Obstruction stimulates COX-2 expression in bladder smooth muscle cells via increased mechanical stretch

JOHN M. PARK,1 TIANXIN YANG,2 LOIS J. AREND,3 JÜRGEN B. SCHNERMANN,4 CRAIG A. PETERS,1 MICHAEL R. FREEMAN,1 AND JOSEPHINE P. BRIGGS5

1Department of Urology, Children’s Hospital and Harvard Medical School, Boston, Massachusetts 02115; Departments of 2Medicine, 3Pathology and 4Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109; and 5National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Park, John M., Tianxin Yang, Lois J. Arend, Jürgen B. Schnermann, Craig A. Peters, Michael R. Freeman, and Josephine P. Briggs. Obstruction stimulates COX-2 expression in bladder smooth muscle cells via increased mechanical stretch. Am. J. Physiol. 276 (Renal Physiol. 45): F129–F136, 1999.—Studies were performed to investigate the regulatory mechanism of bladder cyclooxygenase-2 (COX-2) expression after outlet obstruction. In situ hybridization of murine bladder tissues using COX-2-specific riboprobes demonstrated that COX-2 expression was induced predominantly in the bladder smooth muscle cells after outlet obstruction. To study the effect of increased mechanical stretch on COX isoform expression, cultured rat bladder smooth muscle cells were grown on silicone elastomer–bottomed plates coated with collagen type I and were subjected to continuous cycles of stretch/relaxation for variable duration. COX-1 mRNA levels did not change with stretch. COX-2 expression increased in a time-dependent manner after stretch, with maximal mRNA and protein levels occurring after 4 h. PGE2 levels increased more than 40-fold in the culture media after stretch, consistent with increased COX activity, and this was reduced to near completion in the presence of a COX-2 inhibitor, NS-398. Exposure to stretch over a 48-h period induced a 4.7 ± 0.6-fold increase in tritiated thymidine incorporation rate. This increase in DNA synthesis was markedly suppressed when the cells were stretched in the presence of NS-398. We conclude that in bladder obstruction COX-2 activation occurs predominantly in the smooth muscle cells in response to mechanical stretch. Our findings also suggest that stretch-activated COX-2 expression may participate in bladder smooth muscle cell proliferation and thereby play a role in pathological bladder wall thickening after obstruction.

cyclooxygenase-1; urinary tract; prostaglandins

PROSTAGLANDINS are thought to play an important role in lower urinary tract function (11, 15). There is evidence to suggest that locally produced PGs may be an endogenous regulator of bladder tone and contractility (3, 12). One of the primary in vivo targets of PGs appears to be the caspasein-sensitive afferent nerve endings (13), and it has been postulated that bladder PGs modulate urine storage and micturition neural reflexes (11). In addition, it has been suggested that PGs may also play a role in urinary epithelial cytoprotection, analogous to their established role in gastric mucosa (15).

The recent discovery that cyclooxygenase (COX), one of the rate-limiting enzymatic steps in PG biosynthesis, exists in two distinct isoforms has stimulated a wave of investigations aimed to further explore the biological significance of PG regulation (25). COX-1, the constitutive isoform, is normally expressed in many tissues and is thought to be involved in regulation of various “house-keeping” functions such as gastric mucosal cytoprotection and platelet aggregation. In contrast, COX-2, the inducible isoform, has a limited pattern of basal expression, but is rapidly induced in response to growth factors, tumor promoters, hormones, bacterial endotoxins, and cytokines. Accumulating evidence suggests that COX-1 and COX-2 serve independent biological functions.

We have previously reported that complete outlet obstruction dramatically induced COX-2 expression in the bladder (19). We postulated that COX-2 might be the primary isoform involved in the synthesis of bladder PGs that modulate micturition reflexes in obstruction (19). In this report, we demonstrate that COX-2 induction occurs predominantly in the smooth muscle cells after bladder outlet obstruction and that the primary stimulus for activation of this gene appears to be increased mechanical stretch. We also provide evidence that stretch-activated COX-2 may be involved in the regulation of bladder smooth muscle cell proliferation and thereby promote pathological bladder wall thickening induced by obstruction.

MATERIALS AND METHODS

Bladder outlet obstruction. Complete bladder outlet obstruction was created in adult CD-1 female mice as described previously (19). Briefly, under ketamine and xylazine intraperitoneal anesthesia, the urethra was completely ligated with a nylon suture, and the bladder was allowed to distend with the animal’s own urine for the indicated duration. Sham-operated animals served as controls.

