COX-2 and prostanoid expression in micturition pathways after cyclophosphamide-induced cystitis in the rat

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Hu, V. Y., S. Malley, A. Dattilio, J. B. Folsom, P. Zvara, and M. A. Vizzard. COX-2 and prostanoid expression in micturition pathways after cyclophosphamide-induced cystitis in the rat. Am J Physiol Regul Integr Comp Physiol 284: R574–R585, 2003. First published October 10, 2002; 10.1152/ajpregu.00465.2002.—The purpose of this study was to determine the role of cyclooxygenase-2 (COX-2) and its metabolites in altered micturition reflexes. Bladders were harvested from euthanized female rats for analyses. Conscious cystometry was used to assess the effects of a COX-2-specific inhibitor, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone (DFU, 5 mg/kg sc), a disubstituted furanone, in CYP-induced cystitis. COX-2 mRNA was increased in inflamed bladders after acute (12-fold) and chronic (9-fold) treatment. COX-2 protein expression in inflamed bladders paralleled COX-2 mRNA expression. Prostaglandin D2-methoxime expression in the bladder was significantly \( P \leq 0.01 \) increased in acute (3-fold) and chronic (5.5-fold) cystitis. Prostaglandin E2 was significantly \( P \leq 0.01 \) increased (2-fold) in the bladder with intermediate (1.7-fold) and chronic (2.6-fold) cystitis. COX-2-immunoreactive cell profiles were distributed throughout the inflamed bladder and coexpressed histamine immunoreactivity. Conscious cystometry in rats treated with CYP + DFU showed increased micturition intervals 4 and 48 h after CYP treatment and decreased intravesical pressures during filling and micturition compared with rats treated with CYP + vehicle. These studies suggest an involvement of urinary bladder COX-2 and its metabolites in altered micturition reflexes with CYP-induced cystitis.

inflammation; prostaglandins; 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone; mast cells; cystometry

CHRONIC PATHOLOGICAL CONDITIONS, inducing tissue irritation or inflammation, can alter the properties of sensory pathways leading to a reduction in pain threshold (alldynia) and an amplification of painful sensations (hyperalgesia) (7, 8, 14). Increased pain sensitivity can result from changes in peripheral nociceptor afferents (13, 23, 43, 45, 76) or changes in the central nervous system mechanisms that process nociceptive inputs (9, 10, 34, 41). Recent experiments involving a chemically [cyclophosphamide (CYP)] induced urinary bladder inflammation have demonstrated alterations in neurochemical (69–72), organizational (69), and electrophysiological (22, 77) properties of micturition reflex pathways. These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in the properties of micturition reflex pathways after CYP-induced cystitis. In addition, CYP-induced cystitis is being explored as a model of visceral pain to gain an understanding of painful lower urinary tract syndromes (6, 29).

Possible mechanisms underlying the neural plasticity after chronic CYP-induced cystitis (22, 69–72, 77) may involve alterations in neurotrophic factors (NTFs), cytokines (42), and/or neural activity arising in the urinary bladder (68). The presence of proinflammatory cytokines, growth factors, and lipopolysaccharide can induce cyclooxygenase-2 (COX-2), an inflammatory early response gene (46). Prostaglandins generated by the COX-2 enzyme are also mediators of altered neuronal activity in inflamed tissues and have been demonstrated to stimulate the micturition reflex, possibly through activation of capsacin-sensitive bladder afferents (2, 37). Prostanoids have been suggested to play a physiological role in contributing to the basal tone of the detrusor and modulating activity of bladder nerves (2, 37).

The concept that target organs can influence the neurons that innervate them is widely accepted and readily demonstrated during embryonic or postnatal development (16, 26, 32, 44, 57, 65, 67). Recent studies from several laboratories have demonstrated the influence of target organ-neuron interactions in the adult animal (15, 58–61, 63, 64). Biochemical studies have suggested a role for nerve growth factor (NGF) in mediating some aspects of bladder afferent neuron plasticity after partial urethral obstruction (15, 58, 59, 64). Recent studies from this laboratory have demonstrated changes in the mRNA expression of a number of NTFs and cytokines in the bladder after CYP-induced cystitis (68). In addition, we have also demonstrated changes in receptor tyrosine kinase A and B,

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the receptors that mediate the effects of NGF- and brain-derived NTF, respectively, in bladder afferent neurons in dorsal root ganglia cells after CYP-induced cystitis (49). In this study, we hypothesize that CYP-induced cystitis upregulates COX-2 and prostanooids in the urinary bladder, which contributes to altered urodynamics function. Studies have also hypothesized that COX-2 and prostaglandin synthesis [prostaglandin E2 (PGE2) and D2 (PGD2)] in the spinal cord may contribute to maintenance of hypersensitivity with peripheral inflammation (36, 66, 75).

