehyde for 4 h at 4°C and cryoprotected with 30% sucrose in phosphate-buffered saline (PBS) at 4°C. Tissue sections were made with a cryostat at a thickness of 10 μm. Every 4th section was stained with hematoxylin and eosin for morphological analysis, and 4–6 sections from each bladder were examined microscopically. The urothelium/suburothelium of the remainder of the bladders was mechanically separated from the detrusor using fine forceps as previously described (40, 75). Tissues were stored at −80°C until analyzed (see below).

Peripheral nociception testing. The effects of cystitis on response to peripheral application of mechanical stimuli were also evaluated. The individual performing testing was unaware of the pretreatments of mice. Mechanical sensitivity of the hind paws was assessed using von Frey monofilaments and the up-down method (10). Mice were placed in individual Plexiglas chambers with a wire mesh floor, and allowed to acclimate for at least 30 min or until cage exploration stopped. Sensitivity of the hind paws was assessed with a series of six von Frey filaments of increasing stiffness. Stimulus-related retraction of the tested paw was considered a withdrawal response, and the 50% paw withdrawal threshold was determined by the nonparametric method of Dixon (10). Previous studies have reported enhanced mechanical sensitivity of both the hind paws and pelvic region of mice in experimental cystitis (48, 66, 74) and similar results are reported, regardless of the area tested (48, 66).

Isolation of protein from urothelium/suburothelium. Tissues were homogenized with T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN). Supernatants were collected by centrifugation at 10,000 g for 15 min at 4°C. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific). Protein samples were mixed 1:1 with Laemmlli sample buffer (Bio-Rad, Hercules, CA), placed in boiling water for 5 min, and stored at −80°C until analyzed.

Semi-quantitative immunoblotting analysis. Protein samples (20 μg/lane) were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% dry fat-free milk in 1× TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20; pH 7.5). After rinsing, membranes were incubated at 4°C overnight with the specific primary antibody. Membranes were then stripped and reprobed with a mouse anti-

Effects of GP1α on severity of cystitis. No signs of cystitis were observed in saline-treated animals (Fig. 1A). Three hours after intravesical instillation of 1 mM acrolein, histological examination of the bladders indicated the presence of cystitis characterized primarily by edema in the submucosal region (Fig. 1 B–D). Occasionally, areas of hemorrhage and mild infiltration of inflammatory cells were also observed. The weights of acrolein-treated bladders were significantly increased (2.34 ± 0.32 mg/g, n = 8) relative to saline-treated controls (1.18 ± 0.06 mg/g, n = 6) (P < 0.05), and this appeared to be primarily due to submucosal edema. Treatment with GP1α (1–10 mg/kg, ip) attenuated the increase of bladder weight in a dose-dependent manner (the correlation coefficient r² between dose of GP1α and inhibition was 0.99), and inhibition reached significance at a dose of 10 mg/kg (P < 0.05 vs acrolein-treated). Inhibition of acrolein-induced increased bladder weight produced by GP1α (10 mg/kg) (1.58 ± 0.15 mg/g, n = 8) was reversed by pretreatment with the selective CB2 antagonist AM630 (2.15 ± 0.1 mg/g, n = 8) (10 mg/kg, P < 0.05 vs acrolein+GP1α-treated). Treatment with AM630 (10 mg/kg) alone did not affect acrolein-induced increase of bladder weight (2.21 ± 0.1 mg/g, P > 0.05 vs acrolein-treated, n = 6).

Treatment with GP1α (10 mg/kg) or AM630 (10 mg/kg) in mice instilled with saline did not affect bladder weight (1.16 ± 0.05 and 1.09 ± 0.09 mg/g, respectively, n = 6, P > 0.05 vs saline-treated controls), and no histological evidence of cystitis was observed (not shown).

Presence of CB2 in urothelium/suburothelium. We investigated whether CB2 is present in mouse urothelium/suburothelium using immunoblotting. As shown in Fig. 2, CB2 protein occurred as a single band at about 45 kDa on denaturing acrylamide gel, as reported previously (49). Exposure to acrolein for 3 h did not alter CB2 abundance in urothelium/suburothelium.
suburothelium. Also, treatment with GP1a or AM630/GP1a did not affect abundance of CB2 (Fig. 2).

**Effects of GP1a on enhanced mechanical sensitivity associated with cystitis.** The basal mechanical sensitivity threshold was about 2.8 g in all groups (Fig. 3). Intravesical instillation of saline did not affect peripheral mechanical sensitivity (Fig. 3). The mechanical sensitivity threshold was reduced 3 h after instillation of acrolein (0.22 ± 0.05 g, n = 8) (P < 0.01 vs saline-treated controls). Treatment with GP1a (10 mg/kg) attenuated increased mechanical sensitivity (1.74 ± 0.35 g, n = 8) (P < 0.05 vs acrolein-treated), and the effect of GP1a was reversed by the selective CB2 antagonist AM630 (10 mg/kg) (0.21 ± 0.04 g, n = 8) (P < 0.05 vs acrolein+GP1a-treated) (Fig. 3). Treatment with AM630 (10 mg/kg) alone did not affect acrolein-induced increase of mechanical sensitivity (0.2 ± 0.04 g, n = 6, P > 0.05 vs acrolein treated).

