Expression of corticotropin-releasing factor and CRF receptors in micturition pathways after cyclophosphamide-induced cystitis

Jennifer LaBerge, Susan E. Malley, Katarina Zvarova, and Margaret A. Vizzard

Departments of Anatomy and Neurobiology and Neurology, University of Vermont College of Medicine, Burlington, Vermont

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LaBerge, Jennifer, Susan E. Malley, Katarina Zvarova, and Margaret A. Vizzard. Expression of corticotropin-releasing factor and CRF receptors in micturition pathways after cyclophosphamide-induced cystitis. Am J Physiol Regul Integr Comp Physiol 291: R692–R703, 2006.—Corticotropin-releasing factor (CRF) is a prominent neuropeptide involved in micturition reflexes, and different roles in these reflexes have been suggested. These studies examined the expression of CRF in the urinary bladder and lumbosacral sacral parasympathetic nucleus (SPN) in response to cyclophosphamide (CYP)-induced cystitis (4 h, 48 h, or chronic) in rats. The expression of CRF receptors, CRF1 and CRF2, was examined in urinary bladder from control and CYP-treated rats. Urinary bladder and lumbosacral spinal cord were harvested from rats killed by isoflurane (4%) and thoracotomy. CRF protein expression in whole urinary bladders significantly (P ≤ 0.01) increased with 48 h or chronic CYP treatment. CRF immunoreactivity (IR) was increased significantly (P ≤ 0.01) in the urothelium and SPN after CYP treatment. CRF IR nerve fibers increased in density in the suburothelial plexus and detrusor smooth muscle whole mounts with CYP-induced cystitis. CRF2 receptor transcript was expressed in the urothelium or detrusor smooth muscle, and CRF2 receptor expression increased in whole bladder with CYP-treatment, whereas no CRF1 receptor transcript was expressed in either urothelium or detrusor. Immunohistochemical studies demonstrated CRF2 IR in urinary bladder nerve fibers and urothelial cells from control animals, whereas no CRF1 IR was observed. These studies demonstrated changes in the expression of CRF in urinary bladder and SPN region with CYP-induced cystitis and CRF receptor (CRF2) expression in nerve fibers and urothelium in control rats. CRF may contribute to urinary bladder overactivity and altered sensory processing with CYP-induced cystitis.

inflammation; sacral parasympathetic nucleus; urothelium; enzyme-linked immunosorbent assay

THE CHEMICALLY (cyclophosphamide, CYP) induced bladder inflammation model is associated with alterations in neurochemical (57, 61), electrophysiological (66), organizational (62), and functional properties (14) of micturition pathways. These changes may be mediated by chemical mediators (e.g., neuropeptides, cytokines, neurotrophins) produced in the bladder, spinal cord, or dorsal root ganglia with cystitis (5, 27, 57, 59, 61).

Corticotropin-releasing factor (CRF) is of particular relevance in the rat, since it is present in descending projections from the pontine micturition center or more specifically from Barrington’s nucleus to the sacral parasympathetic nucleus (SPN; see Refs. 30, 31, 49, 56). Historically, Barrington’s nucleus has been viewed as the supraspinal switching center that regulates storage and elimination of urine (25, 33, 41). Recent studies have led to the suggestion that Barrington’s nucleus may contain neurons that control a broad range of pelvic organ functions (28, 29, 34, 38, 55). CRF is prominently expressed in the descending pathway from Barrington’s nucleus to the SPN in the lumbosacral spinal cord, and prominent CRF immunoreactivity (IR) is expressed in the SPN of adult rats (37, 53, 54). Our recent studies have demonstrated an age-dependent upregulation of CRF IR in the SPN region and specifically in association with preganglionic parasympathetic neurons (48). CRF-immunoreactive varicosities on or closely apposed to preganglionic parasympathetic cell bodies or proximal neurites also increased with postnatal age (48). We suggested that upregulation of CRF IR in bulbospinal projections to the SPN may contribute to mature voiding reflexes (48). Opposing roles for CRF in mature bladder function have been proposed (19, 34).

Patients with interstitial cystitis (IC), a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency, and suprapubic and pelvic pain (35), and an involvement of C-fibers has been suggested (8). The overall hypothesis of this work is that CYP-induced cystitis results in changes in CRF expression in the urinary bladder or SPN region, contributing to bladder overactivity and changes in sensory processing. As initial steps in addressing this hypothesis, we determined: 1) CRF protein expression in the urothelium by enzyme-linked immunoassays (ELISAs) after CYP-induced cystitis of varying duration; 2) cellular expression of CRF and CRF receptors (CRF1, CRF2) in urinary bladder of control rats or after CYP-induced cystitis using immunohistochemistry with an emphasis on urothelial cell and nerve fiber expression; 3) CRF1 receptor and CRF2 receptor transcript expression in urinary bladder; 4) CRF2 receptor expression in whole urinary bladder with CYP-treatment using Western blotting techniques; and 5) intensity of CRF IR in the urothelium and SPN after CYP-induced cystitis using semiquantitative image analysis.