The overall aims of the present studies were to 1) measure urinary bladder COX-2 and PGE2 and PGD2 expression after acute (4 h), intermediate (48 h), and chronic (10 day) CYP treatment, 2) determine the cellular localization of COX-2 protein in the urinary bladder with CYP treatment, and 3) determine the effects of a specific COX-2 inhibitor, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(SH)-furanone (DFU) (50, 56), on urodynamics after CYP treatment.

**METHODS**

Experiments were performed on adult female Wistar rats (150–250 g). Rats were housed two per cage and given food and water ad libitum. Sawdust bedding in rodent cages was changed twice weekly. The animal room was maintained at a constant temperature of 22°C and a 12:12-h alternating light-dark cycle (light phase 0700–1900). All experiments were conducted according to the guidelines for animal care and research involving animals (1). Cystometry data were obtained during the light period. All experiments were conducted between 1000 and 1800.

**CYP-induced cystitis: acute, intermediate, or chronic.**

Chemical cystitis was induced by CYP, which is metabolized of a specific COX-2 inhibitor, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(SH)-furanone (DFU) (50, 56), on urodynamics after CYP treatment.

**Preparation of immunoassay samples.** Adult rats were euthanized as described above, and the bladder (n = 4–6 for each time point and control) was rapidly dissected and weighed. Individual bladders were solubilized in tissue protein extraction reagent (Pierce, Rockford, IL; 1 g tissue/20 ml) with 1 mM EDTA and 100 μM ibuprofen according to the manufacturer’s specifications. Bladder tissue was disrupted with a Polytron homogenizer until homogeneous, extracted with acetone, and then centrifuged (10,000 rpm for 10 min). The supernatants were used for PGE2 and PGD2-methoxime (PGD2-Mox) quantification. PGD2 was converted to a stable methoxime derivative according to the manufacturer’s specifications to prevent further degradation. Total protein was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce).

**Principle of the competitive enzyme immunoassay.** For competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI), microtiter plates (R & D Systems) were precoated with rabbit IgG mouse monoclonal antibody. After addition of the tracer (acetylcholinesterase, conjugate) and the sample or standard solution, the second antibody (polyclonal) was applied. Sample and standard solutions were run in duplicate. Ellman’s reagent, containing the acetylcholinesterase substrate, was then added. The intensity of the color change, determined spectrophotometrically, is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of free prostaglandin. The PGE2 standard provided with this system generated a linear standard curve from 7.8 to 1,000 pg/ml (r² = 0.932, P ≤ 0.001). The PGD2-Mox standard provided with this system generated a linear standard curve from 7.8 to 1,000 pg/ml (r² = 0.976, P ≤ 0.001). The absorbance values of standards and samples were corrected by subtraction of the background value (absorbance due to nonspecific binding). Samples were diluted [1:50 (PGE2)-1:100 (PGD2-Mox)] to bring the absorbance values onto the linear portion of the standard curve. No samples fell below the minimum detection limits of the assay. Curve fitting of standards and evaluation of prostaglandin content of samples was performed using a least-squares fit.

**COX-2 Western blot analysis.** Whole rat bladders were homogenized in tissue protein extraction reagent with Complete protease inhibitor tablets (Roche, Indianapolis, IN)
using a Polytron homogenizer. After low-speed centrifugation to remove debris, aliquots of the homogenates were removed for protein assay using the Coomassie Plus Protein Assay Reagent Kit (Pierce). Homogenized bladders were stored at −80°C until use. COX-2 controls (5 and 10 ng) and samples (40 μg) were suspended in sample buffer for fractionation on 10% Tris-glycine gels and subjected to SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane; efficiency of transfer was evaluated using Ponceau S reagent in 0.05% trichloroacetic acid. Membranes were blocked overnight (with shaking at 4°C) in Tris-buffered saline + 0.05% Tween (TBST), 5% nonfat dry milk, and 3% bovine serum albumin. After they were rinsed (3 times for 10 min each) in TBST, membranes were incubated in rabbit polyclonal anti-murine COX-2 antibody (1:1,000; Cayman Chemical) for 2 h at room temperature and then rinsed three times (10 min each with shaking) in TBST. Washed membranes were then incubated in horseradish peroxidae-conjugated goat anti-rabbit antibody IgG (1:5,000 in TBST; Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature for enhanced chemiluminescence detection (Pierce). The blot was exposed to Biomax film (Kodak) and developed. For data analysis, the intensity of each band corresponding to COX-2 was analyzed by semiquantitative image analysis using Un-Scan It software (Silk Scientific, Orem, UT). Background intensities were subtracted from bands of interest.

