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Cannabinoid receptor 2 is increased in acutely and chronically inflamed bladder of rats

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Abstract

Cannabinoid receptors 1 and 2 (CB1 and CB2) are G-protein coupled receptors that are expressed throughout the body. Cannabinoid receptors are expressed in the urinary bladder and may affect bladder function. The purpose of this study was twofold: to confirm the presence of cannabinoid receptors in the bladder, the L6/ S1 spinal cord, and dorsal root ganglia (DRG), and to determine the effects of acute and chronic bladder inflammation on expression of cannabinoid receptors. Acute or chronic bladder inflammation was induced in rats by intravesical administration of acrolein. Abundance of CB1 and CB2 protein and their respective mRNA was determined using immunoblotting and quantitative real-time PCR, respectively. We confirmed the presence of CB1 and CB2 receptor protein and mRNA in bladder, L6-S spinal cord, and DRG. Acute bladder inflammation induced increased expression of CB2, but not CB1, protein in the bladder detrusor. Chronic bladder inflammation increased expression of bladder CB2 protein and mRNA but not CB1 protein or mRNA. Expression of CB1 or CB2 in spinal cord or DRG was unaffected by acute or chronic bladder inflammation. CB1 and CB2 receptors are present in the bladder and its associated innervation, and CB2 receptors are up-regulated in bladder after acute or chronic inflammation. CB2 receptors may be a viable target for pharmacological treatment of bladder inflammation and associated pain.

Cannabinoid receptors 1 and 2 (CB1 and CB2) are members of the G-protein coupled receptor (GPCR) superfamily. CB1 is expressed primarily in the central nervous system (CNS) and is the most abundant GPCR in the brain [19]. CB2 is expressed by leukocytes, including mast cells [6], lymphocytes, monocytes, and neutrophils [2], and CB2 is also expressed at low levels in the CNS in both microglia and some neurons [8,22]. Cannabinoid receptors have also been detected in peripheral tissues, including urinary bladder [10]. Endocannabinoids and cannabinoid agonists decrease motility in normal and inflamed bladder, suggesting that CB receptors may have functional effects on the bladder [4,12,13]. Hyperalgesia associated with turpentine-induced acute bladder inflammation was prevented by administration of a cannabinoid agonist [6]. While there is interest in the use of cannabinoids to treat bladder disorders, effects of inflammation on expression of cannabinoid receptors in the bladder have not been described.

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We investigated expression of CB receptor proteins and mRNA in bladder and associated innervation in eight to ten weeks old female Sprague-Dawley and Wistar rats. Rats were anesthetized with isoflurane (2–5%) in oxygen. Bladders were catheterized with PE 10 tubing (Intramedic, Sparks, MD), emptied by abdominal compression, and acrolein (400 µl, 1mM, Ultra Scientific, Kingstown, RI) or saline (0.9%, 400 µl) was instilled. Acute cystitis was induced by one dose of acrolein, and chronic cystitis was established with three doses of acrolein at 72 hour intervals. Controls animals received intravesical sterile saline (1 or 3 instillations). All procedures were approved by the Institutional Animal Care and Use Committee of University of Wisconsin, Madison and conducted in a manner consistent with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Treated and control rats were euthanized 48 hours after one instillation (acute) or 72 hours after the third instillation (chronic) for immunoblotting studies. Bladder, dorsal root ganglia (DRG; L5,L6 and S1), spinal cord (segments L3 to L5 and L6 to S), spleen (control for CB2 protein expression), and hippocampus (control for CB1 protein expression) were harvested. Bladder afferent innervation arises mainly from L6-S1 DRG and L6-S segments of spinal cord. The present study also analyzed expression of cannabinoid receptor protein in L5 DRG and L3–L5 segments of spinal cord as control tissues. Bladder mucosa (urothelium and lamina propria) was separated from the smooth muscle (detrusor) using forceps and scissors. Proteins were extracted from tissue samples, resolved in SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Membranes were incubated with blocking solution - 5% non-fat milk in TBS-T (20 mM tris-HCl, 150 mM NaCl, 0.1% Tween 20) - at room temperature for 2 hours. Membranes were washed with 1% TBS-T and incubated overnight at 4°C with antibody against CB1 (1:5000, Affinity BioReagents, Golden, CO) or CB2 (1:2000, Cayman, Ann Arbor, MI). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, Santa Cruz, Santa Cruz, CA) for 1 hour at room temperature. Antibody detection was performed by chemiluminescence substrate (Pierce Biothecnology, Rockford, IL). Membranes were stripped and re-probed with beta-actin antibody (1:10000, Cell Signaling Technology, Inc., Danvers, MA) loading control. Protein content was estimated by comparing optical densities with the Image J program (NIH, Bethesda, MD)