MATERIALS AND METHODS

Adult female Wistar rats (150–250 g) were purchased from Charles River Canada (St. Constant, Canada). Chemicals used in these studies were purchased from Sigma ImmunoChemicals (St. Louis, MO). Primary antibodies for immunohistochemistry are described in Table 1. Secondary antibodies for immunohistochemistry were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA; Table 1).

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Immunohistochemistry; WB, Western blotting; HRP, horseradish peroxidase.

CYP-induced Cystitis

Acute and chronic CYP-induced cystitis rat models were examined in these studies. For chronic CYP (Sigma)-induced cystitis studies, rats received drug injection (75 mg/kg ip) every 3rd day for 10 days. For acute CYP-induced cystitis studies, rats received a single injection (150 mg/kg ip) and survived for 4 or 48 h. Control rats received volume-matched injections of saline (0.9% ip) or no treatment. All injections were performed under isoflurane (2%) anesthesia. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (no. 03– 030; 06 – 014). 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.

Tissue Harvesting, Sample Preparation, and Assay

Procedures for tissue processing and ELISAs were identical to those described in detail previously. Adult rats were killed with isoflurane anesthesia (4%) and thoracotomy, and the bladder (n = 8–10 for each group) was rapidly dissected and weighed. Individual bladders were solubilized in T-PER Tissue Protein Extraction Reagent (1 g tissue/20 ml; Pierce, Rockford, IL) with Complete (protease inhibitor cocktail tablets; Roche Diagnostics). Bladder tissue was disrupted with a Polytron homogenizer and then centrifuged (10,000 revolutions/min for 5 min). Total protein was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce). The supernatant was used for CRF quantification using commercially available rat speciﬁc CRF ELISA kits (Peninsula Laboratories, member of the Bachem group, San Carlos, CA) in accordance with the manufacturer’s instructions.

Rat CRF ELISA

Rabbit anti-rat CRF antibody was adsorbed to microtiter (R&D Systems, Minneapolis, MN) plates. After addition of the sample or standard solution, the second antibody (detection antibody) was applied. Sample and standard solutions were run in duplicate. This antibody complex was detected with a horseradish peroxidase-labeled immunoglobulin. Enzyme activity was quantified by the change in optical density, using tetramethyl benzidine as substrate. The CRF antibody complex was detected with a horseradish peroxidase-labeled immunoglobulin. Enzyme activity was quantified by the change in optical density, using tetramethyl benzidine as substrate. The CRF content of samples were performed using a least-squares fit.

Immunohistochemistry

Urinary bladder and lumbosacral spinal cord sections. Adult rats were killed as described in Tissue Harvesting, Sample Preparation, and Assay, and the bladder (n = 6 for each time point and control) was rapidly dissected and weighed. The spinal cord (cervical to sacral) was removed and pinned on a Sylgard dish and segmented. Spinal segments (L6–S1) were identified and placed in paraformaldehyde (4%) for 24 h. Spinal segments (L6–S1) were identified and then placed in sucrose (20%) for 24 h cryoprotection and then sectioned (20 µm) in the transverse plane using a cryostat. Spinal cord segments were identified based on at least two criteria as follows: 1) the T13 dorsal root ganglion (DRG) exits after the last rib and 2) the L4 vertebra is the last moveable vertebrae followed by the fused sacral vertebrae. Segmentation was later confirmed by immunostaining the autonomic nucleus in the L6–S1 spinal segments with neuronal nitric oxide synthase (nNOS) IR (64). Our previous work (64) demonstrated that all nNOS IR cells in the rat SPN were retrogradely labeled from the major pelvic ganglion and therefore represented preganglionic para-sympathetic neurons. Only spinal segments exhibiting nNOS IR in the autonomic nucleus were subsequently used in analysis, and all rats in the present study exhibited nNOS-immunoreactive cell bodies in the L6–S1 autonomic nucleus. A 1:4 series of spinal cord sections throughout the rostral-caudal extent of L6–S1 spinal segments were immunostained for CRF IR. Comparisons of CRF IR among different experimental groups were made from spinal cord sections matched to the same rostral-caudal location in the L6–S1 spinal segments. Sections of the bladder wall (20 µm) from control and experimental treatments (acute, intermediate, chronic CYP-induced cystitis) were examined for CRF IR. Groups of experimental animals were processed simultaneously to decrease the incidence of variation in staining and background that can occur between animals and on different processing days. Bladder sections were directly mounted on gelatin (0.5%) microscope slides for on-slide processing, whereas spinal cord sections were processed free floating, as previously described (60). Briefly, all sections were incubated overnight at room temperature with primary antibody (Table 1) in 1% goat serum and 0.1 M PBS and then washed (3 × 10 min) with 0.1 M PBS, pH 7.4. The tissues were then incubated with Cy3-conjugated species-specific secondary antibodies (Table 1) for 2 h at room temperature in 0.1 M PBS, goat serum, and Triton X-100 (PBGT). For double labeling of CRF IR and nNOS-immunoreactive cells bodies in the autonomic nucleus in the L6–S1, spinal cord sections were incubated in a cocktail of primary antibodies (Table 1) overnight as above and then incubated with a cocktail of species-specific secondary antibodies (Table 1) as above. To demonstrate CRF IR in neuronal fibers in the urinary bladder, urinary bladder sections were incubated with a cocktail of primary and secondary antibodies against CRF and the pan-neuronal marker protein gene product 9.5 (PGP9.5; Table 1). After being washed (3 × 10 min with 0.1 M PBS), the slides were coverslipped with Citifluor