**Immunochemistry.** Adult rats were euthanized as described above, and the bladder (n = 6 for each time point and control) was rapidly dissected and weighed. Sagittal sections of the bladder wall (20 μm) extending from the bladder dome to trigone from control and experimental treatments (acute, intermediate, and chronic CYP-induced cystitis) were examined for COX-2 immunoreactivity. The tissue was postfixed in 4% paraformaldehyde, placed in ascending concentrations of sucrose (10–30%) in 0.1 M PBS for cryoprotection, sectioned (20 μm) on a freezing cryostat, and directly mounted on gelled (0.5%) microscope slides for on-slide processing, as previously described (67a). Briefly, sections were incubated overnight at room temperature or for 72 h at 4°C with rabbit or goat anti-COX-2 polyclonal antibody (Table 1) in 1% serum and 0.1 M potassium PBS (KPBS) and then washed (3 times for 10 min each) with 0.1 M KPBS, pH 7.4. The tissues were then incubated with Cy2- or Cy3-conjugated species-specific secondary antibodies for 2 h at room temperature. After they were washed (3 times for 10 min each with KPBS), the slides were coverslipped with Citifluor. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

To determine the cellular localization of COX-2 protein in the urinary bladder, some bladder sections were also immunostained with antibodies against ED-1, myeloperoxidase (MPO), or histamine (Table 1). The monoclonal antibody ED-1 recognizes a cytoplasmic antigen (CD86) associated with phagolysosomes present in monocytes, most tissue macrophages, and some dendritic cell subpopulations (12). MPO is a naturally occurring enzyme contained in the primary granules of polymorphonuclear (PMN) cell infiltrates. Increased urinary levels of histamine and tryptase have been demonstrated in interstitial cystitis (IC) (47), suggesting a role for mast cells in this syndrome. Thus histamine immunoreactivity was used to identify mast cells. For double-staining procedures, urinary bladder sections were processed simultaneously for COX-2 immunoreactivity and ED-1, MPO, or histamine immunoreactivity (Table 1). We alternated the use of Cy3- and Cy2-conjugated secondary antibodies for each antibody examined; no differences in immunostaining were observed with different secondary antibodies. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

**Assessment of positively stained cells.** Staining in experimental tissue was compared with staining in experimental negative controls. Control rats and rats treated with COX-2 immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained. Positively stained cells were not further divided into categories of different staining intensities. Positively stained cells were identified by individuals blinded to the identity of experimental or control treatment groups. The severity of urinary bladder inflammation was graded according to the scoring scale proposed by Saban et al. (54). The urinary bladder from experimental and control groups was scored separately for COX-2, ED-1, MPO, and histamine immunoreactivity.

**Data analysis.** Six to 10 urinary bladder sections from control and experimental groups were examined under an Olympus fluorescence photomicroscope with a multiband filter set for simultaneous visualization of Cy3 and Cy2 fluorochromes. Cy2 was visualized by using a filter with an excitation range of 447–501 nm and an emission range of 510–540 nm; Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range of 560–655 nm.

**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 microscope (Optical Analysis). Exposure times were held constant when images were acquired from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 6.0.1 (Adobe Systems, San Jose, CA), where groups of images were assembled and labeled.

**Intravesical catheter placement.** A lower midline abdominal incision was performed under general anesthesia with ketamine (60 mg/kg ip)-xylazine (10 mg/kg ip), and polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) with the end flared by heat was inserted into the dome of the bladder and secured in place with a 6-0 nylon purse-string suture (78). The distal end of the tubing was sealed, tunneled subcutaneously, and externalized at the back of the neck, out of the animal’s reach. Abdominal and neck incisions were closed with 4-0 nylon sutures. Animals were maintained for 72 h after surgery to ensure complete recovery.