In situ hybridization. Sense and antisense cRNA probes labeled with digoxigenin were transcribed from a PCR-amplified COX-2 cDNA fragment flanked by T3 and T7 RNA polymerase sites. This COX-2 PCR product corresponds to bp 600–1124 region of the rat COX-2 gene (9), and it was identified to be that of COX-2 by sequencing. The PCR-based technique of riboprobe synthesis has been shown to reduce extraneous nucleotide sequences and minimize nonspecific background signals (34). Probe target specificity was verified by hybridization of a nylon membrane that had been dotted

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.
with 200 ng of COX-2 plasmid cDNA. The full-thickness bladder tissues from sham-operated and 6-h obstructed adult female CD-1 mice were harvested and fixed in 4% paraformaldehyde at 4°C for 2 h. Six-hour obstruction was previously shown to be associated with maximal stimulation of steady-state COX-2 mRNA level (19) and therefore was selected for in situ hybridization assay. The tissues were incubated overnight in 30% sucrose at 4°C for cryoprotection. 10-micrometer thick frozen sections were obtained after the tissues were embedded in Tissue-Tek OCT compound (Miles) and mounted onto poly-L-lysine-treated Superfrost/Plus slides (Fisher Scientific). Hybridiizations were performed under stringent conditions for 16–18 h at 65°C. Following hybridization, tissues underwent RNase A treatment to remove any nonhybridized riboprobes. Subsequent washing steps included 2× SSC and 1× SSC at room temperature for 15 min each, 0.5× SSC at 65°C for 1 h, 0.5× SSC at room temperature for 15 min, and finally 1× bovine serum albumin in PBS (with 0.3% Tween-20) at room temperature for 1 h. Slides were incubated with alkaline-phosphatase conjugated anti-digoxigenin antibody and color developed with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrates. Sections were counterstained with methyl green.

Bladder smooth muscle cell culture. The method for rat bladder smooth muscle cell isolation and culture was modified from the one described by Gunther et al. (7) for aortic smooth muscle cells. Bladders were harvested from newborn Lewis rats (Charles River), and muscle layers were cleared of epithelium and other extraneous tissues under the dissecting microscope. The tissues were minced into 1- to 3-mm pieces, washed few times in sterile PBS, and incubated for 90 min in a 37°C oscillating water bath in the presence of 0.125 mg/ml trypsin (type I, 150 U/mg, Sigma), 0.250 mg/ml soybean trypsin elastase (type III, 90 U/mg, Sigma), 1.0 mg/ml collagenase (EC 1.1.1.13, type 1, 150 U/mg, Sigma), 1.0 mg/ml trypsin, and 0.1 mg/ml streptomycin (standard culture medium). Cells were grown in humidified 5% CO2-95% air atmosphere at 37°C. Cells were subcultured several times with a serological pipette, filtered through a 100-µm cell strainer, and centrifuged. The pellet was resuspended in medium 199 (M 199; Gibco-BRL, Grand Island, NY) supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (standard culture medium). Cells were grown in humidified 5% CO2-95% air atmosphere. For the current study, cells between passages 2 and 5 were used. Their identity as smooth muscle cells was established with >95% positive immunofluorescence staining for smooth muscle α-actin and desmin (both primary antibodies obtained from Sigma). Methodological controls for immunostaining included incubation without the primary antibody and with an unrelated polyclonal antibody.

Application of cyclical stretch-relaxation to cultured bladder smooth muscle cells. Cells were grown to near confluence on six-well silicone elastomer-bottomed culture plates (Bioflex; Flexcell, McKeesport, PA) that had been coated with collagen type I. Medium was changed to a “quiescence” medium, containing M 199 with 0.5% fetal bovine serum. After incubating for 48 h in the quiescent medium, cells were subjected to cyclical stretch/relaxation using the Flexercell Strain Unit FX-3000 (Flexcell). This unit is a modification of the one initially described by Banes et al. (2). Under the computer control, vacuum (~15 to 20 kPa) was repetitively applied (5-s stretch and 5-s relaxation, maximal stretch of ~25% at the periphery) to the silicone elastomer-bottomed culture plates, which were maintained in a humidified incubator with 5% CO2-95% air atmosphere at 37°C. Cells were subjected to stretch/relaxation for variable duration up to 24 h as indicated (n = 4 for each time point).