Table 1. Primary and secondary antibody sources and dilutions

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Working Dilution</th>
<th>Secondary Antibody</th>
<th>Source</th>
<th>Working Dilution</th>
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<td>Cy3-goat anti-rabbit</td>
<td>Jackson Immunoresearch Laboratories, (West Grove, PA)</td>
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<td>CRF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>1:100; IHC</td>
<td>Cy3-donkey anti-goat</td>
<td>Jackson Immunoresearch Laboratories</td>
<td>1:500</td>
</tr>
<tr>
<td>CRF&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Abcam (Cambridge, MA)</td>
<td>1:200; IHC</td>
<td>Cy3-goat anti-rabbit</td>
<td>Jackson Immunoresearch Laboratories</td>
<td>1:500</td>
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<tr>
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<td>1:500; WB</td>
<td>Donkey anti-goat HRP</td>
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<tr>
<td>PGP9.5</td>
<td>Abcam</td>
<td>1:15; IHC</td>
<td>Cy2-goat anti-mouse</td>
<td>Jackson Immunoresearch Laboratories</td>
<td>1:50</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cy2-goat anti-mouse</td>
<td>Jackson Immunoresearch Laboratories</td>
<td>1:100</td>
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CRF, corticotropin-releasing factor; CRF<sub>1</sub> and CRF<sub>2</sub>, CRF receptors; nNOS, neuronal nitric oxide synthase; PGP9.5, protein gene product 9.5; IHC, Immunohistochemistry; WB, Western blotting; HRP, horseradish peroxidase.
Urothelium and bladder musculature were processed for suburothelial structures associated with it, but we use the term in cold fixative (2% paraformaldehyde coated dish. The bladder was incubated for 1.5 h at room temperature. Harvesting, Sample Preparation, and Assay

Assessment of Positively Stained Urinary Bladder Regions

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Urinary bladder or lumbosacral spinal cord sections exhibiting IR that was greater than the background level observed in experiment-matched negative controls were considered positively stained. In this study, we have focused on CRF, CRF1, and CRF2 IR in the urothelium and bladder nerves in the detrusor and suburothelial plexus and SPN region.

Visualization and Quantitative Analysis of CRF IR in Urinary Bladder Sections, Whole Mounts, and SPN

Six to 10 urinary bladder or spinal cord sections from control and experimental groups were examined under an Olympus fluorescence photomicroscope (Optical Analysis, Nashua, NH) with a multiband filter set for visualization of the Cy3 fluorophore. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range from 610 to 655 nm. Grayscale images acquired in tiff format where imported into Meta Morph image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA) as previously described (67). Images were calibrated for pixel size by applying a previously created calibration file calibrated for pixel size by applying a previously created calibration file.

CRF IR in urothelium. The free-hand drawing tool was selected, and the urothelium was drawn and measured in total pixel area (see Fig. 2, A, C, E, and G). A threshold within an intensity range of 100–250 grayscale values was applied to the region of interest (see Fig. 2, A, C, E, and G) in the least brightly stained condition first. The same threshold was subsequently used for all images. Average intensity was calculated within the outlined area. The average intensity represents the average value of all of the pixels above the threshold value. Percent CRF expression above threshold in the total area selected was then calculated and averaged for all samples from control (n = 10) and CYP-treated (n = 8 each) rats. Percentage CRF expression in CRF-treated rats was therefore expressed as a percentage of control.

CRF IR in SPN. The same process was applied to CRF IR in the SPN region with two differences. First, a rectangle of fixed dimension (500 × 500 pixels) was placed over the SPN region in sections from all groups examined because, unlike the urothelium, the SPN could not accurately be outlined using the free-hand drawing tool (see Fig. 4, A–D). Second, average intensity was calculated within the 500 × 500-pixel square. The average intensity represents the average value of all of the pixels above the threshold value. Percent CRF expression in the SPN above threshold in the area selected was then calculated and averaged for all samples from control (n = 10) and CYP-treated (n = 6 each) rats.

CRF IR nerve fibers. The density of CRF IR nerve fibers in the suburothelial plexus or detrusor was determined using a method modified from Brady et al. (7). Grayscale images acquired in tiff format where imported into Image J (1), and images were thresholded as described above. Images were acquired from the same area of detrusor in control and treated rats. A rectangle of fixed dimension (500 × 500 pixels) was placed on the section according to a random selection of x and y coordinates. This process was repeated seven times for each image of detrusor or urothelium. The average density of CRF IR nerve fibers was then calculated for all CRF IR nerve fibers in urinary bladders from control and CYP-treated rats (n = 8).