**Cystometry.** Control rats and rats treated with CYP (acute, 4 h or intermediate, and 48 h) and also treated with a specific COX-2 inhibitor, DFU, or vehicle (98% ethanol) were evaluated with cystometry. We chose not to evaluate the effects of DFU or vehicle in animals treated chronically with CYP, because daily treatment with DFU or vehicle for 10 days

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Rabbit anti-COX-2</td>
<td>1:1000</td>
<td>Cayman (Ann Arbor, MI)</td>
</tr>
<tr>
<td>Goat anti-COX-2</td>
<td>1:1000</td>
<td>Santa Cruz (Santa Cruz, CA)</td>
</tr>
<tr>
<td>Mouse anti-ED-1</td>
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<tr>
<td>Rabbit anti-histamine</td>
<td>1:400</td>
<td>Accurate (Westbury, NY)</td>
</tr>
<tr>
<td>Goat anti-MPO</td>
<td>1:1000</td>
<td>Santa Cruz (Santa Cruz, CA)</td>
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</tbody>
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**R576 COX-2 AND PROSTANOIDS IN CYP-INDUCED CYSTITIS**  

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resulted in unacceptable (>20%) weight loss as well as large areas of necroses from repeated drug or vehicle injections in pilot studies. For conscious cystometry, a rat was placed unrestrained in a cage. Before the start of the recording, the bladder was emptied of urine by syringe aspiration or drained by gravity, and the catheter was connected via a T tube to a pressure transducer (model PT300, Grass, West Warwick, RI) and microinjection pump (model 222, Harvard Apparatus, South Natick, MA). We infused a 0.9% saline solution at room temperature into the bladder at a rate of 10 ml/h. Intravesical pressure was recorded continuously using a Neurodata Acquisition System (Grass model 15, Astro-Med, West Warwick, RI) (78). At least four reproducible micturition cycles were recorded after the initial stabilization period of 25–30 min. The following cystometric parameters were recorded in each animal: filling pressure (pressure at the beginning of the bladder filling), threshold pressure (bladder pressure immediately before micturition), micturition pressure (the maximal bladder pressure during micturition), micturition interval (time between micturition events), void volume, and presence or absence of nonvoiding bladder contractions (NVCs). For the present study, NVCs were defined as increases in bladder pressure of ≥7 cmH2O without release of urine. Cystometry records were evaluated for the presence or absence of NVCs. The number of rats exhibiting NVCs per total number of animals was expressed as a ratio (Table 2). At the end of the evaluation, rats treated acutely (4 h) with CYP were returned to their cages so that they could be evaluated 48 h after CYP treatment with or without DFU. At the conclusion of the experiment (48 h after CYP treatment), the animal was euthanized (2% isoflurane anesthesia). Urodynamic assessment (micturition, threshold, and micturition, pressure) was performed 4 and 48 h after CYP administration in the same rats. The concentration of DFU used as a specific COX-2 inhibitor was based on previous studies using DFU to inhibit colonic (35) or hindpaw inflammation (50).

**Drug treatment.** The effects of a specific COX-2 inhibitor, DFU (5 mg/kg sc; Merck Frosst, Quebec, Canada) (50, 56), or vehicle (98% ethanol sc) on lower urinary tract function in rats treated with CYP (4 and 48 h) were evaluated. One day before CYP injection (150 mg/kg ip) and for each day thereafter, rats were injected with DFU (n = 4) or vehicle (n = 4) under isoflurane anesthesia (2%). Urodynamic assessment was performed 4 and 48 h after CYP administration in the same rats. The concentration of DFU used as a specific COX-2 inhibitor was based on previous studies using DFU to inhibit colonic (35) or hindpaw inflammation (50).

**Statistics.** Values are means ± SE. Comparisons of COX-2 mRNA levels or prostaglandin protein concentration in urinary bladder samples after acute (4 h), intermediate (48 h), or chronic (10 days) CYP-induced cystitis were made using analysis of variance. Animals, processed and analyzed on the same day, were tested as a block in the analysis of variance. Thus day was treated as a blocking effect in the model. Two variables were tested in the analysis: 1) experimental manipulation vs. control situation and 2) the effect of day (i.e., tissue from groups (experimental and control) of animals were processed on different days). Comparisons of urodynamic parameters between control and experimental treatments were made using Fisher’s exact test. Comparisons of histological severity between control and experimental treatments were made using Wilcoxon’s rank sum test. When F ratios exceeded the critical value (P≤ 0.05), Dunnett’s post hoc test was used to compare the control mean with each experimental mean.