Treatment with GP1a (10 mg/kg) or AM630 (10 mg/kg) in mice instilled with saline did not affect the mechanical sensitivity (2.61 ± 0.33 and 2.7 ± 0.23 g, respectively, n = 6, P > 0.05 vs saline-treated controls).

**Effects of GP1a on MAPK activation.** The basal content of p-ERK1/2 in the urothelium/suburothelium was low as shown in Fig. 4A. Phosphorylation of ERK1/2 was increased 3 h after intravesical instillation of acrolein (6.31 ± 0.32, n = 8) (Fig. 4B, P < 0.01 vs saline-treated), and this effect of acrolein was attenuated by treatment with GP1a (10 mg/kg) administered 10 min before acrolein (4.13 ± 0.63, n = 8) (Fig. 4B, P < 0.05 vs acrolein-treated). The effect of GP1a on p-ERK1/2 abun-
phosphorylation of p38 (Fig. 4). GP1a did not affect p38. Treatment with GP1a or AM630 (Fig. 4).

Increased phosphorylation of JNK (P < 0.05 vs acrolein-treated) and enhanced mechanical sensitivity-inhibited by 10.220.33.4 on July 9, 2017 http://ajpregu.physiology.org/ Downloaded from activation of CB2 in urothelium; 2) treatment with a selective CB2 agonist, GP1a, reduced carrageenan-induced edema in hind paws of rats, and this effect was reversed by a selective CB2 antagonist (12). Quartilho et al. (60) reported similar observations using a different CB2 agonist (AM1241) to prevent carrageenan-induced edema, and the inhibitory effect of AM1241 was completely blocked by a selective CB2 antagonist, whereas a selective CB1 receptor antagonist had no effect. These authors further demonstrated that activation of CB2 not only prevented, but also reversed, inflammation-induced edema (60). In other studies, treatment with a selective CB2 agonist reduced arachidonic acid-induced edema in the ear (31), and edema of hind paw elicited by intraplantar injection of lipopolysaccharide (38). These studies clearly support the anti-inflammatory effects of CB2, although the precise mechanisms underlying CB2 activation-induced inhibition of inflammation remain to be clarified.

There is very limited information available regarding the inhibitory activity of CB2 relative to inflammation of visceral structures. Storr et al. (64) reported that treatment with a
selective CB2 agonist inhibited (while a selective CB2 antagonist exacerbated) severity of experimental colitis. Treatment with a selective CB2 agonist attenuated the inflammatory responses in cardio and hepatic ischemia/reperfusion injuries in mice (33, 51–52). In the present study, treatment with the selective CB2 agonist GP1a reduced severity of bladder inflammation, and the inhibitory effect of GP1a was reversed by the selective antagonist AM630. Previous studies have shown that CB2 is present in bladders, particularly in urothelium, in various species (26, 32, 49, 69–70). We provide further evidence that CB2 is expressed in the urothelium of mouse bladders. While urothelium has historically been viewed as a simple barrier separating the bladder wall from urine, increasing evidence also suggests that the urothelium plays a critical role in physiological and pathophysiological processes in the bladder (6, 71–72, 75–76). Specifically, urothelial cells have the capacity to secrete a variety of signaling molecules such as PGE2, nerve growth factor, nitric oxide, and cytokines in response to various stimuli (6, 71–72, 75–76). Conceivably, chemical mediators derived from urothelial cells could significantly influence bladder function and processes involved in bladder inflammation. CB2 is coupled to inhibitory G proteins, and activation of CB2 inhibits adenyl cyclase (17) and release of inflammatory cytokines in inflammatory cells (39, 52) and tumor cells (43). It is possible that activation CB2 may inhibit production and release of inflammatory mediators from urothelial cells, reducing the local inflammatory response of the bladder after acrolein treatment. However, inflammatory responses are complicated, and many biological

![Image of graph showing bladder weight and mechanical sensitivity threshold induced by acrolein or saline with treatment effects]

**Fig. 4.** Semiquantitative immunoblotting analysis of phosphorylation of ERK1/2 (open bar), p38 (gray bar), and JNK (solid bar) in urothelium/suburothelium. Representative immunoblots are shown. Phosphorylation of ERK1/2 was increased 3 h after intravesical instillation of acrolein, and this effect of acrolein was attenuated by treatment with GP1a. The effect of GP1a was reversed by treatment with the selective antagonist AM630. n = 6 (saline-treated controls), n = 8 (other treatment groups). *P < 0.01 vs. saline-treated. #P < 0.05 vs. acrolein-treated. @P < 0.05 vs. acrolein+GP1a-treated.

