Expression of cyclooxygenase-2 in urinary bladder in rats with cyclophosphamide-induced cystitis

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Submitted 30 April 2007; accepted in final form 28 May 2007

Klinger MB, Dattilio A, Vizzard MA. Expression of cyclooxygenase-2 in urinary bladder in rats with cyclophosphamide-induced cystitis. Am J Physiol Regul Integr Comp Physiol 293: R677–R685, 2007. First published May 30, 2007; doi:10.1152/ajpregu.00305.2007.—These studies examined the expression of cyclooxygenase-2 (COX-2) in rats with cyclophosphamide (CYP) to induce acute (4 h), intermediate (48 h), or chronic (10-day) cystitis. Western blot analysis and immunohistochemistry were used to demonstrate COX-2 expression. In whole mount preparations of urinary bladder, nerve fibers in the suburothelial plexus with acute or chronic CYP-induced cystitis. COX-2-IR nerve fibers exhibited increased density in the suburothelium, inflammatory cell infiltrates were present throughout the detrusor. Although COX-2 expression was increased in CYP-induced cystitis, COX-2 expression significantly (P ≤ 0.01) increased in the urothelium + suburothelium and detrusor smooth muscle with acute, intermediate, and chronic (10-day) CYP-induced cystitis, but expression in urothelium + suburothelium was significantly greater. CYP-induced up-regulation of COX-2 was shown by immunostaining in the urothelium + suburothelium was similar to that observed with Western blot analysis and also demonstrated COX-2 inflammatory cell infiltrates (CD86+) and nerve fibers (PGP+) in the suburothelial plexus. Although COX-2 expression was significantly (P ≤ 0.01) increased in detrusor smooth muscle, immunohistochemistry failed to demonstrate an obvious change in COX-2 immunoreactivity (IR) in detrusor muscle, but COX-2 inflammatory infiltrates were present throughout the detrusor. COX-2-IR nerve fibers exhibited increased density in the suburothelial plexus with acute or chronic CYP-induced cystitis. COX-2-IR macrophages (CD86+) were present throughout the urinary bladder with acute and chronic CYP-induced cystitis. These studies demonstrate cellular targets in the urinary bladder where COX-2 inhibitors may act.

urothelium; inflammatory infiltrates; detrusor; nerves

EXPERIMENTS INVOLVING A CHEMICALLY [cyclophosphamide (CYP)]-INDUCED URINARY BLADDER INFLAMMATION HAVE DEMONSTRATED ALTERATIONS IN NEUROCHEMICAL (6, 10, 34), ORGANIZATIONAL (35, 37), AND ELECTROPHYSIOLOGICAL (41) PROPERTIES OF MICTURITION REFLEX PATHWAYS. POSSIBLE MECHANISMS UNDERLYING THE NEURAL PLASTICITY FOLLOWING CYP-INDUCED CYSTITIS MAY INVOLVE ALTERED EXPRESSION OF NEUROTROPIC FACTORS (27, 35), CYTOKINES (25), AND OR NEURAL ACTIVITY IN THE URINARY BLADDER. THE PRESENCE OF PROINFLAMMATORY CYTOKINES AND GROWTH FACTORS CAN INDUCE CYCLOOXYGENASE-2 (COX-2), AN INFLAMMATORY RESPONSE GENE (26). CYP-INDUCED CYSTITIS INDUCES A NUMBER OF NEUROTROPIC FACTORS (27, 35) INCLUDING NERVE GROWTH FACTOR IN THE URINARY BLADDER AND PELVIC GANGLIA.

In addition to COX-2 being induced in response to inflammatory stimuli (14, 16, 21, 26, 32, 40), studies have demonstrated upregulation of COX-2 in the urinary bladder as a result of urinary bladder outlet obstruction (29, 30) and postnatal development (30). Complete bladder outlet obstruction in mice significantly upregulated COX-2 expression in detrusor smooth muscle cells, and this has been suggested to be a result of mechanical stretch (29). During development, embryonic expression of COX-2 transcript in bladder is 100-fold higher compared with postnatal or adult bladder (30). In our previous studies, COX-2 mRNA was increased in the whole urinary bladder after acute and chronic CYP treatment (16). COX-2 protein expression in inflamed bladders paralleled that of COX-2 mRNA (16). 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 capsaicin-sensitive bladder afferents (2, 23, 24). Prostaglandins have been suggested to play a physiological role in contributing to the basal tone of the detrusor and modulating activity of bladder nerves (23, 24). A number of COX-2 inhibitors have also been shown to increase bladder capacity in experimental cystitis induced by CYP (16, 20).