## RESULTS

**COX-2 mRNA and protein expression in urinary bladder with CYP-induced cystitis.** Acute (4 h) CYP-induced cystitis significantly (P≤ 0.01) increased (12-fold) COX-2 mRNA in the urinary bladder (Fig. 1). Similarly, chronic (10 day) CYP-induced cystitis significantly (P ≤ 0.01) increased (9.4-fold) COX-2 mRNA in the urinary bladder (Fig. 1). In contrast, 48 h after initial CYP treatment, there was a trend toward COX-2 mRNA increasing in the urinary bladder (4-fold). COX-2 protein expression in urinary bladders also increased (2- to 3-fold) with acute and chronic CYP treatment (6- to 15-fold; Fig. 2). In contrast to the modest increase in COX-2 mRNA in the urinary bladder 48 h after CYP treatment, COX-2 protein was dramatically increased (15- to 40-fold) in the urinary bladder (Fig. 2).

**Prostaglandin expression in urinary bladder with CYP-induced cystitis.** PGD2-Mox protein expression in the urinary bladder significantly (P ≤ 0.01) increased with acute (4 h, 3-fold) and chronic (10 day, 5.5-fold) CYP-induced cystitis (Fig. 3). In contrast, no change in PGD2-Mox expression was detected in the urinary bladder 48 h after initial CYP treatment (Fig. 3). PGE2 protein expression in the urinary bladder was not changed with acute CYP treatment but was significantly (P≤ 0.01) increased with intermediate (48 h, 1.7-fold) or chronic (10 day, 2.6-fold) CYP-induced cystitis (Fig. 4).

**Urodynamic effects of selective COX-2 inhibitor, DFU, in rats with CYP-induced cystitis.** CYP treatment in the rat (31, 40) resulted in bladder hyperactivity with increases in filling, threshold, and micturition pressures and micturition frequency (Table 2). In conscious rats treated 4 h before the cystometry with CYP, DFU treatment resulted in a significant (P ≤ 0.01) reduction in the filling, threshold, and micturition pressures (Table 2, Fig. 5). There were also signif-

<table>
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<tr>
<th>Group</th>
<th>Bladder Weight, g</th>
<th>Fill Pressure, cmH2O</th>
<th>Threshold Pressure, cmH2O</th>
<th>Micturition Interval, s</th>
<th>Micturition Pressure, cmH2O</th>
<th>NVC</th>
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<tr>
<td>Control</td>
<td>0.31 ± 0.07</td>
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<td>724.0 ± 19.1</td>
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<td>CYP (4 h) + vehicle</td>
<td>30.0 ± 1.9</td>
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<td>94.6 ± 3.8</td>
<td>98.8 ± 4.4</td>
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<td>CYP (4 h) + DFU</td>
<td>17.9 ± 0.8</td>
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<td>82.4 ± 2.9</td>
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</tr>
<tr>
<td>CYP (48 h) + vehicle</td>
<td>34.6 ± 0.4</td>
<td>34.7 ± 1.1</td>
<td>128.3 ± 5.5</td>
<td>117.0 ± 2.7</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>CYP (48 h) + DFU</td>
<td>18.1 ± 0.5</td>
<td>22.5 ± 0.8</td>
<td>428.3 ± 43.4</td>
<td>89.9 ± 1.8</td>
<td>3/4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. CYP, cyclophosphamide (150 mg/kg ip); DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2-(5 H)-furanone (5 mg/kg sc); NVC, nonvoiding contractions (expressed as ratio of number of animals exhibiting NVCs per total number of animals). *P < 0.01 vs. vehicle; †P < 0.01 vs. control.
icant ($P \leq 0.01$) increases in the micturition interval (3.3-fold) and void volume (2.5-fold) compared with vehicle-treated rats (Table 2, Fig. 5). No change was observed in the number of NVCs observed during the testing period with DFU treatment (Table 2). In conscious rats treated 48 h before cystometry with CYP, DFU also significantly ($P \leq 0.01$) reduced the filling, threshold, and micturition pressures and increased the micturition interval (3.3-fold) and void volume (3-fold) compared with rats treated with vehicle (Table 2, Fig. 6). A greater frequency of NVCs was observed in rats treated with CYP (48 h) and DFU than in vehicle-treated rats (Table 2). DFU treatment significantly ($P \leq 0.05$) decreased the number and distribution of COX-2-immunoreactive cell profiles in the urinary bladder (Table 3).