RNA Extraction, Reverse Transcription, and PCR

Urinary bladders were dissected from female rats; the urothelium and suburothelial structures were removed from the underlying bladder smooth muscle with the aid of fine forceps and a dissecting microscope, and all tissues were snap-frozen on dry ice before processing. In this study, use of the word urothelium refers to the urothelium and suburothelial structures. Total RNA from the different tissues was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test “B,” Friendswood, TX), as previously described (6). The quality and quantity of the resulting RNA were assessed with spectrometry at 260/280 nm, and 2 μg of total RNA from each sample were used to synthesize first-strand cDNA using SuperScript II RT with the SuperScript II Preamplification System (Invitrogen, Carlsbad, CA) in a 20-μl final reaction volume. After digestion with RNase H to remove residual RNA, the cDNA was amplified with 200 μM dNTP, 0.2 M primers, and 1.25 units AmpliTaq Gold DNA Polymerase with the following parameters: initial denaturation, 94°C, 5 min; denaturation, 94°C for 45 s; annealing, primer-specific annealing temperature for 30 s; extension, 72°C for 45 s (30–35 cycles); final extension, 72°C for 5 min. The oligonucleotide primers for CRF receptors, CRF1, and CRF2, were used as described by Porcher et al. (36; see Ref. Table 2). The amplified products were resolved on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. cDNA synthesis in the absence of RNA template or RT or amplification without template, primers, or DNA polymerase were used as controls.

Western Blotting for CRF2 Receptor IR in Urinary Bladder from Control and CYP-treated Rats

The bladder was harvested from control (n = 4) and CYP-treated (n = 4 each condition) rats and homogenized, and aliquots were removed for protein assay as described above. Samples (20 μg) were suspended in sample buffer for fractionation on Tris-glycine gels (Invitrogen) and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated. Membranes were blocked overnight followed by rinsing in Tris-buffered saline plus 0.05% Tween. Membranes were then incubated in rabbit anti-CRF2 receptor (Table 1) overnight at 4°C. Washed membranes were incubated in horseradish peroxidase-conjugated donkey anti-goat IgG (Table 1) for 2 h at room temperature for enhanced chemiluminescence detection (Amersham, Piscataway, NJ). Blots were exposed to Biomax film (Kodak, Rochester, NY), and developed. The intensity of each band was analyzed (Un-Scan It; Silk Scientific, Orem, UT), and background intensities were subtracted. Western blotting of actin (Santa Cruz Biotechnology, Santa Cruz, CA) and averaged for all samples from control (n = 4) and CYP-treated (n = 8 each) rats.

Table 2. Primer sequences for CRF1 and CRF2 receptors

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Accession No.</th>
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<tr>
<td>CRF1 forward</td>
<td>TGG CTG AGA AAC ATC ATC CAC TGG</td>
<td>L25438</td>
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<tr>
<td>CRF1 reverse</td>
<td>TAA TTG TAG GCG GCT GTC AGC AAC</td>
<td></td>
</tr>
<tr>
<td>CRF2 forward</td>
<td>AGC GGC ATC AAG TAG AAC AGC AC</td>
<td>U16253</td>
</tr>
<tr>
<td>CRF2 reverse</td>
<td>GGA TTC GGT AAT GGA GGT CAT AC</td>
<td></td>
</tr>
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</table>
CRF expression in urinary bladder samples after acute (4 or 48 h) or chronic (10 days) CYP-induced cystitis were made using ANOVA. Percentage data from image analysis were arcsine transformed to meet the requirements of this statistical test. 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 Dunnett’s post hoc test was used to compare the control means with each experimental mean.

**Figure Preparation**

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) where groups of images were assembled and labeled.

**RESULTS**

**CRF Protein Expression in Urinary Bladder with CYP-induced Cystitis**

CRF protein expression in whole urinary bladders as determined by ELISAs significantly (P ≤ 0.01) increased (4.0-fold) with acute (48 h) CYP treatment and chronic CYP treatment (3.0-fold; Fig. 1). No change in CRF protein expression was observed 4 h after CYP treatment (Fig. 1).

**CRF IR in Urothelium with CYP-Induced Cystitis**

The expression of CRF IR was low in urothelium from urinary bladder sections from control (Fig. 2, A and B) and acute (4 h; Fig. 2, C and D) CYP treatment. Little if any CRF IR was present in suburothelial structures (Fig. 2, A and B) or detrusor smooth muscle of control rats. Acute (48 h; Fig. 2, E and F) CYP-induced cystitis increased (5.0-fold; P ≤ 0.01) CRF IR expression (Fig. 3) in the urothelium, and some diffuse CRF staining was observed in suburothelial structures. Similar to that observed for control rats, no CRF staining was observed in the detrusor smooth muscle with CYP treatment. No changes in CRF IR were observed in the urothelium with chronic CYP treatment (Fig. 2, G and H).

