Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation

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Arms L, Girard BM, Malley SE, Vizzard MA. Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation. Am J Physiol Renal Physiol 305: F111–F122, 2013. First published April 17, 2013; doi:10.1152/ajprenal.00139.2013.—Chemokines are proinflammatory mediators of the immune response, and there is growing evidence for chemokine/receptor signaling involvement in prioncepcion. Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic pain syndrome characterized by pain, pressure, or discomfort perceived to be bladder-related with at least one urinary symptom. We have explored the expression and functional roles of CCL2 (monocyte chemoattractant protein-1) and its high-affinity receptor, CCR2, in micturition reflex function and somatic sensitivity in rats with urinary bladder inflammation induced by cyclophosphamide (CYP) treatment of varying duration (4 h, 48 h, chronic). Real-time quantitative RT-PCR, ELISAs, and immunohistochemistry demonstrated significant (P ≤ 0.01) increases in CCL2 and CCR2 expression in the urothelium and in Fast Blue-labeled bladder afferent neurons in lumbosacral dorsal root ganglia with CYP-induced cystitis. Intravesical infusion of RS504393 (5 μM), a specific CCR2 antagonist, reduced voiding frequency and increased bladder capacity and void volume in rats with CYP-induced cystitis (4 h), as determined with open outlet, conscious cystometry. In addition, CCR2 blockade, at the level of the urinary bladder, reduced referred somatic sensitivity of the hindpaw and pelvic region in rats with CYP treatment, as determined with von Frey filament testing. We provide evidence of functional roles for CCL2/CCR2 signaling at the level of the urinary bladder in reducing voiding frequency and somatic sensitivity following CYP-induced cystitis (4 h). These studies suggest that chemokines/receptors may be novel targets with therapeutic potential in the context of urinary bladder inflammation.

Chemokine/receptor signaling; conscious cystometry; von Frey filaments; urothelium

BLADDER PAIN SYNDROME (BPS)/Interstitial Cystitis (IC) is a chronic pain syndrome characterized by pain, pressure, or discomfort perceived to be bladder-related with at least one urinary symptom (28, 29). Although the etiology and pathogenesis of BPS/IC are unknown, numerous theories, including infection, inflammation, autoimmune disorder, toxic urinary agents, urothelial dysfunction, and neurogenic causes have been proposed (22, 34, 50, 54, 55). We have hypothesized that pain associated with BPS/IC involves an alteration of visceral sensation/bladder sensory physiology. BPS/IC patients have a lower threshold for sensing bladder volume and often experience pain at normal bladder volumes, suggesting altered sensory processing within the urinary tract and/or its innervation (24, 48). The majority of biopsies from BPS/IC patients reveal some degree of inflammation; therefore; inflammatory mediators, including, but not limited to, chemokines, may contribute to inflammation-induced changes, such as urinary bladder sensory dysfunction (55).

Chemokines, chemotactic cytokines, have well-established roles in the innate immune system, and they are also emerging as nociceptive mediators and contributors to neuron-glia communication (47, 52, 56, 75). Under physiological conditions, neuronal chemokine expression is limited; however, following mechanical injury or inflammation, chemokine expression increases in multiple cell types, including neurons, glia, macrophages, T-cells, and urothelium (8, 9, 43, 52, 65, 69, 76). Specifically, numerous studies suggest a role for the chemokine, CCL2 (monocyte chemoattractant protein-1, MCP), and its high-affinity receptor, chemokine (C-C motif) receptor 2 (CCR2), in hypersensitivity following neuronal inflammation or mechanical injury (1, 20, 25, 45, 51, 65, 67) in the central (i.e., spinal cord) and peripheral (i.e., dorsal root ganglia, DRG) nervous system. Blockade of CCR2 reduces established pain behaviors resulting from chronic nerve injury (20, 25, 51, 67) and exogenous application of CCL2, either centrally or peripherally, can elicit exaggerated sensory behavioral responses in rodents (20, 25, 51, 65, 67). In addition, CCR2-null mice fail to develop somatic sensitivity following partial sciatic nerve ligation (1), whereas mice with CCL2 overexpression in astrocytes develop exaggerated thermal hyperalgesia following complete Freund’s adjuvant-induced inflammation (45).

Importantly, BPS/IC patients demonstrate increased serum expression of chemokines (53). Additionally, recent rodent studies, including those by the Vizzard laboratory, have demonstrated chemokine expression, regulation, and function in the urinary bladder following cyclophosphamide (CYP)-induced bladder inflammation in rats (5, 79). CYP induces increased voiding frequency and somatic sensitization (15, 27, 59), and it is associated with neurochemical (70, 71, 73, 83), organizational (71, 74), and electrophysiological (33, 78) plasticity within the urinary bladder, bladder afferent neurons in lumbosacral DRG, and central micturition pathways. Potential mediators of urinary bladder inflammation and subsequent urinary bladder dysfunction and referred somatic sensitivity are numerous and include chemokines (5, 53, 68), cytokines (23, 41, 44), neuropeptides (14, 70), neuroactive compounds (13), and growth factors (72, 77, 82).