**COX-2 immunoreactivity in urinary bladder and colocalization with histamine-immunoreactive cell profiles: CYP-induced cystitis.** After acute (4 h), intermediate (48 h), or chronic (10 day) administration of CYP, bladder weight significantly increased ($P \leq 0.01$) compared with that of control animals: 250 ±15 mg (4 h CYP), 337 ± 20 mg (48 h CYP), and 370 ± 10 mg (chronic CYP) vs. 84 ± 12 mg (control). As previously demonstrated (68, 71, 72) and confirmed in this study, gross microscopic analysis of bladders from animals treated with CYP 4 or 48 h before examination showed a few, scattered regions of mucosal erosion on the luminal surface (Table 3). Chronic (10 day) administration of CYP increased the severity of the bladder changes, resulting in more extensive regions of mucosal erosion, ulcerations, edema, and, in some instances, petechial hemorrhages (Table 3). Histological changes evident after chronic CYP treatment included edema of the lamina propria and plasma cell infiltrates in the lamina propria, submucosa, and perivascular tissue (Table 3). We previously demonstrated (69) that some of these cellular infiltrates include macrophages as detected with an ED-1 antibody that recognizes an unidentified cytoplasmic antigen, unique to all phagocytic cells of monocyte/macrophage origin (51) and PMNs, as shown by significant increases in MPO activity (69). In the present studies, the number and distribution of ED-1-, MPO-, and histamine-immunoreactive cell profiles increased with the duration of CYP treatment (chronic > intermediate > acute; Fig. 7, Table 3).

In urinary bladder from control animals, COX-2-immunoreactive cell profiles were not observed (Fig. 7). After acute (4 h), intermediate (48 h), or chronic (10 day) administration of CYP, the number and distribu-
tion of COX-2-immunoreactive cell profiles increased in all regions of the urinary bladder examined (Fig. 7, Table 3). In animals treated with CYP (4 or 48 h) and DFU, the number and distribution of COX-2-immunoreactive cell profiles were dramatically reduced compared with urinary bladders from vehicle-treated rats (Fig. 7). To determine the cellular localization of COX-2 protein in the urinary bladder with CYP treatment, urinary bladder sections were double stained with antibodies to identify macrophage (ED-1-immunoreactive), neutrophils (MPO-immunoreactive), or mast cell (histamine-immunoreactive) profiles (Table 1). These cellular infiltrates are present in the inflamed urinary bladder (53, 69). In agreement with previous studies (69), numerous ED-1- and MPO-immunoreactive cell profiles were present in the urinary bladder after CYP treatment (Table 3); however, ED-1- or MPO-immunoreactive cell profiles did not coexpress COX-2 immunoreactivity. Histamine-immunoreactive cell profiles were also widely distributed in the urinary bladder after CYP treatment (Table 3), and virtually all histamine-immunoreactive cell profiles examined also expressed COX-2 immunoreactivity (Fig. 8).

DISCUSSION

These studies examined the contribution of the inducible enzyme COX-2 and its metabolites PGE$_2$ and PGD$_2$-Mox to changes in lower urinary tract function after acute (4 h), intermediate (48 h), or chronic (10 day) CYP-induced cystitis.
day) CYP-induced cystitis. Increases in urinary bladder COX-2 mRNA and PGD2-Mox expression were bi-phasic (4 h and 10 days), whereas no changes were detected 48 h after initial CYP treatment. In contrast, PGE2 and COX-2 protein expression generally increased with the duration of CYP-induced cystitis. In agreement with previous studies, CYP treatment in rats resulted in bladder hyperactivity, with an increase in bladder pressure and micturition frequency. Treatment with a selective COX-2 inhibitor, DFU (5 mg/kg sc), reversed or minimized these changes. COX-2-immunoreactive cell profiles were distributed throughout the urinary bladder after CYP-induced cystitis, and DFU treatment reduced the number and distribution

Fig. 5. Cystometrogram recordings in control rats (A) and rats treated for 4 h with CYP + vehicle (B) and CYP + 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone (DFU, 5 mg/kg sc; C).

Fig. 6. Cystometrogram recordings in rats treated for 48 hours with CYP + vehicle (A) and CYP + DFU (5 mg/kg sc; B).
of these cell profiles. COX-2-immunoreactive cell profiles in the inflamed urinary bladder coexpressed histamine immunoreactivity but failed to express ED-1 or MPO immunoreactivity. This suggests that mast cells in the inflamed bladder are the cellular source of COX-2.