**CRF Expression in Lumbosacral SPN**

**Distribution and general characteristics.** As previously described (48) and confirmed in this study, CRF IR was expressed in distinct regions in lumbosacral spinal levels (L6–S3) in adult rats. CRF IR was most intense in the region of the SPN, but less intense CRF IR was also present in the medial and lateral dorsal horn (laminae I-II), dorsal commissure, and in a small fiber bundle extending ventrally from Lissauer’s tract in lamina I along the lateral edge of the dorsal horn in the dorsal part of the SPN. CRF IR was largely absent from the ventral horn. CRF IR was expressed in nerve fibers in specific regions of the spinal cord and had a punctate staining quality (Fig. 4). In the SPN region, CRF IR was observed in close approximation to nNOS IR cells (presumptive preganglionic neurons; Fig. 4C). No CRF IR was observed in cell bodies in the spinal cord.

We focused the current analysis of CRF expression on the SPN in the lumbosacral spinal cord, since previous studies (48) from this laboratory and the current study have demonstrated the most abundant CRF IR in the adult SPN region compared with other spinal cord regions. We have confirmed that the CRF IR in the lateral horn of L6–S1 is associated with the autonomic nucleus by using segmentation protocols previously used and validated (6, 61, 64) and by also staining spinal cord sections for nNOS IR (48), a marker for preganglionic autonomic neurons (Fig. 4C). Consistent with our previous studies (48), dense CRF IR was present in the SPN region of the rat lumbosacral spinal cord (Figs. 4 and 5, A and B). With CYP-induced cystitis, the intensity of CRF IR in the SPN region was significantly (P ≤ 0.05) increased (2.0-fold; Fig. 6) after chronic CYP-treatment (Fig. 5, G and H).

**CRF Expression in Nerve Fibers from Whole Mounts of Urothelium/Suburothelium and Detrusor Smooth Muscle**

To determine if CRF IR was present in the suburothelial nerve plexus, whole mount preparations of urinary bladder were prepared to aid in the visualization of the nerve plexus. In the detrusor whole mount preparation (Fig. 7A), very few CRF IR nerve fibers were observed in the detrusor from control rats. In contrast, CRF IR nerve fibers in the suburothelial plexus (Fig. 7C) in the urothelium/suburothelium whole mount preparation were more abundant in control rats. In contrast, with CYP-induced cystitis (48 h), the density of CRF IR nerve fibers in the detrusor (Fig. 7B) and suburothelial plexus (Fig. 7D) significantly (5.0-fold) increased. The density of CRF IR nerve fibers increased in the trigone and neck regions of the detrusor, whereas the CRF IR nerve fibers in the suburothelial plexus displayed a generalized increase in density. CRF IR nerve fibers in the detrusor or urothelium whole mounts exhibited colocalization with the pan-neuronal marker PGP9.5 (Fig. 7, E and F). In some detrusor whole mount samples, small, rounded cells without dendritic processes (presumptive mast cells) were observed to exhibit CRF and CRF2 receptor IR (data not shown). These cells show a similar morphology to mast cells previously described (14).
CRF$_1$ and CRF$_2$ in Control Whole Mounts of Urothelium/suburothelium or Detrusor Smooth Muscle

We examined the distribution of CRF$_1$ receptor and CRF$_2$ receptor in whole mounts of urothelium/suburothelium and detrusor smooth muscle. CRF$_2$ receptor IR was observed in urothelial cells (Fig. 8A) in the bladder dome and in the suburothelial nerve plexus near the urinary bladder neck (Fig. 8B). No CRF$_2$ receptor IR nerve fibers were observed in the suburothelial nerve plexus in the bladder dome. CRF$_2$ receptor IR was also present in single nerve fibers and nerve trunks scattered throughout the detrusor smooth muscle (Fig. 8C). In contrast, no CRF$_1$ receptor IR staining was observed in any region of the urinary bladder examined.

CRF$_1$ and CRF$_2$ Transcript Expression in Control Urothelium/suburothelium or Detrusor Smooth Muscle

From RT-PCR analyses, both urinary bladder detrusor smooth muscle and urothelium demonstrated similar patterns of CRF$_2$ receptor transcript expression (Fig. 9). In contrast,
neither detrusor smooth muscle nor urothelium/suburothelium expressed CRF₁ receptor transcript expression (Fig. 9), in agreement with the immunohistochemistry studies described above.

Upregulation of CRF₂ Receptor Expression in Whole Urinary Bladder from CYP-treated Rats

Western blotting of whole urinary bladder from control and CYP-treated rats demonstrated significant \((P \leq 0.05)\) upregulation of CRF₂ receptor expression 48 h after CYP treatment (Fig. 10). CRF₂ receptor expression tended to increase with CYP treatment with 4-h and chronic CYP treatment; however, this trend was not significant (Fig. 10).