This study addresses the contribution of CCL2/CCR2 interactions in rat micturition reflexes and somatic (e.g., hindpaw, pelvic) sensitivity during control (no inflammation) or CYP-induced inflammation. We determined 1) expression of CCL2 and CCR2 in the urinary bladder and bladder primary afferents using immunohistochemistry, quantitative PCR (qRT-PCR), and ELISAs with CYP-induced cystitis of varying duration; and 2) the effects of CCR2 blockade with a highly selective CCR2 chemokine receptor antagonist, RS504393, on micturition reflex activity and somatic sensitivity in both CYP-treated
and control (no inflammation) rats using conscious cystometry and von Frey filament sensitivity testing, respectively.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (150–250 g), purchased from Charles River Canada (St. Constant, PQ, Canada), were housed two per cage and maintained in standard laboratory conditions with free access to food and water. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC no. 08-085). Animal care was under the supervision of the University of Vermont’s Office of Animal Care Management, in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

CYP-Induced Cystitis in Female Rats

Rats were anesthetized with isoflurane (2%) and received intraperitoneal injection(s) of CYP (Sigma Aldrich, St. Louis, MO) to produce urinary bladder inflammation. To induce chronic bladder inflammation, CYP was injected (150 mg/kg ip) with euthanasia occurring on the 8th day (5, 19, 38). Control rats had bladder inflammation, CYP was injected (75 mg/kg ip) every third day for 8 days with urinary bladder inflammation. To induce chronic bladder inflammation, CYP was injected (Sigma Aldrich, St. Louis, MO) to produce CYP-Induced Cystitis in Female Rats.

CCL2 and CCR2 Transcript Expression in Urinary Bladder: qRT-PCR

Bladders (n = 5 or 6 rats per group) were harvested, and the urothelium was separated from the detrusor. The bladder was cut open along the midline and pinned to a silicon-coated plate (Sygrid, Dow Corning, Midland, MI), the urothelium was removed with the aid of fine forceps and a dissecting microscope, and all tissues were snap-frozen on dry ice before processing, as previously described. Thus dissected, the urothelium also includes suburothelial structures; the term “urothelium” in this article refers to both urothelial and suburothelial structures. Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test “B”; Friendswood, TX), as previously described (5, 26). One to 2 μg of RNA per sample were used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20-μl final reaction volume. Amplification of cDNA was performed using oligonucleotide primers specific for CCL2, CCR2, or 18S (Table 1).

The qRT-PCR standards for all transcripts were prepared with the amplified CCL2, CCR2, and 18S cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the inserts were verified by automated fluorescent dyeoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically.

Real-time qRT-PCR was performed using SYBR Green I detection (5, 26). cDNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using the HotStart-IT SYBR Green qPCRMaster Mix (USB, Cleveland, OH) containing 5 mM MgCl2, 0.4 mM dATP, dGTP, dCTP, and dTTP, HotStart-IT Taq DNA polymerase, and 300 nM of each primer in a final 25-μl reaction volume. The real-time qRT-PCR was performed on an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) (5, 26) using the following standard conditions: 1) 94°C for 2 min; and 2) amplification over 40 cycles at 94°C for 15 s and 58–60°C depending on primer set for 30 s. The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by ramping the temperature of the reaction samples from 60 to 95°C. A single DNA melting profile was observed under these dissociation assay conditions, demonstrating amplification of a single unique product free of primer dimers or other anomalous products.

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. Data were analyzed at the termination of each assay using the Sequence Detection Software version 1.3.1 (Applied Biosystems, Norwalk, CT). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number, and the threshold cycle was determined by the software as the amplification cycle at which the ΔRn first intersects the established baseline. All data are expressed as the relative quantity of the gene of interest normalized to the relative quantity of reference gene (18S). Control samples were set equal to 100%.

Preparation of Tissue Samples for ELISAs

Rats from all experimental groups (control, 4 h, 48 h, and chronic; n = 6–8) were euthanized with isoflurane (4%), a thoracotomy was performed, and the urinary bladder was harvested. Individual bladders were immediately weighed and solubilized in tissue protein extraction agent (T-PER; Roche, Indianapolis, IN), a mild zwitterionic dialyzable detergent in 25 mM bicarbonate, 150 mM sodium chloride (pH 7.6), supplemented with a protease inhibitor mix (16 μg/ml benzamidine, 2 μg/ml leupeptin, 50 μg/ml lima bean trypsin inhibitor, and 2 μg/ml pepstatin A, Sigma-Aldrich), and aliquots were removed for protein assays (5, 79). Tissue was homogenized using a Polytron homogenizer, centrifuged (10,000 rpm for 10 min), and the resulting supernatant was used for CCL2 protein quantification. Total protein was determined using the Coomassie Plus protein assay reagent kit (Thermo Fischer Scientific, Rockford, IL). CCL2 was quantified using standard 96-well ELISA plates (R&D Systems, Minneapolis, MN), according to the manufacturer’s recommendations.

ELISAs for CCL2 in Urinary Bladder

Microtiter plates (R&D Systems) were coated with anti-CCL2 antibody. Sample and standard solutions were run in duplicate. Horseradish peroxidase-streptavidin conjugate was used to detect the antibody complex. Tetramethylbenzidine was the substrate, and the enzyme activity was measured by the change in optical density. The standards provided with these protocols generated a linear standard curve from 3.9–250 pg/ml (R2 = 0.998, P = 0.0001) for samples. The absorbance values of standards and samples were corrected by the subtraction of the background value (absorbance resulting from nonspecific binding). Samples were not diluted, and no samples fell below the detection limits of the assays.