An increase in COX-2 expression induced by complete bladder outlet obstruction has been specifically localized to the detrusor smooth muscle cells (29). With respect to COX-2 upregulation induced by CYP-induced cystitis (16), there is currently no information that addresses the cellular sources in the urinary bladder that express COX-2 with bladder inflammation of varying duration. The purpose of this study was to determine 1) COX-2 protein expression in the urothelium + suburothelium compared with detrusor smooth muscle by Western blot analysis after CYP-induced cystitis of varying duration; 2) cellular location of COX-2 in urinary bladder of control rats or after CYP-induced cystitis using immunohistochemistry with an emphasis on urothelial cell, nerve fiber, and inflammatory cell infiltrate expression; and 3) intensity of COX-2 immunoreactivity in the urothelium after CYP-induced cystitis using semiquantitative image analysis. In contrast to COX-2 expression in detrusor smooth muscle cells with outlet obstruction (29), the present study demonstrates robust expression of COX-2 protein in the urothelium, inflammatory cell infiltrates, and, to a lesser extent, detrusor smooth muscle with CYP-induced cystitis.

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 were purchased from commercial sources (Tables 1). Secondary antibodies were purchased from Jackson ImmunoResearch, West Grove, PA (Table 1). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Cyclophosphamide-induced cystitis: acute, intermediate or chronic.

Chemical cystitis was induced in adult female Wistar rats by CYP treatment. CYP is metabolized to acrolein, an irritant eliminated in the urine (9). CYP was administered in one of the following ways (8): i) 4 h (150 mg/kg ip) prior to euthanasia of the animals to elicit acute inflammation \( (n = 21) \); ii) 48 h (150 mg/kg ip) prior to euthanasia to examine an intermediate inflammation \( (n = 21) \); or iii) administered every 3rd day for 10 days to elicit chronic inflammation \( (n = 21) \; 75 \text{ mg/kg ip} \). All injections of CYP were performed under isoflurane (2%) anesthesia. Control animals \( (n = 21) \) were gender matched to the experimental groups and received a corresponding volume of saline (0.9%) or distilled water injected intraperitoneally under isoflurane (2%) anesthesia. Animals were euthanized by isoflurane anesthesia (4%) plus thoracotomy at the indicated time points, and the urinary bladder was harvested and weighed. The University of Vermont Institutional Animal Care and Use Committee approved all experimental procedures (license no. 06-014) involving animal use. Animal care was under the supervision of University of Vermont College of Medicine’s Office of Animal Care in accordance with American Association for Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. All efforts were made to minimize animal stress/distress and suffering and to use the minimum number of animals.

Western blot analysis for COX-2. The urothelium + suburothelium was dissected from the detrusor smooth muscle using fine forceps under a dissecting microscope (10). Urothelium was dissected from the detrusor smooth muscle using fine forceps and placed in Krebs solution. The bladder was cut open along the midline and pinned to a Sylgard-coated dish. The bladder was incubated for 1.5 h at room temperature in cold fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.1 M cacodylate buffer, pH 7.4). Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. The intensity of each band was analyzed, and background densities were subtracted using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). COX-2-IR in the urinary bladder was examined for the presence of COX-2 immunoreactive cells and markers of intermediate inflammation (5). COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1). For single staining expressing COX-2-IR in the suburothelial region and throughout the urinary bladder, additional immunostaining for COX-2 and markers of intermediate filaments (vimentin) and Schwann cells (S100), interstitial cells (c-kit), macrophages, dendritic cell populations, monocytes (CD68), and activated B lymphocytes (CD80) was performed (Table 1).
incidence of staining variation that can occur between tissues processed on different days. Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Urinary bladder sections or whole mounts exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