DISCUSSION

We demonstrate several novel findings with respect to CRF expression in lower urinary tract (LUT) tissues and its plasticity with CYP-induced cystitis. CRF protein expression in whole urinary bladders significantly increased with CYP treatment (48 h or chronic). CRF IR was increased significantly in the urothelium and SPN after CYP treatment, although the time course of this upregulation was tissue dependent. The density of CRF IR nerve fibers increased in the suburothelial plexus and detrusor with CYP-induced cystitis. CRF₂ receptor transcript was expressed in the urothelium or detrusor smooth muscle in control rats, and CRF₂ receptor expression was upregulated significantly with CYP treatment, whereas no CRF₁ receptor transcript was expressed in either tissue examined. CRF₂ receptor IR was present in urinary bladder nerve fibers and urothelial cells from control animals. Immunohistochemical studies confirmed the selective distribution of CRF₂ receptor to LUT tissues. The present studies demonstrated the plasticity in CRF and CRF₂ receptor expression in urinary bladder and SPN with CYP-induced cystitis.

IC is a chronic inflammatory bladder disease syndrome characterized by urinary frequency, urgency, and suprapubic and pelvic pain (10, 35). 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 (10, 13, 17, 35, 40). Alterations in peripheral afferent (sensory)/efferent (autonomic and motor) and central interneuronal pathway functions as well as changes in the urinary bladder may underlie detrusor overactivity that accompany CYP-induced cystitis. Pain associated with IC may involve an alteration of visceral sensation/bladder sensory physiology. These changes may be mediated, in part, by inflammatory changes in the urinary bladder.
Chemical cystitis was induced in female Wistar rats by CYP that is metabolized to acrolein, an irritant eliminated in the urine (9, 24). 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 (9, 24, 47). The most common urological complication associated with CYP treatment in humans is cystitis, with or without hemorrhage (9, 24, 47, 65). Gross histological findings from the current study and previous studies (16, 63, 66) of animals treated with CYP demonstrate extensive regions of urothelial erosion, ulcerations, edema, and associated increases in bladder weight and petechial hemorrhages. Recent studies (58) have also demonstrated extensive monocyte/macrophage infiltration in the inflamed urinary bladder and a significant increase in myeloperoxidase activity. Our previous studies (14, 27, 58, 59) have demonstrated that chronic CYP treatment generates a more pronounced inflammatory response compared with acute (4 or 48 h) CYP treatment evidenced by

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Fig. 5. Semiquantitative analysis of CRF IR in the SPN of the L6–S1 spinal cord after CYP-induced cystitis. A box encompassing the SPN region (A, C, E, and G) was used consistently in all measurements from all conditions to outline the region of interest. A threshold within an intensity range of 100–250 grayscale values was applied to the region of interest (A, C, E, and G). The same threshold was subsequently used for all images. %CRF expression above threshold in the total area selected was then calculated. Grayscale versions of CRF IR in control SPN (A), 4 (D) and 48 (F) h after CYP treatment, or chronic CYP treatment (G) with the selected region outlined in green are shown. Images are thresholded, and the presence of yellow within the outlined region represents staining above threshold in control (A), 4-h (C), 48-h (E), or chronic CYP-treated (G) rats. Fluorescence images of CRF expression in SPN region of control (B), 4-h (D), 48-h (F), and chronic (H) CYP-treated rats. For all images, exposure times were held constant, and all tissues were processed simultaneously. With chronic CYP-induced cystitis, the intensity of CRF IR in the SPN region increased significantly (G and H). CC, central canal. Calibration bar represents 300 μm.
histological observations and diffuse inflammatory cell infiltration and increased expression of PGE$_2$ and cyclooxygenase-2 (COX-2) in the urinary bladder. Previous studies have suggested that mast cells in the inflamed urinary bladder are the cellular source of COX-2 (14). In contrast, increases in PGD$_2$ in the urinary bladder were biphasic, occurring with both acute (4 h) and chronic CYP treatment but not acute (48 h) treatment (14). Animal studies have also indicated that CYP treatment (acute treatment) in the rat induces increased frequency of voiding in awake rats and urinary bladder hyperreflexia in anesthetized rats (14, 21–23, 26), and these changes are maintained with acute (48 h) and chronic CYP treatment. Thus CYP treatment represents a noxious, chemical irritation of the urinary bladder that also induces bladder overactivity. Repeated intraperitoneal injection of rats with CYP results in a progressive inflammatory condition with sustained bladder overactivity.

Previous studies have demonstrated that a number of neurotrophic factors (15, 59), neuropeptides (6, 57), and cytokines (27, 67) are upregulated in the urinary bladder after CYP-induced cystitis. Subtractive techniques to block these factors improve bladder function and reduce pain-related behaviors (6,

Fig. 6. Histogram of the intensity of CRF IR in the SPN region presented as a percentage of control for all tissues examined ($n = 6$–10) from the semiquantitative analysis described in Fig. 5. *$P \leq 0.01$.