Immunohistochemical Localization of CCR2 in Cryostat Sections of Urinary Bladder from Control and CYP-Treated Rats

The bladders were rapidly dissected from control and CYP-treated rats (n = 16; 4 each group), placed in 4% paraformaldehyde followed...
by overnight incubation in 30% sucrose in 0.1 M PBS for cryoprotection. Tissue was frozen in optimal cutting temperature compound, sectioned (20 μm) on a freezing cryostat, and mounted directly onto gelled (0.5%) microscope slides (5, 19). Sections were incubated overnight at room temperature in rabbit anti-CCR2 (1:2,000; Aviva Systems Biology, San Diego, CA) diluted in 1% goat serum and 0.1 M phosphate buffer. After overnight incubation, sections were washed (3 × 10 min) with 0.1 M PBS (pH 7.4). Sections were then incubated with goat anti-rabbit Cy3-conjugated secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature followed by washes (3 × 10 min) with PBS and coverslipping with antifade medium (Citifluor, Fisher Scientific, Pittsburgh, PA). Immunohistochemical controls included incubation of tissue sections with 1% goat serum and 0.1 M phosphate buffer alone (no primary antibody) or rabbit isotype control (1:500; Cell Signaling Technology, Danvers, MA), followed by normal washing and incubation with secondary antibodies to evaluate background staining levels. In the absence of primary antibody or in the presence of the isotype control, no positive immunostaining above background levels was observed. Control tissues that were incubated in the absence of secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of secondary antibody, no positive immunostaining was observed. Finally, immunosabsorptions with CCR2 peptide (5 μg/ml; Aviva Systems Biology) and antisera in urinary bladder sections resulted in no staining above background (data not shown).

Visualization and Semiquantitative Analyses of CCR2 in Urothelium and Detrusor Smooth Muscle

CCR2-immunoreactivity (IR) in bladder sections was visualized, and images were captured using an Olympus fluorescence microscope (Optical Analysis, Nashua, NH). The filter was set to an excitation range of 560–569 nm and an emission range of 610–655 nm for visualization of Cy3. Images were captured, acquired in tagged image file format (TIFF), and imported into image analysis software (MetaMorph, version 4.5r4; University Imaging, Pittsburgh, PA). Immunohistochemical controls included incubation of tissue sections with 1% goat serum and 0.1 M phosphate buffer alone (no primary antibody) or rabbit isotype control (1:500; Cell Signaling Technology, Danvers, MA), followed by normal washing and incubation with secondary antibodies to evaluate background staining levels. In the absence of primary antibody or in the presence of the isotype control, no positive immunostaining above background levels was observed. Control tissues that were incubated in the absence of secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of secondary antibody, no positive immunostaining was observed. Finally, immunosabsorptions with CCR2 peptide (5 μg/ml; Aviva Systems Biology) and antisera in urinary bladder sections resulted in no staining above background (data not shown).

Assessment of Immunohistochemical Staining in Urinary Bladder Regions

Immunohistochemistry and subsequent evaluation of CCR2-IR in bladder sections or whole mount preparations were performed on control and experimental tissues simultaneously to reduce the incidence of staining variation that can occur between tissues processed on different days. Staining in experimental tissue was compared with that in experiment-matched negative controls. Urinary bladder sections or whole mounts exhibiting immunoreactivity that was greater than background level in experiment-matched negative controls were considered positively stained.

Immunohistochemical Localization of CCR2 in Suburothelial Nerve Plexus in Urinary Bladder Whole Mounts

The urinary bladder was dissected rapidly and placed into oxygenated (95% O2 and 5% CO2) physiological saline solution (119.0 mmol NaCl, 4.7 mmol KCl, 24.0 mmol NaHCO₃, 1.2 mmol KH₂PO₄, 1.2 mmol MgSO₄·7H₂O, 11.0 mmol glucose) (38, 79). Starting at the urethra, a midline incision was made through the bladder, and then it was pinned flat onto a silicon-coated plate (Sylgard, Dow Corning, Midland, MI), maximally stretched, and then fixed in 2% paraformaldehyde + 0.2% picric acid for 1.5 h. After fixation, the urothelium was separated from the detrusor layer using fine-tip forceps, iris scissors, and a dissecting microscope, as previously described (38, 79). Notches were made in the region of the bladder neck to track orientation and assess regional immunoreactivity of the bladder. Urothelium and bladder musculature were processed for CCR2-IR, as described above. In some whole mount preparations processed for CCR2-IR, nerve fibers in the suburothelial nerve plexus were also stained with the pan-neuronal marker protein gene product (PGP9.5, 1: 3,000; AbD Serotec, Raleigh, NC) to determine potential expression of CCR2 in suburothelial nerve fibers and visualized with Cy2-conjugated species-specific secondary antibodies (1:200; Jackson ImmunoResearch Laboratories).

Visualization of CCR2-IR in Suburothelial Plexus in Bladder Whole Mounts

Whole mount tissues from control and experimental groups (n = 4 for control and CYP treatment groups) were examined using an Olympus fluorescence photomicroscope (Optical Analysis) with a multiband filter set for simultaneous visualization of the Cy3 and Cy2 fluorophores. Cy2 was viewed using a filter with an excitation range of 447–501 nm and an emission range from 510 to 540 nm.

Retrograde Labeling of Bladder Afferent Neurons in Lumbosacral DRG

Five to seven days prior to CYP injection or no treatment, Fast Blue (FB; 4%, wt/vol; Polysci, Gross-Ulmstadt, Germany) was injected into the bladder to retrogradely label bladder afferent neurons in lumbosacral (L1, L2, L6, S1) DRG in control (n = 5) and 4 h CYP-treated (n = 7) rats. As previously described (18, 39), a total volume of 40 μl divided into six to eight injections was injected into the dorsal surface of the bladder wall with particular care to avoid injections into the bladder lumen, major blood vessels, or overlying fascial layers. At each injection site, the needle was kept in place for several seconds after injection, and the site was washed with saline to minimize contamination of adjacent organs with FB. The 4 h CYP treatment group was selected for analysis of CCR2 expression in FB-labeled (presumptive bladder) afferent neurons and to determine the bladder function effects of pharmacological blockade of CCR2 in cystometry experiments (described below) because CCR2 transcript and protein was significantly increased in whole urinary bladder with acute (4 h) bladder inflammation. Furthermore, longer duration of CYP treatment did not result in additional increases in expression.