Revertase-transcription-polymerasechain reaction. Semiquantitative RT-PCR assays were performed as described previously (19). Briefly, RNA extraction was performed using Tri-Reagent (Molecular Research, Cincinnati, OH) according to the manufacturer’s instructions. Reverse transcription was performed using Moloney murine leukemia virus-reverse transcriptase (Gibco) with oligo-dT as the first strand primer (Gibco). cDNA was precipitated with linear acrylamide, ammonium acetate, and ethanol, and redissolved in Tris-EDTA buffer. Primer selection was based on previously published rat COX-1 (6), COX-2 (9), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (27) sequences, found through GenBank database search. The COX-1 primers used were as follows: sense, 5′-CTG CTG AGA AGG GAG TTC CAT-3′ (bp 602–621); antisense, 5′-GTC ACA CAC ACG GTT ATG CT-3′ (bp 981–1000), amplifying a 398-bp product. An ~584-bp COX-2 fragment was amplified using the following primers: sense, 5′-ACG TTC TAT CAC TGG CAT CC-3′ (bp 1229–1248); antisense, 5′-GAA GGG ACA CCC TTT CAC AT-3′ (bp 1794–1813). An ~571-bp GAPDH product was amplified using the following primers: sense, 5′-CCA TCT TCC AGG AGC G-3′ (bp 245–263); and antisense, 5′-CGT CTG CAC CTT CTT GA-3′ (bp 816–797). PCR reactions were performed in a total volume of 25 µl, containing 22 µl of PCR SuperMix (Gibco), 0.5 µl each of sense and antisense primer (20 pmol/µl), 0.1 µl of [32P]dCTP (3,000 Ci/mmol, Amersham, Arlington Heights, IL), and 2 µl of cDNA. PCR amplification was performed for 30 cycles at 94°C (denature), 58°C (anneal), and 72°C (extend) for 40 s each. PCR products were subjected to size separation by PAGE. Additional confirmation of PCR products was made by sequence-specific restriction enzyme digest, as shown in our previous report (19). All samples were normalized to GAPDH expression, and a limiting dilution method was used to make semiquantitative comparisons. Band intensity was determined with ImageQuant Software on PhosphorImager System (Molecular Dynamics, Sunnyvale, CA).

Immuno blot analysis. The cell lysates were obtained in the presence of protease inhibitors and stored at −80°C. The protein concentration was determined by spectrophotometric assays using commercial colorimetric reagents (Bio-Rad). Total and cytosolic protein samples were heated to 100°C for 5 min to cause denaturation. Proteins (25 µg total per lane) were subjected to electrophoresis under reducing conditions in 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was initially blocked for 2 h in Tris-buffered saline (pH 7.5) containing 5% nonfat dry milk, followed by incubation for 60 min with a rabbit anti-murine polyclonal COX-2 antibody (Cayman Chemical, Ann Arbor, MI) at 1:500 dilution. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) at 1:2,500 dilution. Blots were developed using the ECL-Plus chemiluminescent reagent (Amersham) and subjected to autoradiography as instructed by the manufacturer.

PG E 2 measurements. PG E 2 in culture media was measured by E LiSA (Cayman Chemical) according to the manufacturer’s instructions. Bladder smooth muscle cells were grown to near confluence on Bioflex plates and rendered quiescent. One hundred microliters of culture medium (from the total volume of 6 ml) was collected from each well prior to stretch. One group of cells was subjected to stretch stimulation, while the other group was exposed to the same stretch but in the presence of a COX-2-specific inhibitor, NS-398 (30 µM). After 4 h, 100 µl of culture medium was again collected from each well. [Methyl-3H]thymidine incorporation assay. Cells were subjected to cyclical stretch/relaxation as described above for
Counter (Wallace, Gaithersburg, MD). was performed with LKB Rackbeta Liquid Scintillation precipitable materials. This was transferred to scintillation dried. One milliliter of 1% SDS in 0.3 N NaOH was added to min each, cells were quickly rinsed with methanol and air dried. One milliliter of 1% SDS in 0.3 N NaOH was added to each well and incubated for 20 min to solubilize the TCA- precipitable materials. This was transferred to scintillation vials and mixed with 10 ml of scintillant LiquiGel. Counting was performed with LKB Rackbeta Liquid Scintillation Counter (Wallace, Gaithersburg, MD).

Statistics All data are presented as means ± SE. Comparisons between the means were made with nonparametric Mann-Whitney test.