Fig. 7. Fluorescence photographs of CRF IR nerve fibers in the detrusor (A and B) or nerve fibers in the suburothelial plexus in whole mount preparations of the urothelium/suburothelium (C and D) in control (A and C) or CYP-treated (B and D) rats. Few CRF IR nerve fibers were observed in the detrusor from control rats (A). In contrast, CRF IR nerve fibers in the suburothelial plexus (C) in the urothelium/suburothelium whole mount preparation were more abundant in control rats. With CYP-induced cystitis (48 h), the density of CRF IR nerve fibers in the detrusor (B) and suburothelial plexus (D) increased. CRF IR nerve fibers in the urothelium or detrusor whole mount preparations colocalized with the pan-neuronal marker protein gene product 9.5 (PGP9.5; arrows; E). CRF IR nerve fibers colocalized with PGP9.5 IR in the same urothelium whole mount (E). F: merged image of D and E that demonstrates complete overlap between CRF and PGP immunostaining in the suburothelial plexus of the urothelium. Several examples of colabeled nerve fibers are indicated (D–F, arrows). Calibration bar represents 80 $\mu$m.
15). We believe that this study is the first to demonstrate upregulation of CRF in the urinary bladder and specifically in the urothelium and bladder nerve fibers after CYP-induced cystitis. Previous gene profiling studies have demonstrated upregulation of CRF receptor in the urinary bladder in murine models of cystitis (39). We extend these studies by demonstrating upregulation of CRF in both acute and chronic CYP-induced cystitis as well as by demonstration of increased CRF expression in specific urinary bladder tissues (urothelium and nerve fibers). Urothelial cells share a number of similarities with sensory neurons, and the urothelium has been suggested (2–4) to have “neuronal-like” properties. Urothelial cells express a number of receptors and ion channels similar to those found in sensory neurons (4, 32). The effectiveness of capsa...
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CRF in normal and inflamed states.

Additional functional studies are needed to define the role of CRF in micturition reflex pathways with CYP-induced cystitis, but previous demonstrations with calcitonin gene-related peptide (57), substance P (57), pituitary adenylate cyclase-activating polypeptide (6), and galanin (68).

Although increases in CRF expression or protein content were demonstrated in the urinary bladder and SPN region of the lumbosacral spinal cord, the time course of this upregulation was different and may reflect different roles for CRF in different parts of the micturition reflex. Increased CRF protein expression in the whole urinary bladder was demonstrated after 48 h or chronic CYP treatment, whereas CRF IR was increased significantly in the urothelium and suburothelial nerve fibers 48 h after CYP treatment. Detrusor smooth muscle from control or CYP-treated rats largely lacked CRF IR, so differences between the ELISA results and the immunohistochemistry and subsequent semiquantitative analysis may be because of upregulation of CRF in the suburothelial region that did not demonstrate CRF IR or may also reflect differences in the sensitivity between these two techniques and the use of different antibodies against CRF. In contrast, increased CRF IR in the SPN region of the lumbosacral spinal cord was only observed with chronic CYP treatment. Comparisons of CRF IR in the SPN region were made among experimental groups with spinal cord sections matched with respect to rostral-caudal location in the L6-S1 spinal segments. It should also be noted that CYP-induced cystitis and cystitis induced by other chemical irritants is associated with central and peripheral upregulation of a number of neuropeptides (6, 57), cytokines (27, 67), and neurotrophic (15, 59) factors that may all contribute to altered sensory processing and bladder overactivity. It is not possible with this present study that focuses solely on CRF and CRF receptor distribution, expression, and plasticity with CYP-induced cystitis, without examining the functional contribution of CRF to LUT reflexes, to attribute a definitive role for CRF in the acute or chronic stages of CYP-induced cystitis. Bladder inflammation can have both acute and chronic stages such as observed in IC, and these stages may induce expression of genes and protein that underlie inflammation, edema, tissue degradation, and tissue remodeling, repair, and adaptive strategies (39). Expression of CRF after acute or chronic CYP-induced cystitis in LUT tissues suggests diverse roles for CRF that remain to be fully elucidated.

The reasons for the differences in the time course of the expression of CRF in the urinary bladder, bladder nerve fibers, and SPN region are not known but may reflect tissue-specific roles in acute and chronic bladder inflammation stages. If one compares CRF expression changes in these tissues using only the immunohistochemistry data and semiquantitative analyses, one observes significant upregulation of CRF IR in the urothelium and suburothelial nerve fibers 48 h after CYP treatment. In contrast, upregulation of CRF IR in the SPN region of the lumbosacral spinal cord was only observed after chronic CYP treatment. Differences may reflect a role for CRF in long-term remodeling of the spinal cord with chronic bladder inflammation and/or may also suggest that CRF is upregulated secondary to the local production of or retrograde transport of target tissue-derived neurotrophic factors (59).

Neurotrophic factors expressed in the inflamed urinary bladder may also contribute to CRF upregulation. Previous studies from several laboratories have suggested that neurotrophic factor expression in the urinary bladder may underlie the changes in the neurochemical (57, 61) and electrical (66) phenotype of bladder afferent neurons after urinary bladder dysfunction. Recent experiments from several laboratories, including our own, have demonstrated the influence of target organ-neuron interactions in the adult animal (11, 15, 43–46, 51, 52). Studies from our laboratory have demonstrated that CYP-induced cystitis alters the expression of nerve growth factor (NGF) and NGF mRNA in the urinary bladder and in major pelvic ganglia (59). A number of other neurotrophic factors, including brain-derived neurotrophic factor, glial-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, are also upregulated in the inflamed urinary bladder (32, 59).