Immunohistochemical Localization of CCR2 in Lumbosacral Dorsal Root Ganglia

In control situations and after CYP treatment (4 h) (n = 5–7), animals were deeply anesthetized with isoflurane (3–4%) and then euthanized via thoracotomy. The lumbosacral DRG were quickly removed and postfixed in 4% paraformaldehyde for 12 h. Tissue was then placed overnight in sucrose (30%) in 0.1 M PBS for cryoprotection. DRG were identified on the basis of at least two criteria: 1) the T13 DRG are present after the last rib and 2) the L6 vertebra was the last moveable vertebra followed by the fused sacral vertebrae (18, 39). Another less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest DRG, L5 (18, 39). DRG
(L1, L2, L5-S1) were sectioned parasagitally at a thickness of 20 μm on a cryostat. Some DRG (L1, L2, L6, S1) were specifically chosen for analysis based upon the previously determined segmental representation of urinary bladder circuitry. The L5 DRG were used as internal controls because bladder afferents are not distributed within the L4-L5 DRG that contain only somatic afferents. Tissues from control animals (no CYP treatment) were handled in an identical manner to that described.

**CCR2-Immunoreactivity in DRG**

DRG sections from both control and 4 h CYP-treated rats were processed for CCR2-immunoreactivity (IR) using an on-slide processing technique using methodology described above with some minor modifications. Tissues from control and experimental animals were processed simultaneously to decrease the possible incidence of variation in staining and background between tissues and between animals. DRG sections were incubated overnight at room temperature with rabbit polyclonal anti-CCR2 antibody (1:1,000; Aviva Systems Biology) in 1% goat serum and 0.1 M KPBS (phosphate buffer with rabbit polyclonal anti-CCR2 antibody (1:1,000; Aviva Systems Biology) and antisera in DRG sections resulted in no staining above background (data not shown). Repeated attempts to localize CCL2-IR in cryostat bladder and DRG sections and in urinary bladder afferent neurons with several different antibodies and substantial trouble-shooting were not successful. Thus, data are not presented for immunohistochemical localization of CCL2-IR in urinary bladder or DRG.

**Data Analysis of CCR2- and FB-Labeled DRG**

Tissues were examined under an Olympus fluorescence photomicroscope for visualization of Cy3 and FB. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range of 610–655 nm. In DRG from control and 4 h CYP-treated rats, CCR2-immunoreactive cell profiles were counted in 6–8 sections of each selected DRG (L1, L2, L5-S1) (18, 39). Only cell profiles with a nucleus were quantified in a blinded fashion (18, 39). DRG sections with FB-labeled cells were viewed with a filter with an excitation wavelength of 340–380 nm and an emission wavelength of 420 nm. Cells labeled with FB and expressing CCR2-IR were similarly counted. Numbers of CCR2-immunoreactive cell profiles per DRG section are presented (means ± SE). The percentage of presumptive bladder afferent cells (FB-labeled) expressing CCR2-IR in each DRG examined is also presented (means ± SE). The results are not corrected for double counting.

**Intravesical Catheter Implant**

A lower midline abdominal incision was performed under general anesthesia with 2–3% isoflurane using aseptic surgical techniques (5, 6, 15, 31, 40). The end of polyethylene tubing (PE-50; Clay Adams, Parsippany, NJ) was flared with heat, inserted into the dome of the bladder, and secured in place with a 6–0 nylon purse-string suture (5, 6, 15, 31, 40). The distal end of the tubing was sealed and tunneled subcutaneously to the back of the neck where it was externalized, out of the animal’s reach (5, 6, 15, 31, 40). Rats received buprenorphine (0.05 mg/kg sc) starting at the time of surgery and then every 8–12 h postoperatively for a total of four doses. Animals were maintained for 96 h after surgery before conscious cystometry was initiated to ensure complete recovery and clearance of postoperative analgesics.

**Open Voiding, Conscious Cystometry With Continuous Intravesical Infusion of Saline and CCR2 Antagonist**

The effects of CCR2 receptor blockade on bladder function in control (no inflammation; n = 6) and CYP-treated rats (4 h; n = 6) were evaluated with intravesical infusion of RS504393 (5 μM; Tocris, Bristol, United Kingdom), a highly specific CCR2 receptor antagonist. Unrestrained and conscious rats were placed in a recording cage over a scale and pan to collect and measure voided urine. To elicit repetitive bladder contractions using conscious cystometry, continuous intravesical infusion of room temperature saline was infused at a constant rate (10 ml/h). At least six reproducible micturition cycles were recorded after an initial stabilization period (15–30 min). Intravesical pressure changes were recorded using a Small Animal Cystometry System (Med Associates, St. Albans, VT) (5, 6, 15, 19, 40). Filling pressure, pressure threshold for voiding, maximal voiding pressure, and intercontraction interval were evaluated. Nonvoiding bladder contractions (NVCs), defined as rhythmic intravesical pressure increases 7 cm H2O above baseline without the release of fluid from the urethra, were also determined per voiding cycle. Bladder capacity was measured as the amount of saline infused into the bladder at the time when micturition commenced (12, 30).