To determine mRNA abundance, treated and control rats were euthanized 2 hours after one (acute) or three acrolein instillations (chronic). Quantitative real-time PCR was performed to determine CB1 and CB2 mRNA in whole bladder, L6 and S1 DRG, and spinal cord (segments L6-S) from controls and rats with acute or chronic cystitis. Total RNA was extracted from sample tissues using TRIzol reagent (1 ml per 50 mg of tissue, Invitrogen, Carlsbad, CA). Samples were homogenized and extracted in chloroform. RNA was precipitated in isopropanol at –20° overnight, centrifuged for 30 minutes, washed with 70% ethanol, and resuspended in DEPC water. Samples were treated with DNase (DNA-free reagent, Ambion, Austin, TX), and RNA concentrations were determined with a spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA). Total RNA was reverse-transcribed to cDNA using SuperScript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR primers for CB1 and CB2 were: CB1 primer forward 5'-CTACTGGTGCTGTGTGTCATC-3', reverse 5'-GCTGTCTTTACGGTGGAATAC [19]; CB2 primer forward 5'-AGCAGGAGTTGGGAGGAGACT-3', reverse 5'-CTGAATCTGCCAGAGACAGCAT – 3'' [22]. Quantitative real-time PCR was performed using an ABI 7300 RT-PCR (Applied Biosystems, Foster City, CA). This instrument detects florescence emitted by SYBR Green that binds to double-stranded DNA. Samples were amplified in duplicate using the following thermal cycling conditions: 94° C for 10 min, 40–45 cycles of amplification at 94 °C for 30 seconds, and 60° C for 1 minute to allow denaturing and annealing-extension. Expression of each gene was normalized to abundance of mRNA for GAPDH.

Statistical analysis of all data was performed using Student's t-test. *p* values less than 0.05 were considered significant, and data are presented as mean $\pm$ S.E.M.

Both CB1 and CB2 protein was detected in rat bladder mucosa, detrusor, spinal cord (L3 to S) and L5, L6 and S1 DRG in rats that received only saline intravesically (Figure 1–Figure 4). CB1 and CB2 mRNA were found in whole bladder, L6/S1 DRG and L6-S spinal cord in control rats (Figure 5 and Figure 6).

Acute cystitis was associated with a significant increase in CB2 protein abundance in the detrusor, but not mucosa (Figure 2). No significant changes in abundance of CB1 protein were observed in any tissues (Figure 1).

Chronic cystitis caused significant increases in CB2 protein in detrusor and mucosa (Figure 4). Cystitis did not induce an increase in CB2 protein in either DRG or spinal cord (Figure 4). Chronic bladder inflammation did not affect expression of CB1 protein in any tissues (Figure 3).

Expression of CB1 and CB2 transcripts was unaffected by acute cystitis in any tissues (Figure 5). However, CB2 mRNA was increased in chronically inflamed bladders. This finding was consistent with increased CB2 protein in the presence of chronic inflammation.