Visualization and quantitative analysis of COX-2-IR in urothelium.
Six to ten urinary bladder sections from control and experimental groups were examined under an Olympus fluorescence photomicroscope with a multiband filter set for simultaneous visualization of the cyanine (Cy)3 and Cy2 fluorophores. Cy3 was visualized with a filter with an excitation range of 560 to 596 nm and an emission range from 610 to 655 nm. Cy2 was viewed by using a filter with an excitation range of 447 to 501 nm and an emission range from 510 to 540 nm. Quantification of COX-2-IR in the urothelium was performed as previously described (19, 42). Grayscale images acquired in TIFF format were imported into Meta Morph image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA). The opened image was first calibrated for pixel size by applying a previously created calibration file. The freehand drawing tool was selected, and the urothelium was drawn and measured in total pixels area. A threshold encompassing an intensity range of 100 to 250 grayscale values was applied first to the condition of interest in the least brightly stained condition. The same threshold was subsequently used for all images. Percent COX-2 expression above threshold in the total area selected was then calculated. Quantification of COX-2 expression in nerve fibers in the suburothelial plexus was performed as previously described (8, 19, 42) and modified from Brady et al. (7). Grayscale images acquired in TIFF format were imported into Image J (1) and images were thresholded. Images were acquired from the trigone region of the suburothelial plexus in control and treated rats. A rectangle of fixed dimension (500 x 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. The average density of COX-2-IR nerve fibers was then calculated.

Statistics. All values are means ± SE. Comparisons of COX-2 densitometry values from Western blots of urinary bladder samples were made using ANOVA. Percentage data from image analysis were arcsin 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 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 acquiring images from control and experimental animals processed on the same day. Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Urinary bladder sections or whole mounts exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

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cessed 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

COX-2 protein expression in detrusor smooth muscle, urothelium + suburothelium with CYP-induced cystitis. COX-2 protein expression in detrusor as determined by Western blot analysis significantly (P ≤ 0.01) increased (sixfold) with acute (4 h), intermediate (48 h; 4.2-fold), or chronic CYP-treatment (6.3-fold; Fig. 1, A and B). COX-2 protein expression in urothelium + suburothelium also significantly (P ≤ 0.01) increased with acute (15-fold), intermediate (16-fold), or chronic (17-fold) CYP treatment (Fig. 1, C and D). Basal COX-2 expression in detrusor was significantly (P ≤ 0.01) greater than that in urothelium + suburothelium. The fold increase in COX-2 expression in the urothelium + suburothelium induced by CYP-induced cystitis was significantly (P ≤ 0.01) greater than in the detrusor smooth muscle.

Fig. 2. Fluorescence images of COX-2 expression in urinary bladder sections of control (A), 4 h (B and C), and chronic (D and E) CYP-treated rats. For all images, exposure times were held constant, and all tissues were processed simultaneously. In control rats, little if any COX-2 expression was visible in the urothelium (U; A). CYP treatment (4 h, B and C; chronic, D and E) upregulated expression of COX-2 in the urothelium in all layers (apical, intermediate, and basal) of the urothelium. CYP-induced cystitis (all time points) also increased COX-2-immunoreactivity (IR) in the suburothelium (SubU; C–E). In normal urinary bladder, the detrusor smooth muscle expressed basal COX-2 but increases with cystitis were not obvious. F: COX-2-IR inflammatory cell infiltrates were present in the urinary bladder of rats with CYP treatment (4 h, C; chronic, D and E). Merged image of double-label immunostaining revealed that COX-2-IR (red) was present in cells that expressed the cytoplasmic antigen, CD86 (e.g., macrophages, dendritic cells) (F, arrows). L, lumen; sm, smooth muscle. Bar = 50 μm.
greater at all time points examined compared with detrusor (Fig. 1E).

COX-2-immunoreactivity in urinary bladder with CYP-induced cystitis. The expression of COX-2-IR was virtually absent in the urothelium of urinary bladder sections from control rats (Fig. 2A). However, some COX-2-IR was present in the detrusor smooth muscle of control rats (Fig. 2A). With acute (4 h; Fig. 2, B and C), intermediate (48 h), and chronic CYP-treatment (Fig. 2, D and E), COX-2-IR was increased primarily in the urothelium and in the suburothelial region. COX-2-IR in the urothelium and suburothelial regions induced by cystitis was nonhomogenous throughout the urothelium: patchy in appearance and intensity. COX-2-IR was present in detrusor smooth muscle with CYP-treatment but changes in COX-2 staining intensity were not obvious. We chose to focus the quantitation of COX-2 expression on the urothelium because expression was dramatically and obviously upregulated in the urothelium with CYP-treatment. COX-2 expression in the urothelium significantly \((P \leq 0.001)\) increased in the urothelium with acute (4 h; 2.4-fold) and chronic CYP-treatment (3.5-fold).