To evaluate the effects of CCR2 blockade on bladder function, rats were anesthetized (1–2% isoflurane) and saline or RS504393 (5 μM), a highly specific CCR2 receptor antagonist (7, 37, 81), was intravesically infused for 30 min. Prior to intravesical drug infusion, the bladder was manually emptied using the Credé maneuver. Bladders were then infused with ~300 μL·mL⁻¹ (a volume less than bladder capacity to not elicit a bladder contraction and expulsion of instillate) of vehicle (1% DMSO in saline; Sigma-Aldrich), or RS504393 (5 μM), according to prior published studies (5, 6, 15, 40). Rats remained anesthetized (1–2% isoflurane) during infusion (30 min) to subdue the micturition reflex and prevent expulsion of drug from the bladder. To avoid potential variation resulting from circadian rhythms, experiments were conducted at similar times of the day (21).

At the conclusion of the study, rats were euthanized as described above. Micturition function before and after vehicle or RS504393 intravesical instillation was evaluated in the same rats (control and 4-h CYP-induced cystitis). A variety of routes of administration and concentrations of RS504393 have been used in diverse applications (7, 37, 81) but was also determined empirically in pilot studies. In pilot studies, a range of RS504393 concentrations (1, 5, and 10 μM) were evaluated. The lowest concentration evaluated was without effect; 5 μM produced effects described in the current study, and 10 μM RS504393 produced results comparable to but not greater than those observed with 5 μM. Thus, the effects of 5 μM RS504393 on bladder function and somatic sensitivity are presented in this study.

**Mechanical Sensitivity Testing**

Mechanical sensitivity testing was performed in separate groups (n = 8–10 each; hindpaw, pelvic region) of rats not used for bladder function determination. Referred (secondary) hyperalgesia and tactile allodynia were tested using calibrated von Frey hairs with forces of 0.1–4 g applied to the hindpaw or pelvic region. Rats were tested in individual Plexiglas chambers with a stainless-steel wire grid floor. Rats were acclimated to the chambers for a period of 2 h (15, 57). The von Frey hairs were applied in an up-down method for 1–3 s with an inter-stimulus interval of 15 s. For pelvic region stimulation, stimulation was confined to the lower abdominal area overlying the urinary bladder. Testing of the plantar region of the hindpaw and lower abdominal area was performed by percutaneous application of von Frey hairs to the indicated areas until the hair bent slightly. The
following behaviors were considered positive responses to pelvic region stimulation: sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area (15, 57). A positive response to hindpaw stimulation was sharp withdrawal of the paw or licking of the tested hindpaw (15, 57). Somatic sensitivity before and after vehicle or RS504393 intravesical instillation was evaluated in the same rats (control and 4 h CYP-induced cystitis). All somatic testing was performed in a blinded manner with respect to treatment. The groups were decoded after data analysis.

Exclusion Criteria

Rats were removed from the study when adverse events occurred that included: 20% reduction in body weight postsurgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or hematuria in control rodents (5, 6, 15, 19, 40). In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements, such as grooming, standing, walking, and defecation rendered bladder pressure recordings unusable during these events.

Materials

RS504393 was prepared as a stock solution in DMSO, aliquoted and stored at −20°C until use. Aliquots were diluted with saline to achieve final concentration.

Figure Preparation

Digital images were obtained using a charge-coupled device camera (MagnaFire SP, Optronics; Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus fluorescence microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and CYP-treated groups processed and analyzed on the same day. Images were imported into a graphics-editing program (Adobe Photoshop, version 8.0, Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

Statistical Analyses

All values represent means ± SE. Data were compared using ANOVA. Percentage data were arcsine transformed to meet the requirements of this statistical test. Cystometry data were compared using repeated-measures ANOVA, where each animal served as its own control. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F ratios exceeded the critical value (P ≤ 0.05), the Newman-Keul’s or Dunnett’s post hoc tests were used to compare group means.

RESULTS

CCL2 Protein Expression in the Whole Urinary Bladder with CYP-Induced Cystitis

CCL2 protein expression in the whole urinary bladder increased significantly (P ≤ 0.01) compared with control urinary bladders (no CYP) with all durations of CYP treatment evaluated as determined with ELISAs (Fig. 1). CCL2 expression in the urinary bladder was greatest (9.5-fold) with 4 h of CYP treatment. With 4 h of CYP treatment, CCL2 expression in urinary bladder was significantly (P ≤ 0.01) greater than that with 48 h or chronic CYP treatment. CCL2 protein expression with 48 h of CYP treatment was significantly (P ≤ 0.01) greater than protein expression with chronic CYP treatment (Fig. 1).

Fig. 1. CCL2 protein expression is increased in whole urinary bladders following cyclophosphamide (CYP) treatment of varying duration, as determined with ELISAs. CCL2 protein expression increased significantly (*P ≤ 0.01) with each time point examined compared with control urinary bladders (no CYP). Sample sizes are n = 6–8 for each group.

CCL2 and CCR2 mRNA Transcript Levels With and Without CYP-Induced Cystitis

Real-time qRT-PCR experiments demonstrated significant increases (P ≤ 0.01) in CCL2 mRNA transcript expression in both the urothelium and detrusor smooth muscle with acute (4 h) and intermediate (48 h) CYP treatment, but not chronic CYP-induced bladder inflammation (Fig. 2A). Increases in CCL2 mRNA levels in urothelium and detrusor were most robust with 4 h of CYP treatment, and these levels were significantly (P ≤ 0.01) higher than those detected with 48 h of CYP treatment (Fig. 2A). CCL2 mRNA expression in urothelium and detrusor returned to control expression with chronic CYP treatment.