These results confirm the presence of CB1 and CB2 protein and transcripts in rat urinary bladder and its innervation. Immunoreactivity for CB1 and CB2 in the mucosa of rat bladder has been reported recently [10]. We also found increased abundance of CB2 protein in acute (detrusor) and chronic (detrusor and mucosa) bladder inflammation. Part of this increase could be related to leukocytic infiltration during inflammation. However, we have observed minimal infiltration of inflammatory cells into the mucosa in histological sections of bladders inflamed by instillation of acrolein [1]. It therefore appears that increased CB2 expression in the mucosa of animals with chronic inflammation is the result of increased abundance within urothelial cells.

Cannabinoids have been demonstrated to have a pronounced anti-inflammatory effect and inhibit pain associated with inflammation [5]. This may be organ-dependent, because the anti-inflammatory activity of cannabinoids in experimental hepatitis was mediated by down-regulation of T-cell activity [11], while in vivo inhibition of arthritis [20] and decreased in vitro release of mediators of inflammation by pancreatic stellate cells [16] was attributed to direct activation of cannabinoid receptors in these tissues. Interestingly, the concentration of anandamide, an endogenous cannabinoid, is increased in the bladder after acute or chronic inflammation [3]. Thus, increased expression of CB2 after inflammation may contribute to suppression of the inflammatory response and associated pain and may be of use in developing new treatments for pain and inflammation associated with bladder disorders.

Abundances of CB2 mRNA and protein fluctuated in a similar manner in the bladders of rats with chronic, but not acute, inflammation. This may be due to timing of sample collection relative to duration of the inflammatory process. We chose to collect tissues for gene expression measurements 2 hours after the last treatment with acrolein in both acute and chronic cystitis. Other studies have shown a rapid up-regulation of mRNA for a variety of proteins in the bladder, occurring within 2 hours after chemical insult, and returning to control levels after 6 hours [9,18]. However, we speculate that an increase of CB2 mRNA may not have been detected because an increase in CB2 mRNA occurred prior to, or after, 2 hours subsequent to a single instillation of acrolein. If endocannabinoid concentrations influence local expression of CB mRNA, it is possible that endocannabinoid concentrations failed to reach sufficiently high levels to affect CB2 transcription until more than 2 hours after the inflammatory insult,

because anandamide concentrations in the bladder did not reach a maximal level until 72 hours after a single intraperitoneal injection of cyclophosphamide in rats [3].

In vitro bladder contraction induced by electrical stimulation was inhibited by CB1 agonists, suggesting a functional role for cannabinoid receptors in the urinary bladder [19]. This may be relevant to inflammation-induced increased bladder contractility, because bladder hyperreflexia resulting from intravesical instillation of turpentine was prevented by anandamide [13] and inhibited by WIN 55,212-2, a synthetic cannabinoid agonist [4]. Intraarterial anandamide also prevented thermal referred hyperalgesia associated with turpentine-induced acute bladder inflammation [7]. Other investigators demonstrated that ajulemic acid (a synthetic analog of tetrahydrocannabinol or THC that is a mixed CB1/CB2 agonist) reduced bladder motility subsequent to intravesical instillation of acetic acid or systemic treatment with cyclophosphamide [12].

The abundance of CB1 or CB2 protein and mRNA in spinal cord and DRG was unaffected by acute or chronic cystitis. In agreement with our findings, Zhang J. *et al.* reported that animal models of peripheral nerve injury, but not peripheral inflammation, induced CB2 expression in rat spinal cord [24]. Indeed, models of neuropathic pain stimulate increased expression of CB1 and CB2 in spinal cord. For example, chronic sciatic nerve injury caused increased CB1 in the dorsal horn of spinal cord in rats [15], and CB2 immunoreactivity was increased in the spinal cord in rats with neural damage (sciatic nerve section or spinal nerve ligation) [23]. The severity and type of injury appear to play important roles in expression of cannabinoid receptors in spinal cord. CB1 and CB2 expression are unaffected in DRG in animal models of neuropathic pain, suggesting that increased CB1 or CB2 formed in the cell body are transported to the nerve terminals [17,23]. The absence of increased CB protein or transcript in spinal cord or DRG in rats with acute or chronic cystitis indicates that treatment with cannabinoid receptor agonists may be effective if administered locally (intravesically), because this route of treatment could avoid the side effects caused by systemic administration of cannabinoid receptor agonists.