COX-2 expression in urinary bladder nerve fibers and inflammatory infiltrates after CYP treatment. To determine whether COX-2-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. COX-2-IR nerve fibers were observed in the suburothelial plexus from control rats (Fig. 3A), and the density significantly \((P \leq 0.01)\) increased with CYP-induced cystitis (4 h; Fig. 3B and chronic; Fig. 3, D and E). COX-2-IR nerve fibers were most obvious in the trigone region. COX-2-IR nerve fibers in suburothelial nerve plexus exhibited colocalization with the pan-neuronal marker, PGP9.5 (Fig. 4). In whole mount and sections of the urinary bladder after CYP treatment of all durations, fusiform cells with short processes in the suburothelial region were observed to exhibit COX-2-IR (Fig. 2, C–F). In whole mounts, these cells and processes were located in a different focal plane compared with the suburothelial region.

Fig. 3. Fluorescence photographs of COX-2-IR nerve fibers in the suburothelial plexus in whole mount preparations of the urothelium/suburothelium in control (A), acute (B), intermediate (C), and chronic CYP-treated rats (D). COX-2-IR nerve fibers in the suburothelial plexus in the urothelium/suburothelium whole mount preparation in the trigone region increased in density with CYP-induced cystitis (4 h, B; and chronic, D). Single COX-2-IR nerve fibers (arrows), as well as COX-2-IR nerve bundles (*) were observed in the suburothelial plexus. E: summary histogram of increase in COX-2-IR nerve fiber density with CYP-induced cystitis. *\(P \leq 0.01\). Bar = 80 \(\mu\)m.
plexus (Figs. 3 and 5B). COX-2-IR cells were abundant within the suburothelial region (Fig. 2, B–E) and scattered throughout the detrusor smooth muscle in bladder sections. These cells displayed a similar morphology to macrophages previously described in the urinary bladder after CYP-induced cystitis (8), but we pursued additional double-labeling studies with markers of other cellular candidates based upon morphology to determine the identity of these cells (Table 2). Double-labeling experiments of urinary bladder sections or whole mount preparations demonstrated that these COX-2-IR cells expressed the CD86 antigen (e.g., macrophages, dendritic cells) (Figs. 2F and 5B) (Table 2) but did not express immunoreactivity for vimentin, GFAP, c-kit, S-100, or CD80 (Table 2).

**DISCUSSION**

These studies demonstrate several novel findings with respect to COX-2 expression in micturition reflexes with CYP-induced cystitis. COX-2 expression is significantly increased in the urothelium + suburothelium and detrusor smooth muscle with acute (4 h), intermediate (48 h), and chronic (10-day) CYP-induced cystitis as determined by Western blot analysis, but expression in urothelium + suburothelium is significantly greater. COX-2 immunostaining in the urothelium + suburothelium generally mirrored that observed through Western blot analysis and also demonstrated COX-2 inflammatory cell infiltrates and nerve fibers in the suburothelial plexus. Although COX-2 expression was significantly increased in detrusor smooth muscle as determined by Western blot analysis, immunostaining failed to demonstrate an obvious change in COX-2-IR in detrusor muscle, but COX-2 inflammatory infiltrates were present throughout the detrusor. COX-2-IR nerve fibers exhibited increased density in the suburothelial plexus in whole mount preparations with acute or chronic CYP-induced cystitis. Migration of macrophages (CD86+) in the suburothelial space and detrusor with CYP-induced cystitis is dramatic, and expression of COX-2-IR is present in both acute and chronic inflammation of the urinary bladder. Inhibitors of COX-2 that have been shown to reduce bladder overactivity (16, 17, 20) with bladder inflammation are likely to exert effects on the urothelium, inflammatory cell infiltrates, and suburothelial nerve plexus among other targets.