RESULTS
Outlet obstruction induces COX-2 expression in the bladder smooth muscle cells. We have previously reported that bladder outlet obstruction induced COX-2 expression predominantly in the microdissected stromal (muscle) layer and postulated that the most likely site of this gene induction was the smooth muscle cells (19). To test this further, we performed an in situ hybridization assay using COX-2-specific riboprobes. When incubated with the antisense riboprobe, bladder tissues from 6-h obstructed CD-1 mice revealed intense signals in the perinuclear regions of the smooth muscle cells. No consistent signal was detected in the uroepithelial or submucosal regions nor in the control slides incubated with the sense riboprobe (Fig. 1, A and B). Some COX-2 signals were detectable in the serosal epithelium. Nonobstructed control bladders (sham operation) also did not demonstrate any signals above background level when incubated with the antisense probe, indicating that increased COX-2 transcript levels occurred specifically in response to outlet obstruction. Higher power microscopic views revealed an elongated and “stretched” appearance of the smooth muscle cells in the obstructed bladders, in contrast to a more round and “nonstretched” morphology of the cells in the nonobstructed control bladders (Fig. 2, A and B).

In vitro cyclical stretch/relaxation activates COX-2 expression in bladder smooth muscle cells. In our previous report, using a model of in vivo regional bladder distention, we provided evidence that increase in local stretch might be the primary stimulus for the COX-2 induction after obstruction. We therefore examined the effect of mechanical stretch in vitro using cultured bladder smooth muscle cells. Exposure of bladder smooth muscle cells to cyclical stretch/relaxation caused a time-dependent increase in COX-2 expression at both the mRNA and protein levels, as measured by semiquantitative RT-PCR on cDNA prepared from microdissected tissues, is confined to the stromal (muscle) layer of bladder after obstruction. COX-2 inducibility has been demonstrated in the smooth muscle cell type of other organ systems. COX-2 expression was activated in the rat aortic smooth muscle cells after balloon distention injury in vivo and in response to serum stimulation in vitro (22). Mechanical stretch/relaxation activated COX-2 expression in cultured renal mesangial cells,
also thought to be of smooth muscle cell lineage (1). The biological significance of COX-2 activation in smooth muscle cells by increased mechanical stretch is yet to be established. Locally generated PGs appear to play a role in maintenance of tone and spontaneous contractile activity in bladder smooth muscle cells (3). In a number of cell types, the increased prostanoid production has been implicated to participate in modulation of cell proliferation and protein turnover (21, 30).

In our previous report, using regional bladder wall distention, we demonstrated in vivo that increased local stretch, rather than intraluminal pressure elevation, was primarily responsible for COX-2 gene activation after outlet obstruction (19). In the current study, we provide complementary in vitro evidence that increased mechanical stretch is a potent stimulus for COX-2 activation in cultured bladder smooth muscle cells. In bladder outlet obstruction, there are several factors that might mediate changes in gene expression, including mechanical factors such as increased wall stretch and luminal pressure, hypoxia, inflammatory infiltrate, and paracrine factors of renal origin. Our findings indicate that mechanical stimulation alone, in the absence of alteration in serum or other hormonal factors, is capable of marked induction in bladder COX-2 expression, comparable to our in vivo findings.
In contrast, COX-1 mRNA levels did not change after stretch, again consistent with our previous findings based on an in vivo model of bladder obstruction. We also demonstrated that COX-2 gene expression by stretch was functionally significant by documenting the increase in PGE$_2$ secretion after stretch. The fact that this increase in PGE$_2$ levels was almost completely suppressed by a COX-2-specific inhibitor, NS-398, provides a further evidence that COX-2 gene activation, rather than COX-1, is primarily responsible for enhanced COX activity after stretch. On the basis of these findings, we postulate that COX-2 is a key regulatory step of increased PG synthesis in bladder outlet obstruction.

This paradigm of mechano-induction has been demonstrated in other organ systems. In cultured rat vascular smooth muscle cells, mechanical strain causes an autocrine secretion of platelet-derived growth factor, which appears to modulate cell proliferation (32). Mechanical stretch activates several immediate early response genes in cultured rat cardiomyocytes (23). Mechanical stretch is a potent inducer of smooth muscle cell relaxant, parathyroid hormone-related peptide in bladder (33), uterus (4), and aortic smooth muscle cells (16, 17). Hollow organs such as bladder, blood vessels, uterus, and heart, whose primary functions include storage and conduit passage, appear to demonstrate
many common responses to mechanical stretch and therefore may possess similar molecular mechanisms of gene regulation in response to mechanical stimulation.