In this study, we have shown that CB1 and CB2 are present in the bladder and its innervation, and that expression of CB2 is increased in the bladders of rats with acute and chronic cystitis. Bladder inflammation and pain is the summation of a number of biological events, including participation of the endocannabinoid system. The endocannabinoid system could play an important role in modulation of severity of bladder inflammation and pain, and it may be possible to take advantage of the cannabinoid system in the bladder to decrease inflammation and resultant pain.

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TISSUE	CONTROL (n=4)	TREATED (n=7)	p VALUE
Detrusor	1 ± 0.12	1.05 ± 0.21	0.86
Mucosa	1 ± 0.12	1.459 ± 0.21	0.15
L5 DRG	1 ± 0.11	1.007 ± 0.06	0.94
L6/S1 DRG	1 ± 0.11	0.941 ± 0.04	0.34
L3/5 Spinal cord	1 ± 0.07	1.064 ± 0.03	0.33
L6/S Spinal cord	1 ± 0.07	1.058 ± 0.05	0.48

(A)

(B)

(C)

Figure 1.

Immunoblotting for CB1 receptor in rats with acute cystitis. Intensity of bands for CB1 was normalized to that of beta-actin in the same sample. Average intensity of bands for control tissues was arbitrarily set at 1, and samples from treated animals were compared to controls. (A) There were no significant changes in CB1 expression. (B) Representative blots of CB1 expression. (C) Bar graph summarizing data. Data presented as mean of optical density ± S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.

TISSUE	CONTROL (n=4)	TREATED (n=7)	p VALUE
Detrusor	1 $\pm$ 0.21	1.42 $\pm$ 0.06	0.036*
Mucosa	1 $\pm$ 0.04	1.01 $\pm$ 0.02	0.741
L5 DRG	1 $\pm$ 0.03	1.02 $\pm$ 0.02	0.149
L6/S1 DRG	1 $\pm$ 0.11	1.22 $\pm$ 0.09	0.175
L3/5 Spinal cord	1 $\pm$ 0.12	1.12 $\pm$ 0.01	0.202
L6/S Spinal cord	1 $\pm$ 0.22	1.02 $\pm$ 0.21	0.941

(A)

(B)

Figure 2.

Immunoblotting for CB2 receptor in rats with acute cystitis. Intensity of bands for CB2 was normalized to that of beta-actin in the same sample. Average intensity of bands for control tissues was arbitrarily set at 1, and samples from treated animals were compared to controls. (A) CB2 expression was significantly increased in bladder detrusor. (B) Representative blots of CB2 expression. Data presented as mean of optical density $\pm$ S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.

TISSUE	CONTROL (n=6)	TREATED (n=7 - 8)	<i>p</i> VALUE
Detrusor	1 ± 0.05	0.984 ± 0.54	0.79
Mucosa	1 ± 0.34	1.372 ± 0.22	0.36
L5 DRG	1 ± 0.14	0.903 ± 0.05	0.47
L6/S1 DRG	1 ± 0.02	0.96 ± 0.13	0.82
L3/5 Spinal cord	1 ± 0.70	1.083 ± 0.08	0.48
L6/S Spinal cord	1 ± 0.02	1.289 ± 0.15	0.11

(A)

(B)

Figure 3.

Immunoblotting for CB1 receptor in rats with chronic cystitis. Intensity of bands for CB1 was normalized to that of beta-actin in the same sample. Average intensity of bands for control tissues was arbitrarily set at 1, and samples from treated animals were compared to controls. (A) There were no significant changes in CB1 expression. (B) Representative blots of CB1 expression. (C) Bar graph summarizing data. Data presented as mean of optical density ± S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.