A number of previous studies have demonstrated roles for COX-2 and prostaglandins in bladder overactivity induced by bladder inflammation (3, 16, 17, 20, 23, 24). It has previously been suggested that prostanoids are key mediators following the induction of CYP-induced cystitis (48 h) (16, 20). Our previous studies (16) have also demonstrated mRNA and protein for COX-2 upregulation in whole urinary bladder with CYP-induced cystitis. PGE2 and PGD2 expression in the urinary bladder with acute, intermediate, and chronic CYP-induced cystitis is also increased (16). Previous studies using selective or nonselective inhibitor of COX-2 demonstrated an increase in bladder capacity in rodents treated with CYP (3, 16, 20). In these studies (3, 16, 20), COX-2 inhibitors were delivered systemically and any understanding of potential sites of action was limited. In addition, our previous study (16) used whole urinary bladder with no emphasis on cell types in the urinary bladder that may express COX-2 with CYP-induced cystitis. In the present study, we demonstrate several potential sites of action of such inhibitors in the urinary bladder, including the urothelium, macrophages, and suburothelial nerve plexus. The functional studies are consistent with an effect of COX-2 inhibitors on bladder afferents or urothelium (increased
bladder capacity, reduced micturition frequency) (16, 20). The present studies also provide anatomical data to support the use of an intravesical route of administration of COX-2 inhibitors (17) in reducing bladder overactivity induced by CYP.

A number of reports have demonstrated upregulation of COX-2 in response to inflammatory stimuli (14, 16, 21, 26, 32, 40). A recent report (22) emphasizes the inclusion of a number of controls to be certain that a signal obtained byWestern blot analysis is indeed reflective of COX-2. We have performed each of these suggested controls. First, we have included a purified, recombinant COX-2 protein as a positive control in our Western blots that is located at 74 kDa. A review of the literature reveals that the mature, enzymatically active form of COX-2 is located in the 70- to 74-kDa range on denaturing acrylamide gel electrophoresis. This is consistent with the COX-2 protein identified from urinary bladders in the present and previous (16) study. The difference in band location for the positive control and the samples may be attributed to several issues; species differences as the positive control is from human, nonglycosylated forms of COX-2 and/or incomplete proteolysis. Second, in Hu et al. (16) we have identified increased COX-2 mRNA in urinary bladder induced by CYP treatment to further support our Western blot analysis results. Third, we have demonstrated upregulation of PGE2 and PGD2, in urinary bladder with CYP treatment as further evidence that COX-2 may affect bladder function through downstream activation of PG (16).

Immunohistochemical studies demonstrated that increases in COX-2 protein determined by Western blot analysis are a reflection of increased urothelial cell expression of COX-2, COX-2-IR macrophages (CD86+), and COX-2-IR nerve fibers in the suburothelial plexus. A number of studies have demonstrated COX-2-IR in nerves (i.e., endoneurium) in response inflammatory stimuli alone or as a result of neural injury (13, 31). In the present study, we have confirmed that COX-2-IR is present in nerve in the suburothelial plexus and that COX-2-IR nerve fibers increase in density in the trigone region of the urinary bladder with acute and chronic CYP-induced cystitis. One limitation to this study is the method used to determine density of innervation. All COX-2-IR structures in the whole mount within the same plane of focus are captured in the frame and density determination is performed on all COX-2-IR structures. Therefore, COX-2-IR macrophages or other COX-2-IR inflammatory cell infiltrates of unknown cellular phenotype may have increased the density determinations that we have attributed to nerve fibers. However, a large proportion of the COX-2-IR nerve fibers were not located in the same focal plane as the macrophages, so we feel that this possibility is limited in the present study. Afferent nerve fibers make a large contribution to this neural plexus although some contribution from efferent sources cannot be ruled out (11). Previous studies have shown COX-2-IR in primary afferent cells in dorsal root ganglia (Vizzard MA, Dattilio A, Klinger MB, unpublished data and Refs. 12 and 13), and therefore there is precedent for COX-2 expression in afferent nerves.

Urothelial cells share a number of similarities with sensory neurons, and the urothelium has been suggested to have neuronal-like properties (4, 5). Urothelial cells express a number of receptors and ion channels similar to those found in sensory

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<th>Antibody</th>
<th>Staining of Cryostat Bladder Sections</th>
<th>Staining of Bladder Whole Mounts</th>
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<td>Vimentin</td>
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<td>CD80</td>
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Double-labeling demonstrated that COX-2-IR cells expressed the CD86 antigen (e.g., macrophages, dendritic cells) but did not express immunoreactivity for vimentin, GFAP, c-kit, S-100, or CD80.
neurons (4, 5, 8, 19, 27), and it was therefore not surprising to observe COX-2 expression in the urothelium after CYP-induced cystitis. COX-2-IR in the urothelium was observed in apical, intermediate, and basal cells. Immortalized human urothelial cells express COX-2 and inducible nitric oxide synthase after stimulation of β-adrenergic receptors (15). The present study adds to the growing list of similarities between urothelial cells and sensory neurons and may also suggest that urothelial cells participate in the transduction of inflammatory signals to the central nervous system (4, 5).