CCR2 mRNA transcript expression also significantly (P < 0.01) increased in the urothelium and detrusor with acute (4 h) and intermediate (48 h) CYP treatment. CCR2 mRNA expression increased significantly (P ≤ 0.01) in the urothelium with 4 h of CYP treatment only, whereas CCR2 mRNA levels increased significantly (P ≤ 0.01) in the detrusor with both 4 h and 48 h CYP treatment (Fig. 2B). CCR2 mRNA expression in urothelium and detrusor returned to control levels with chronic CYP treatment.

CCR2-IR in Urinary Bladder With Acute (4 h) CYP-Induced Cystitis

Low expression of CCR2-IR was present in the urothelium of control bladders (no CYP treatment) (Fig. 3, A and C). CCR2-IR increased significantly (P ≤ 0.01) in the urothelium in all layers (basal, intermediate, apical) with 4 h of CYP treatment in both cryostat urinary bladder sections (Fig. 3, B, E, and F) and whole mount preparations (Fig. 3D). Low expression of CCR2-IR was also present in the detrusor smooth muscle of control rats, but expression was not altered with CYP treatment of any duration examined (4 h, 48 h, chronic) (data not shown). CCR2-IR was not observed in whole mount preparations of the suburothelial nerve plexus in control or CYP-treated rats (data not shown).

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was a significant ($P \leq 0.01$) increase in the percentage of bladder afferent cells that expressed CCR2-IR in L1 (70.2% ± 4.0) (Fig. 4C), L2 (69.2% ± 3.9), L6 (78.3% ± 12.2) (Fig. 4D), and S1 (75.3% ± 7.8) DRG. No differences were observed in the number of CCR2-IR cells in DRG examined between control and control with FB injection or between CYP-treated and CYP-treated with FB injection (data not shown).

**CCR2 Receptor Blockade With Intravesical Infusion of RS504393 Using Conscious Cystometry in Rats With or Without CYP-Induced Cystitis (4 h)**

**Control (no inflammation).** Conscious cystometry was performed in control rats before intravesical infusion of RS504393 (5 μM), a CCR2 antagonist, to establish baseline voiding frequency, bladder capacity, and void volume (data not shown). Drug treatment with RS504393 did not change bladder capacity, as measured as the amount of saline infused in the bladder at the time when micturition commenced, void frequency or threshold, filling or peak micturition pressures (data not shown) in control rats. Intravesical infusion of vehicle in control rats was also without effect on the cystometric parameters evaluated.

**4-h CYP treatment.** Conscious cystometry was performed in rats with 4-h CYP treatment before intravesical infusion of RS504393 to establish baseline voiding frequency, bladder capacity, and void volume associated with acute (4 h) CYP treatment (Fig. 5A). As previously demonstrated (5, 14, 15, 31, 71), CYP treatment (4 h) increased void frequency and decreased bladder capacity (3.4-fold), intercontraction interval (3.5-fold), and void volume (3.2-fold) compared with control rats (no CYP treatment). Following intravesical infusion of the CCR2 receptor antagonist, RS504393 (5 μM), the same CYP-treated rats exhibited decreased voiding frequency and significantly ($P \leq 0.01$) increased bladder capacity (as measured as the amount of saline infused in the bladder at the time when micturition commenced), intercontraction interval, and void volume (Figs. 5B and 6, A and B). There were no changes in threshold, baseline, or maximum micturition pressures following intravesical instillation with the CCR2 receptor infusion in 4-h CYP-treated rats (Fig. 6C). Intravesical infusion of vehicle in CYP-treated rats was also without effect on the cystometric parameters evaluated, consistent with previous studies (6, 40). NVCs (increases in baseline pressure with an amplitude ≥7 cmH$_2$O without the expulsion of urine) were infrequently observed in the evaluated rats with CYP treatment. Therefore, any effects of the CCR2 receptor antagonist on NVCs were not evaluated.

**Hindpaw and Pelvic Somatic Sensitivity With CYP-Induced Cystitis and Effects of CCR2 Antagonist, RS504393**

Somatic sensitivity in the hindpaw was significantly ($P \leq 0.01$) increased with the monofilament forces tested (0.1 to 4 g) following 4 h of CYP treatment compared with control rats (Fig. 7A), as previously described (15). Similarly, pelvic somatic sensitivity was also significantly ($P \leq 0.01$) increased following 4-h CYP treatment at all monofilament forces evaluated (0.1 to 4 g) (Fig. 7B). Intravesical infusion of RS504393 (5 μM) significantly ($P \leq 0.01$) decreased the somatic sensitivity in the hindpaw (Fig. 7A) and pelvic region (Fig. 7B) in

**CCR2-Immunoreactivity (IR) Is Increased in Lumbosacral DRG Cells With 4-h CYP-Induced Cystitis**

CCR2 expression was observed in the cytoplasm of DRG cells in all levels examined (L1-L2; L5-S1) and DRG cells exhibiting CCR2-IR was similar (8–15 cells/section) across DRG levels examined (Fig. 4A). The number of cells that expressed CCR2-IR (Fig. 4A) increased (17–45 cells/section) significantly ($P \leq 0.01$) in L1, L2, L6, and S1 DRG examined after 4 h CYP. CCR2 expression was significantly ($P \leq 0.01$) greater in L6 DRG with 4 h CYP treatment compared with L1 or L2 DRG with 4 h CYP treatment (Fig. 4A). CCR2-IR was observed primarily in small-diameter DRG cells (19.6 ± 2.4 μm), but some larger DRG cells (24.3 ± 3.2 μm) also exhibited CCR2-IR. No changes in CCR2-IR were observed in L5 DRG (Fig. 4A).