TISSUE	CONTROL (n=5 - 6)	TREATED (n=7 - 8)	<i>p</i> VALUE
Detrusor	1 ± 0.12	1.96 ± 0.16	0.001*
Mucosa	1 ± 0.06	1.39 ± 0.07	0.001*
L5 DRG	1 ± 0.12	0.86 ± 0.06	0.28
L6/S1 DRG	1 ± 0.01	0.97 ± 0.13	0.86
L3/5 Spinal cord	1 ± 0.06	1.07 ± 0.07	0.47
L6/S Spinal cord	1 ± 0.03	1.10 ± 0.25	0.74

(A)

Figure 4.

Immunoblotting for CB2 receptor in rats with chronic cystitis. Intensity of bands for CB2 was normalized to that of beta-actin in the same sample. Average intensity of bands for control tissues was arbitrarily set at 1, and samples from treated animals were compared to controls. (A) CB2 expression was significantly increased in bladder mucosa and detrusor. (B) Representative blots of CB2 expression. (C) Bar graph summarizing data. Data presented as mean of optical density ± S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.

CB1

TISSUE	CONTROL (n=5 - 6)	TREATED (n=3 - 6)	p VALUE
Bladder	1 $\pm$ 0.38	0.93 $\pm$ 0.29	0.89
L6/S1 DRG	1 $\pm$ 0.29	0.87 $\pm$ 0.05	0.77
L6/S Spinal cord	1 $\pm$ 0.12	1.26 $\pm$ 0.34	0.48

CB2

TISSUE	CONTROL (n=3 - 6)	TREATED (n=2 - 6)	p VALUE
Bladder	1 $\pm$ 0.30	0.60 $\pm$ 0.15	0.25
L6/S1 DRG	1 $\pm$ 0.31	1.38 $\pm$ 1.06	0.68
L6/S Spinal cord	1 $\pm$ 0.18	1.23 $\pm$ 0.36	0.57

(A)

(B)

Figure 5.

CB1 and CB2 mRNA in rats with acute cystitis. Gene expression was normalized to abundance of mRNA for GAPDH in the same sample. Average abundance of mRNA from control tissues was arbitrarily set at 1. Relative changes in CB1 and CB2 mRNA in treated animals were compared to control. (A) There were no significant changes in abundance of CB1 and CB2 mRNA. (B) Bar graph summarizing data. Data presented as mean $\pm$ S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.

CB1

TISSUE	CONTROL (n=4 - 5)	TREATED (n=4 - 6)	p VALUE
Bladder	1 $\pm$ 0.3	0.20 $\pm$ 0.03	0.06
L6/S1 DRG	1 $\pm$ 0.15	0.72 $\pm$ 0.27	0.42
L6/S Spinal cord	1 $\pm$ 0.17	0.72 $\pm$ 0.19	0.33

CB2

TISSUE	CONTROL (n=4 - 5)	TREATED (n=4 - 6)	p VALUE
Bladder	1 $\pm$ 0.36	3.14 $\pm$ 0.70	0.03*
L6/S1 DRG	1 $\pm$ 0.31	0.77 $\pm$ 0.31	0.62
L6/S Spinal cord	1 $\pm$ 0.34	1.8 $\pm$ 0.81	0.35

(A)

(B)

Figure 6.

CB1 and CB2 mRNA in rats with chronic cystitis. Gene expression was normalized to abundance of mRNA for GAPDH in the same sample. Average abundance of mRNA from control tissues was arbitrarily set at 1. Relative changes in CB1 and CB2 mRNA in treated animals were compared to control. (A) There was a significant increase in abundance of CB2 mRNA in bladder. (B) Bar graph summarizing data. Data presented as mean $\pm$ S.E.M. DRG, dorsal root ganglia; SC, spinal cord; C, control; T, treated.