A number of previous studies have also demonstrated COX-2 expression in macrophages (33, 38, 40). Surprisingly, in our previous study (16), we failed to demonstrate COX-2-IR in macrophages, although present in the bladder after CYP-induced cystitis, but demonstrated COX-2-IR in mast cells in the inflamed bladder. In the present study, the immunostaining confirms that a proportion of COX-2-IR in the suburothelial space and detrusor express the antigen CD86. Although our present study does not rule out a contribution from mast cells, the prominent cellular staining in the suburothelial region largely represents macrophages. The reason for this difference is not known but likely reflects our choice of COX-2 antiserum as has been suggested in the COX-2 literature (40). We used a monoclonal COX-2 antibody in the present study because the polyclonal antibody used in our previous study was inconsistent in its staining for this study.

Previous studies have demonstrated robust COX-2 expression in the detrusor smooth muscle after complete bladder outlet obstruction in mice, and this increase has been attributed to mechanical stretch (29, 30). In contrast, no COX-2 expression was present in the urothelium or suburothelial region in control tissues or after outlet obstruction (29, 30). The present study clearly demonstrates a larger COX-2 contribution from the urothelium and suburothelial region in response to CYP-induced cystitis. This difference probably is a reflection of COX-2 protein induced by an inflammatory stimulus vs. a mechanical stimulus. In both bladder inflammation and outlet obstruction, COX-2 is either demonstrated or hypothesized to contribute to bladder overactivity/hyperactivity (16, 20, 29, 30), although the source of COX-2 and resultant production of prostaglandins is likely to be different. It is interesting that COX-2 is highly expressed in the urinary bladder during development and that bladder outlet obstruction represents reactivation of this gene (30). The urinary bladder during early postnatal development exhibits spontaneous bladder contractions (28, 30), but the contribution of COX-2 to this function is presently unknown.

COX-2 expression can be stimulated by growth factors, proinflammatory cytokines, and chemokines (18, 39). Changes in neurotrophic factor expression in the urinary bladder with proinflammatory cytokines, and chemokines (18, 39). Changes in COX-2 expression in the urinary bladder after complete bladder obstruction, COX-2 is either demonstrated or hypothesized to contribute to bladder overactivity/hyperactivity (16, 20, 29, 30), although the source of COX-2 and resultant production of prostaglandins is likely to be different. It is interesting that COX-2 is highly expressed in the urinary bladder during development and that bladder outlet obstruction represents reactivation of this gene (30). The urinary bladder during early postnatal development exhibits spontaneous bladder contractions (28, 30), but the contribution of COX-2 to this function is presently unknown.

COX-2 expression can be stimulated by growth factors, proinflammatory cytokines, and chemokines (18, 39). Changes in neurotrophic factor expression in the urinary bladder with cystitis, including β-nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and neurotrophin-3 and -4 (27, 35) have been demonstrated. Robust changes in a number of urinary bladder cytokines including IL-1β, IL-2, IL-4, IL-6 and more modest changes in TNF-α and TNF-β with CYP-induced cystitis have also been demonstrated (25). Most recently, we have demonstrated upregulation of the chemokine, fractalkine, and fractalkine receptor in the urinary bladder and specifically in the urothelium with CYP-induced cystitis (42). Separately or in combination, neurotrophic factors, proinflammatory cytokines, and chemokines expressed in the inflamed urinary bladder may also contribute to COX-2 upregulation.

In summary, these studies have demonstrated significant changes in COX-2 expression in the urinary bladder after CYP-induced cystitis examined at three time points (acute, intermediate, and chronic). Specifically, COX-2 expression is significantly increased in the urothelium, in nerve fibers in the suburothelial plexus, and in macrophages in the suburothelial space. Although Western blot analysis demonstrates COX-2 expression in detrusor smooth muscle, this change likely reflects COX-2 expression in inflammatory cell infiltrates as immunostaining for COX-2 in detrusor smooth muscle did not exhibit robust changes in COX-2 expression. A number of cellular sources in the urinary bladder express COX-2 after CYP-induced cystitis that may be induced by neurotrophic factors, cytokines, and chemokines in the inflamed bladder. The present study defines some bladder cellular sources that are likely targets of COX-2 inhibitors.

GRANTS

This work was funded by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-051369, DK-006481, and DK-065989.

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