**CCR2-IR in Fast Blue-Labeled Bladder Afferent Cells**

To determine whether CCR2-IR was expressed in bladder afferent cells in DRG from control rats or from those treated with CYP (4 h), FB was injected into the urinary bladder to retrogradely label bladder afferent cells in the L1, L2, L6, and S1 DRG (Fig. 4, C and D). In control animals (Fig. 4B), 25.2–27.4% of bladder afferent cells expressed CCR2-IR in L1, L2, L6 and S1 DRG. After 4 h CYP (Fig. 4, B–D), there

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Fig. 2. CCL2 and CCR2 mRNA transcript expression is increased in urothelium and detrusor smooth muscle following CYP treatment of varying duration as determined by quantitative PCR (qRT-PCR). A: CCL2 mRNA levels increased significantly (**$P \leq 0.01$** with 4 h and 48 h CYP treatment in both the urothelium and detrusor smooth muscle. CCL2 mRNA levels with 4-h CYP treatment were significantly (**$P \leq 0.01$**) higher than those detected at the 48-h time point. B: CCR2 mRNA levels were significantly (**$P \leq 0.01$**) increased in the urothelium with 4 h of CYP treatment, and these levels were significantly (**$P \leq 0.01$**) higher than those detected at the 48 h and chronic time points. In the detrusor smooth muscle, CCR2 mRNA levels increased significantly with 4 h and 48 h CYP treatment. Sample sizes are $n = 5$ or 6 for each group.
rats with CYP-induced cystitis (4 h). Intravesical infusion of RS504393 in control rats (no CYP treatment) produced no change in somatic sensitivity in the hindpaw or pelvic region (data not shown).

**DISCUSSION**

Models of neuropathic pain, neuronal injury, and tissue inflammation have demonstrated nociceptive roles for chemokine/receptor signaling, particularly for CCL2 and its high-affinity receptor, CCR2. The present studies demonstrate novel findings with respect to the contribution of CCL2/CCR2 interactions with bladder inflammation-induced changes in bladder function and somatic sensitivity in female rats. We demonstrate that CYP-induced cystitis increases: 1) CCL2 and CCR2 transcript and protein expression in the rat urinary bladder and 2) the number of bladder-associated CCR2-immunoreactive bladder afferent cells in the lumbosacral DRG. Blockade of CCR2 receptor interactions with the highly selective receptor antagonist RS504393 (5 μM) at the level of the urinary bladder, increased bladder capacity and decreased void frequency and reduced somatic sensitivity of the hindpaw and pelvic region following CYP treatment. Our laboratory and others have previously demonstrated chemokine/receptor expression (CX3CL1/CX3CR1; CXCL12/CXCR4) (5, 68, 79) and function (CXCL12/CXCR4) (5) associated with urinary bladder inflammation. These results extend previous findings (20, 65, 76, 80, 81) by demonstrating that CCL2/CCR2 interactions contribute to inflammation-induced bladder dysfunction and increased referred somatic sensitivity. These studies provide additional support for chemokines/receptors as potential lower urinary tract targets with therapeutic potential in the context of urinary bladder inflammation.

BPS/IC is viewed as one type of chronic pain syndrome characterized by pain, pressure, or discomfort perceived to be bladder related with at least one urinary symptom, such as urinary frequency (22, 32, 34, 49, 50, 55). We hypothesize that pain associated with BPS/IC involves alteration of visceral sensation/bladder sensory physiology. Altered visceral sensation from the bladder (i.e., pain at low or moderate bladder filling) that accompanies BPS/IC may be mediated by many factors, including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (alldynia). These changes may be mediated, in part, by inflammatory changes in the bladder. Potential mediators of bladder inflammation are numerous and include chemokines (5, 53, 68, 79), cytokines (23, 41, 44), neuropeptides (14, 70), neuroactive compounds (13), and growth factors (72, 77, 82). Interestingly, elevated chemokine levels have been detected in the serum from patients with BPS/IC (53);
therefore, further investigation of chemokine/receptor signaling related to bladder dysfunction is warranted.

Our overall hypothesis is that inflammation-induced changes within the sensory limb (e.g., bladder afferent cells, urothelium) of the micturition reflex pathway can lead to bladder dysfunction. Changes within the afferent limb can occur at peripheral sites such as the urinary bladder and bladder-associated DRG neurons, as well as at central locations, including spinal cord and supraspinal regions. The present studies provide evidence of functional roles for CCL2/CCR2 signaling at the level of the urinary bladder in reducing voiding frequency and somatic sensitivity following CYP-induced cystitis (4 h). Immunohistochemical experiments...
detected robust increases of CCR2-IR in CYP-induced cystitis (4 h) in the urothelium of urinary bladders and a relative increase in the overall percentage of CCR2-positive bladder-associated DRG neurons. CCR2 blockade in the urinary bladder with intravesical infusion of RS504393 (5 μM) reduced void frequency and increased bladder capacity and void volume in rats with CYP-induced cystitis (4 h). In addition, CCR2 blockade, at the level of the urinary bladder, reduced somatic sensitivity of the hindpaw and pelvic region in rats treated with CYP. CCL2/CCR2 is another chemokine/receptor pair (after CXCL12/CXCR4) now identified to have a functional contribution to urinary bladder function and referred somatic sensitivity during inflammation-induced bladder hyperreflexia.