**Fig. 5.** Treatment with U0126 (30 mg/kg), an inhibitor of ERK1/2 phosphorylation, attenuated the increase of bladder weight (A) and the reduction in mechanical sensitivity threshold-induced by acrolein (B). n = 6. *P < 0.05, **P < 0.01 vs. saline-treated, respectively. #P < 0.05 vs. acrolein-treated.
events are involved. CB2 activation may also affect other cellular components, such as infiltration by immune cells, contributing to the anti-inflammatory effects of CB2, and this will be the focus of future studies.

It has been shown that activation of CB2 exerts anti-nociceptive effects (35–36, 45, 54). Treatment with CB2 agonists reduced the second phase of nocifensive behaviors elicited by intraplantar injection of formalin in mice (4) and allodynia elicited by L5-L6 spinal nerve ligation in rats (4). These effects of CB2 agonists were prevented by selective CB2 antagonists (4). In other reports, treatment with CB2 agonists suppressed carrageenan-evoked thermal and mechanical hyperalgesia in rodents (12, 30, 55) and cancer-induced pain (43–44). Little is known about the effects of CB2 activation on visceral pain. One hallmark of visceral pain is perception of pain arising from somatic sites distant from the area of visceral injury (referred hyperalgesia) (7–8, 42), and this is a relatively common finding in patients with IC/PBS (67). Sensitization of primary afferents at the site of injury plays an important role in development of referred hyperalgesia (8, 78). CB2 is present in dorsal root ganglia (DRG) afferent neurons, and CB2 expression in DRG is upregulated by pathological pain states (34). Gratze et al. (26) demonstrated that CB2 is present in sensory nerve fibers in the bladder. Activation of CB2 inhibits capsaicin-induced increase of intracellular calcium in DRG afferent neurons (61), and release of calcitonin gene-related peptide from afferent nerve fibers in bladders (32) and spinal cord slices (4). Mackley et al. (55) reported that enhanced C-fiber activity in response to electrical stimulation was inhibited by a CB2 agonist. We found that treatment with the CB2 agonist GP1a inhibited bladder inflammation-associated enhanced peripheral mechanical sensitivity. Conceivably, activation CB2 attenuates production and release of inflammatory mediators from urothelial cells that are involved in sensitization of afferent nerves. Further, activation of CB2 may exert a direct inhibitory effect on afferent nerves. The results from the present study, together with previous findings, suggest that suppression of referred mechanical hyperalgesia associated with bladder inflammation by the CB2 agonist may result from the combined effects of reduced inflammation and inhibition of afferent nerve activity following CB2 activation.

The MAPK are a group of protein serine/threonine kinases that mediate signal transduction from the cell surface to the nucleus in response to a variety of extracellular stimuli (9). The MAPK consist of three major families of protein kinases, including ERK, p38, and JNK (9, 18), and activity of MAPK depends on their phosphorylation status (9, 18). There is substantial evidence that ERK1/2 is involved in inflammatory responses (62), as well as in the development of inflammatory and neuropathic pain (16, 37, 41, 77). Blockade of ERK1/2 phosphorylation reduces mechanical hyperalgesia of inflamed paws in rodents (24, 37) and development of referred hyperalgesia after intracolonic instillation of capsaicin or mustard oil in mice (23). Treatment of rats with CYP induced phosphorylation of ERK1/2 in bladders (14, 59), and ERK1/2 phosphorylation occurred primarily in urothelium (14). Furthermore, treatment with ERK1/2 phosphorylation inhibitor U0126 decreased bladder hyperreactivity and improved bladder capacity in CYP-treated rats, suggesting that ERK1/2 participates in inflammatory responses in the bladder (14). We confirmed that bladder inflammation induced phosphorylation of ERK1/2 in mice urothelium. Also, our findings that bladder inflammation induced phosphorylation of JNK, but not p38, in urothelium are consistent with previous observations (11, 13). We further demonstrated that GP1a selectively inhibited phosphorylation of ERK1/2 without affecting phosphorylation of JNK, and treatment of mice with U0126 inhibited bladder inflammation and the associated mechanical hyperalgesia of hind paws. Therefore, our results suggest that the ERK1/2 pathway may partly mediate the inhibitory effects of CB2 activation on bladder inflammation and the associated enhanced pain sensation.

**Perspectives and Significance**

Our findings demonstrate that CB2 is present in mouse urothelium/suburothelium and acute bladder inflammation does not appear to alter CB2 protein abundance. We further demonstrate that treatment with a selective CB2 agonist inhibits bladder inflammation and associated referred mechanical hyperalgesia. The inhibitory effects of CB2 appear to be at least partly mediated by reducing bladder inflammation-induced activation of ERK1/2 MAPK pathway. The results of the current study indicate that the CB2 is a potential therapeutic target for treatment of bladder inflammation and pain in patients. It should be noted that bladder inflammation and associated enhanced pain sensation involve complicated biological processes and other cellular components, and signaling pathways may also contribute to inhibitory effects of CB2 activation. These areas will, therefore, be the focus of future studies.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: Z.-Y. W. and D. E. B. conception and design of research; Z.-Y. W. and P. W. performed experiments; Z.-Y. W. and P. W. edited and revised manuscript; Z.-Y. W. and D. E. B. provided final version of manuscript.

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