Conflicting roles for CRF in micturition reflex pathways have been described previously. CRF is expressed prominently in the descending pathway from Barrington’s nucleus to the SPN in the lumbosacral spinal cord, and prominent CRF IR is expressed in the SPN of adult rats (37, 53, 54). Intrathecal administration of CRF decreases the amplitude of bladder contractions induced by electrical stimulation of Barrington’s nucleus (34), suggesting that CRF in descending projections from Barrington’s nucleus to the SPN is inhibitory. On the other hand, recent studies now demonstrate that intrathecal or systemic CRF induces bladder overactivity (19, 20). Different putative roles for CRF in these studies may be related to the use of halothane-anesthetized (34) or conscious (19, 20) rats for functional studies. Klausner et al. (19, 20) suggest that CRF’s role in voiding function and dysfunction is related to a central stress response and may underlie bladder overactivity in the clinical syndrome of IC. On the other hand, Pavcovich et al. (34) suggest that CRF’s role in the voiding reflex is in maintaining continence. Our previous study (48) that described an age-dependent upregulation of CRF IR in the SPN is more consistent with a role in maintaining continence, since the mature voiding reflex must alternate between two modes of operation, voiding, and storage. However, the present studies demonstrate time- and tissue-dependent upregulation of CRF in bladder reflex pathways with CYP-induced cystitis, but whether or not this upregulation has any functional significance remains to be determined. It may be the case that CRF plays different roles in bladder function during development and inflammatory conditions and may be dependent on the presence of mast cells, as previously suggested (39, 50). Clearly, additional functional studies are needed to define the role of CRF in normal and inflamed states.
This study also has the first demonstration of CRF2 receptor transcript in the urinary bladder (urothelium/suburothelium or detrusor) of control rats and CRF2 receptor IR in urothelial cells and nerve fibers located both in the detrusor and suburothelial plexus. In contrast, neither CRF1 receptor transcript nor CRF1 receptor IR was expressed in bladder from control rats. In the small intestine, CRF1-2 receptors were distributed within the neuronal elements of both nerve varicosities and nerve cell bodies in addition to epithelial cells (36). The CRF2 receptor distribution in the small intestine (36) is consistent with the observations in the present study. However, CRF1-2 receptors were also located in the tunica muscularis of the small intestine (36), whereas no CRF1 receptor expression was observed in the detrusor smooth muscle in the present study. Western blotting of whole urinary bladders from CYP-treated rats (48 h) demonstrated a significant upregulation of CRF2 receptor expression compared with control urinary bladder. Previous studies have demonstrated modulation of CRF2 receptor transcript in the rat cardiovascular system after lipopolysaccharide or glucocorticoid administration (18). It is worth noting that CRF is probably not the natural ligand for CRF2 receptor, and a number of studies argue that that natural ligand for CRF2 receptor is urocortin (18). Interestingly, a recent report suggests that urocortin may be an endogenous anti-inflammatory factor and demonstrates that treatment with urocortin decreases the severity of inflammatory colitis in a murine model (12). These studies clearly have implications for IC. Whereas CRF may not be a natural ligand for CRF2 receptor (18), recent studies suggest that the peptide cocaine-and-amphetamine regulated transcript (CART) protein may function through CRF receptors. Recent studies (69, 70) from this laboratory have demonstrated CART expression in intramural neurons and nerve fibers of the urinary bladder during development and in adult rats. Furthermore, CART peptide also facilitates detrusor contractility (71).

In conclusion, these studies have demonstrated significant time- and tissue-dependent changes in CRF expression in LUT tissues after CYP-induced cystitis (acute and chronic). This study has demonstrated that inflammation of the urinary bladder induces changes in bladder CRF expression in the urothelium and in urinary bladder nerve fibers with acute (48 h) CYP treatment. Central changes in CRF expression in the SPN of the lumbo-sacral spinal cord were also observed with chronic CYP treatment. The CRF2 receptor is expressed in urinary bladder and specifically in the urothelium and bladder nerve fibers, and its expression is increased in whole urinary bladder with CYP-induced cystitis. In contrast, no CRF2 receptor transcript or IR was observed in LUT tissues. This study demonstrates that CRF and CRF2 receptor expression is altered in LUT tissues with CYP-induced cystitis and adds to a growing number of neuropeptides, cytokines, and neurotrophic factors with altered expression in LUT tissues with CYP-induced cystitis. The interactions among these neuroactive compounds in the context of acute and chronic bladder inflammation remain to be determined. Ongoing studies are determining the functional contribution of CRF and urocortin to LUT function after cystitis using CRF1 and CRF2 receptor antagonists.

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