CCL2/CCR2 interactions at the level of the urothelium and suburothelial nerve plexus in the urinary bladder are likely to contribute to bladder dysfunction and increased somatic sensitivity following CYP-induced cystitis. Intravesical instillation of RS504393 likely makes direct contact with the urothelium that expresses CCR2 and the increased urothelial permeability due to CYP treatment makes it likely that intravesical RS504393 also contacts suburothelial nerves. In the present study, we did not observe CCR2-IR in the suburothelial nerve plexus in whole mount preparations of the urinary bladder from control or CYP-treated rats, but basal as well as increased CCR2-IR was present in bladder afferent cell bodies in the L1, L2, L6, and S1 DRG. The reasons for the failure to demonstrate CCR2-IR in the peripheral terminals of bladder afferent cells are not known but may be a reflection of low expression in the terminals, difficulty in visualizing CCR2 expression in small structures or lack of distal transport. These results are consistent with previous studies examining different chemokine/receptor (CXCL12/CXCR4; CX3CL1/CX3CR1) expression in micturition reflex pathways with and without bladder inflammation (5, 79). Additionally, focal nerve demyelination induces a change in CCR2-IR in associated DRG, but neuronal CCL2-IR remained unchanged at the lesion site (8). CCL2/CCR2 interactions may influence nucleus-bound processes, such as inflammatory gene expression, more than peripheral branch processes, such as neurotransmitter release and/or excitability (36). Ex vivo studies examining the impact of chemokine/receptor activation and blockade on proinflammatory gene
expression and neuronal excitability in DRG cells are necessary to clarify this point.

Recent evidence suggests that urothelial cells have “neuron-like” properties, including sensory, transduction, and signaling capabilities (2, 4, 10, 11, 61–64). Urothelial cells share a number of similarities with sensory neurons, including the expression of receptors, including purinergic, norepinephrine, acetylcholine, neuropeptide- and protease-activated receptors, acid-sensing ion channels, neurotrophin receptors, and transient receptor potential channels. Current and prior studies demonstrate chemokine receptor expression (2, 10, 11, 17, 46, 61–64). The current studies do not differentiate between direct urothelial or nerve-mediated CCR2 effects vs. indirect urothelial-mediated communication with the detrusor smooth muscle, suburothelial nerve plexus and/or interstitial cells as previously suggested (2, 10). It is possible that urothelial CCL2/CCR2 signaling facilitates the release of urothelial-derived mediators, such as ATP or nitric oxide, which may then influence underlying structures, such as the suburothelial nerve plexus and/or detrusor smooth muscle (2, 10, 11). These possibilities need further investigation.

Alternatively, or in addition to urothelial-mediated mechanisms, CCL2/CCR2 interactions in bladder-associated DRG neurons may contribute to inflammatory induced changes in bladder sensory physiology and function. Numerous studies suggest a role for the chemokines CCL2 and CCR2 in hyper-sensitivity following neuronal inflammation or injury (1, 20, 25, 45, 51, 65, 67) in the peripheral nervous system. In the present study, CYP treatment triggered a robust increase in the percentage of bladder afferent cell bodies expressing CCR2-IR. These results complement previous findings demonstrating an increase in the percentage of primary sensory afferent cells expressing CCL2 and/or CCR2 following focal nerve demyelination, sciatric nerve ligation, or chronic constriction injury (8, 36, 42, 65, 76, 80). Jung and Miller (35) demonstrate that depolarization of cultured sensory neurons is sufficient to induce CCR2 mRNA expression, suggesting that heightened sensory neuron activity during states of injury or inflammation may contribute to elevated levels of neuronal CCR2 expression. Increased receptor expression may explain why peripheral nerve damage or inflammation can also change the functional properties of sensory neuron populations, such that an increasing percentage of DRG neurons respond to CCL2 application or neurons respond with increased intracellular calcium ion currents and/or frequency of excitatory postsynaptic currents (8, 25, 42, 51, 60, 76). Therefore, it is possible that CCL2 released, in vivo, by DRG neurons, glial cells, and urothelial cells could contribute to nociceptive sensations/behaviors by autocrine or paracrine signaling mechanisms. Exogenous application of CCL2, either intrathecally or in peripheral tissues, or genetic overexpression of CCL2 elicits heightened sensory behaviors in rodents (20, 25, 45, 51, 65, 67). In contrast, CCR2 antagonists attenuate established pain behaviors following nerve injury, and CCR2-null mice fail to develop certain neuropathic pain behaviors after sciatic nerve ligation (1, 20, 25, 51, 67).

Blockade of chemokine/receptor signaling may represent a potential therapeutic target for inflammation-associated bladder dysfunction. In addition, the presence of certain chemokines and other inflammatory molecules in a patient’s urine may be useful biomarkers for BPS/IC or other bladder dysfunction syndromes, such as overactive bladder (OAB). Similar to BPS/IC, the etiology of OAB remains elusive; however, on the basis of patient biopsies, an inflammatory contribution has been suggested (3, 16, 58). Tyagi et al. (66) detected a 10-fold increase of CCL2 and the soluble fraction of the CD40 ligand (CD40L) in the urine of OAB patients vs. controls. Various cytokines, epidermal growth factor, and the oncogene GRO-2 were also elevated (3–5-fold) in the urine of OAB patients (66). Whether certain chemokine/receptor interactions and downstream signaling pathways are redundant or unique across diverse bladder dysfunction or pelvic pain syndromes remains to be determined. Identification of urinary biomarkers in BPS/IC, OAB, or other bladder dysfunction would improve diagnostic strategies and reduce invasiveness to the patient, improving exclusionary criteria, reducing time to diagnosis and aid in patient selection for pharmacological trials.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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