Chemical cystitis was induced in female Wistar rats by CYP, which is metabolized to acrolein, an irritant eliminated in the urine (11, 33). Within the urinary tract, the urinary bladder is the organ most affected by the toxic actions of CYP because of its reservoir function and the longer exposure to the toxic metabolite acrolein (11, 33, 62). The most common urological complication associated with CYP treatment in humans is cystitis, with or without hemorrhage (11, 33, 62, 73). The histological findings from the present study as well as previous studies (22, 72, 77) of animals treated with CYP further demonstrate the damage caused by CYP to the urinary bladder. Recent studies (69) have also demonstrated extensive monocyte/macrophage infiltration into the inflamed urinary bladder as well as a significant increase in MPO activity. Therefore, chronic CYP treatment represents a noxious, chemical irritation of the bladder mucosa. In addition, the re-

Table 3. Histological severity of CYP-induced cystitis

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>COX-2-IR</th>
<th>ED-1-IR</th>
<th>MPO-IR</th>
<th>Histamine-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.17 ± 0.17</td>
<td>1.17 ± 0.17</td>
<td>1.17 ± 0.17</td>
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<tr>
<td>Acute (4 h) CYP</td>
<td>6</td>
<td>2.17 ± 0.17*</td>
<td>2.17 ± 0.17*</td>
<td>2.67 ± 0.21*</td>
<td>2.67 ± 0.21*</td>
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<tr>
<td>Intermediate (48 h) CYP</td>
<td>6</td>
<td>2.50 ± 0.22*</td>
<td>2.50 ± 0.22*</td>
<td>2.67 ± 0.21*</td>
<td>2.67 ± 0.21*</td>
</tr>
<tr>
<td>Chronic (10 day) CYP</td>
<td>6</td>
<td>2.83 ± 0.17*</td>
<td>2.83 ± 0.17*</td>
<td>2.83 ± 0.17*</td>
<td>2.83 ± 0.17*</td>
</tr>
<tr>
<td>CYP (48 h) + DFU</td>
<td>5</td>
<td>1.50 ± 0.29*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The histological severity of CYP-induced cystitis was graded by a score of 0–3. Values are means ± SE expressed as scores on a scale of 1–3: 1, mild (low number of inflammatory cell infiltrates in the lamina propria and little or no interstitial edema); 2, moderate (infiltration of moderate numbers of inflammatory cell infiltrates in the lamina propria and moderate interstitial edema); 3, severe (diffuse presence of large numbers of inflammatory cell infiltrates in the lamina propria and severe interstitial edema). IR, immunoreactivity; ND, not determined. *P < 0.05.
Results of the present studies together with our previous studies (42, 68, 69) demonstrate that chronic CYP treatment generates a more pronounced inflammatory response than acute (4 h) or intermediate (48 h) CYP treatment, evidenced by histological observations and diffuse inflammatory cell infiltration.

IC is a chronic inflammatory bladder disease syndrome characterized by urinary frequency, urgency, and suprapubic and pelvic pain. Although the etiology and pathogenesis of IC are unknown, numerous theories, including infection, autoimmune disorder, toxic urinary agents, deficiency in bladder wall lining, and neurogenic causes, have been proposed (18, 19, 52, 55). CYP-induced cystitis results in a dramatic reorganization of micturition reflex circuitry that is characterized by changes in neurochemical, electrophysiological, and organizational properties (22, 69–72, 77). These changes suggest considerable reorganization of reflex connections in the spinal cord and bladder afferents after bladder inflammation. Previous studies have demonstrated alterations in NTF and cytokine mRNA and/or protein after acute (4 h), intermediate (48 h), and chronic (10 day) CYP-induced cystitis. Inflammation-induced changes in NTFs, cytokines, and/or neural activity arising in the bladder (68) may mediate changes in the micturition reflex. In addition, an involvement of COX-2 and prostanoids in cystitis (30, 74) and postoperative ileus (56) has been suggested.