Our study suggests that stretch-induced COX-2 activation might play an important role in mediating bladder smooth muscle cell proliferation. Inhibition of COX-2 enzymatic activity with a specific pharmacological inhibitor, NS-398, significantly attenuated mitogenic response after stretch. This finding is consistent with an emerging notion that COX-2-driven PG products participate in the regulation of tissue proliferation and remodeling. Several observations regarding COX-2 biology support this hypothesis. In various organ systems, the temporal pattern of COX-2 induction has been shown to follow that of immediate-early genes. These early response genes are activated rapidly and transiently by extracellular stimulation to encode proteins that regulate other gene transcription (8). COX-2 expression is specifically regulated during fetal development of the bladder (19), kidney (10), and brain (26). COX-2 expression is stimulated by various mitogens and oncogenes (5). Constitutive COX-2 overexpression has been implicated as playing an important role in intestinal adenocarcinoma progression by affecting apoptotic pathways (28) and metastatic potential (29). COX-2 products may regulate fibroblast proliferation and thereby tissue fibrosis (31). Given these findings, it seems likely that stretch-activated COX-2 expression might also play a role in modulating bladder smooth muscle cell proliferation.

Fig. 3. Effect of in vitro mechanical stretch on COX-1 and COX-2 expression in bladder smooth muscle cells. Cultured bladder smooth muscle cells were harvested from newborn Lewis rats and grown on silicone elastomer-bottomed culture plates (Bioflex). These were subjected to cyclical stretch/relaxation (0.1 Hz, 25% maximal elongation) for variable duration in serum-deficient media. Semiquantitative RT-PCR was performed to assess COX-1 and COX-2 mRNA levels with normalization to GAPDH levels. Results are representative of 4 similar experiments.

Fig. 4. Increased COX-2 protein levels in bladder smooth muscle cells after stretch. Cell lysates (25 µg total protein per lane) were separated using 12% SDS-PAGE and were transferred onto a nitrocellulose membrane. These were hybridized with 1:500 rabbit anti-murine polyclonal COX-2 antibody. Blots were developed using ECL-Plus chemiluminescent reagent. Two samples from four independently performed experiments are shown.

Fig. 5. Increased COX activity after stretch. Culture media were sampled before and after 4-h stretch, and PGE2 levels were measured by ELISA. To assess the contribution of stretch-induced COX-2, bladder smooth muscle cells were subjected to 4-h stretch in presence of a COX-2-specific inhibitor, NS-398 (30 µM), and PGE2 levels were assessed in culture media as above (n = 6 per group). P < 0.01 for 0-h stretch vs. 4-h stretch. P < 0.01 for 0-h stretch vs. 4-h stretch with NS-398.

Fig. 6. Effect of COX-2 inhibition by NS-398 (30 µM) on stretch-induced bladder smooth muscle cell proliferation. Cells were plated on collagen-coated silicone elastomer plates and subjected to 48-h stretch/relaxation. During the final 8 h, [methyl-3H]thymidine (1 µCi per ml culture medium) was added. Thymidine incorporation rate was measured as described in MATERIALS AND METHODS (n = 6 per group). P < 0.01 for nonstretch vs. stretch. P < 0.01 for stretch vs. stretch + NS-398.
When the bladder becomes obstructed (as in the case of benign prostatic hyperplasia and posterior urethral valves), there is a progressive functional impairment (14, 20). One of the early changes after obstruction is the development of hyperactive voiding, which may lead to incontinence and urinary tract infection. Persistent outflow obstruction also leads to an increase in local tissue mass, in part due to smooth muscle cell proliferation (18, 20, 24). Continued cell growth causes a progressive bladder wall thickening, compromising its urine storage function. Under normal circumstances, the bladder can store a wide range of fluid volumes without significant changes in luminal pressures. This compliance of the bladder as a fluid reservoir is critical for maintenance of renal integrity, as it allows glomerular filtration and tubular function to proceed without being subjected to high hydrostatic pressure gradients.

It is well established clinically that the key determinant of renal outcome in obstructive uropathy is the bladder’s ability to store fluid at low pressures. Therefore, it is important to understand the pathophysiological mechanism of tissue proliferation that occurs in response to outflow obstruction.

Our study provides a framework within which COX-2 regulation may be studied as a potential mechanism of pathophysiological alteration in bladder outflow obstruction. It most likely contributes to increases in extracellular PG levels that mediate hyperactive voiding syndromes by sensitizing capsaicin-sensitive afferent nerve endings. In addition, a subset of COX-2 products may mediate nuclear events such as DNA synthesis associated with cell proliferation.

Address for reprint requests: J. M. Park, Dept. of Urology, Children's Hospital, 300 Longwood Ave., Boston, MA 02115.

Received 26 January 1998; accepted in final form 8 October 1998.

REFERENCES