Urinary bladder inflammation and/or hypertrophy may change neural activity arising in the urinary bladder. Lecci et al. (30) suggested that prostanoids are key mediators after the induction of CYP-induced cystitis (48 h). The present studies expand on these results by demonstrating upregulation of COX-2 mRNA, protein, and prostaglandin (PGE2 and PGD2-Mox) expression in the urinary bladder with acute, intermediate, and chronic CYP-induced cystitis. In addition, the present study demonstrates improvement in bladder function with administration of a specific COX-2 inhibitor, DFU. This study suggests that COX-2 expression in urinary bladder mast cells contributes to altered lower urinary tract function with CYP-induced cystitis. Nu-
numerous studies have suggested an involvement of mast cells in IC (4, 17, 48). Recent studies with mast cell-deficient mice have delineated changes in gene expression during allergic cystitis dependent on the presence of mast cells (53). The results of the present study further suggest a role for mast cells in contributing to lower urinary tract dysfunction with CYP-induced cystitis. The influence of target organ-neuron interactions during embryonic and postnatal development is well established. Recent experiments from several laboratories including our own have demonstrated the influence of the target organ on neuron interactions in the adult animal (15, 16, 28, 58, 60, 61, 64). Partial urethral obstruction leads to increased resistance to urine flow and, in turn, to increased bladder work and, ultimately, to bladder hypertrophy. This is also accompanied by hypertrophy of afferent neurons in the L2-S1 dorsal respiratory ganglia and postganglionic efferent neurons in the major pelvic ganglia (15, 59). The hypertrophied bladder exhibits markedly increased levels of NGF, and autoimmunization against NGF reduces the major pelvic ganglia neuronal hypertrophy (15, 59, 61). This suggests that neurotrophin(s) released in the hypertrophied bladder is partly responsible for the change in neuronal morphology. A recent study from this laboratory demonstrated changes in NTF expression in the urinary bladder with cystitis, including β-NGF, brain-derived NTF, glial-derived NTF, and neurotrophin-3 and -4 (68). Thus a variety of NTFs may contribute to neuroplasticity of micturition reflexes after cystitis. The present studies add prostaglandins, upregulated in the urinary bladder with CYP-induced cystitis, to the list of mediators that may contribute to altered micturition reflexes with cystitis. In addition to target organ influences on lower urinary tract function, there is a growing body of literature indicating that the hormonal milieu can contribute to CYP-induced cystitis and may affect the etiology of the disease process (5, 27) as well as the micturition threshold (24).

Changes in COX-2 mRNA and PGD2-Mox expression in the urinary bladder were biphasic, with acute (4 h) and chronic (10 day) CYP-induced cystitis resulting in significant upregulation. In contrast, COX-2 protein and PGE2 expression in the urinary bladder generally increased with the severity of the inflammation (chronic > intermediate > acute). Previous studies with a rodent model of postoperative ileus have similarly demonstrated a biphasic response of COX-2 mRNA and have suggested that distinct cellular sources of COX-2 underlie this observation (56). In contrast, in the present study, COX-2-immunoreactive cell profiles were colabeled with histamine immunoreactivity at all time points examined (acute, intermediate, and chronic) with induction of CYP-induced cystitis.

In agreement with previous studies (31, 40), CYP treatment in rats resulted in bladder hyperactivity with an increase in bladder threshold pressure to induce a micturition contraction. Pretreatment and daily treatment with a selective COX-2 inhibitor, DFU, reversed or minimized these changes. These urodynamic changes may be mediated by the actions of prostaglandins on urinary bladder smooth muscle (21, 38) or urethra and may also involve an action on urinary bladder nerves, located in close proximity to the urothelium (3). Prostaglandins stimulate the micturition reflex after systemic or direct application of prostaglandins on the mucosal or serosal bladder surface (21, 38). This action is most likely mediated by capsaicin-sensitive bladder afferents, because pretreatment with capsaicin or with tachykinin receptor antagonists blocks this stimulation (2, 37). It has previously been suggested that prostaglandins play physiological role(s) in lower urinary tract function. Prostaglandins released by urinary bladder distension during filling may regulate the threshold for activating the micturition reflex through activation of capsaicin-sensitive bladder afferent nerves (2, 37, 39). The present results additionally suggest that prostaglandins synthesized in the urinary bladder after induction of CYP-induced cystitis may play a pathophysiological role. In the context of bladder inflammation, prostaglandins may also activate or sensitize bladder afferents, thereby changing the micturition reflex threshold. Thus some changes in lower urinary tract function with CYP-induced cystitis may be explained, at least in part, by changes in urinary bladder prostanoid production. In the present study, changes in lower urinary tract function induced by CYP-induced cystitis were reduced or eliminated by treatment with a COX-2 specific inhibitor, DFU.

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REFERENCES


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51. Rinaman L, Card JP, and Enquist LW. Spatiotemporal responses of astrocytes, ramified microglia, and brain macro-


