Cyclooxygenase-2 is expressed in bladder during fetal development and stimulated by outlet obstruction

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Park, John M., Tianxin Yang, Lois J. Arend, Ann M. Smart, Jurgen B. Schnermann, and Josephine P. Briggs. Cyclooxygenase-2 is expressed in bladder during fetal development and stimulated by outlet obstruction. Am. J. Physiol. 273 (Renal Physiol. 42): F538–F544, 1997.—Studies were undertaken to assess expression of inducible cyclooxygenase (COX)-2 in bladder during fetal development and COX-1 and COX-2 expression after outlet obstruction. Bladder tissue or bladder progenitor tissue was harvested from CD-1 murine embryos at embryonic ages E11.5, E14.5, E17.5, and E20.5 (newborn), and from adult. Bladder obstruction was created in adult female mice by ligating the urethra, and bladders were harvested after 3–24 h of obstruction. Gene expression was assessed by semiquantitative reverse transcription-polymerase chain reaction and Western blotting. COX-2 was highly expressed at the early stages of bladder development and declined progressively throughout gestation. In adult bladder, both COX-1 and COX-2 were detectable at low levels under basal conditions. An ~30-fold increase in COX-2 mRNA was seen after 24 h of obstruction. In contrast, COX-1 did not change with obstruction. COX-2 mRNA levels peaked at 6 h of obstruction. In regional bladder-distention models, COX-2 induction was confined to the area of distention. Bladder outlet obstruction stimulates COX-2 expression dramatically, reactivating a gene that is highly expressed during fetal development.

MATERIALS AND METHODS

Dissection of fetal bladder tissues. Murine gestation is typically 20–21 days, with the day of conception being designated as embryonic day 0 (E0.0). The developing bladder or its progenitor tissue was dissected from murine CD-1 embryos at gestational ages E11.5, E14.5, E17.5, and E20.5 (newborn), and from adult animals. After E14.5, the anatomic configuration of the developing bladder has assumed that of mature animals. At E11.5, however, the true bladder does not yet exist; the nephric ducts and developing ureteric bud empty into the cloaca, which is the bladder progenitor (36). In E11.5 fetuses, therefore, the nephric ducts and ureteric buds were first identified as anatomic landmarks, and the portion of the cloaca into which these structures drained was identified under the dissection microscope using dark-field illumination. A segment of this tissue, ~1–2 mm2, at the site of the nephric duct orifices was dissected free. At later gestational stages, 1–2 mm2 full-thickness bladder tissue was harvested from each fetus. Three separate litters were analyzed for each gestational time point.

Bladder outlet obstruction. Adult female CD-1 mice weighing 30–40 g were anesthetized with an intraperitoneal ketamine (0.5 mg/kg) injection. Complete obstruction was created by ligating the urethra with 6–0 nylon, resulting in a progressive bladder distention. Female mice were used exclusively because of relative ease of creating surgical obstruc-
tion. Although urethral obstruction is possible in males, it requires a more extensive manipulation of bladder and urethra due to the presence of various male sex organs (prostate, seminal vesicle), which may create a greater degree of local inflammation. Currently, there is no data, either clinical or experimental, that implicate a differential obstructive response in bladder between sexes. No specific hydration or diuresis was performed. Animals recovered from anesthesia and were maintained with an ad libitum supply of standard mouse diet and water. Sham-operated mice served as negative controls (n=3) for bladder obstruction, where bladder and urethra were identically manipulated and dissected but left unobstructed. Unoperated, normal mice served as additional negative controls (n=3). Bladder tissues were harvested after 3, 6, 12, and 24 h of obstruction (n=6 for 24-h obstruction and n=3 for other time points). To localize the site of COX variation, epithelial and stromal layers were separated by microscopic dissection. Whole bladders were also obtained for both normal (n=3) and obstruction as above (n=2 for each 3, 6, and 24 h time points) for histological analysis and Western immunoblot analysis.

Regional bladder distention. A model of local bladder distention was used to study the role of wall stretch in COX-2 induction (40). A polyethylene ring (8 mm length, 4 mm width) was placed around the proximal half of the bladder, and the urethra was ligated as above, resulting in bladder distention confined to the distal half (n=4). After 4 h of obstruction, tissue was harvested for reverse transcription-polymerase chain reaction (RT-PCR) from the proximal and distal portions of the bladder. Tissue was also harvested from similar regions of the negative control (no obstruction) and positive control (conventional obstruction) bladders (n=2 for each).

Tissue handling. Dissected tissues (1-3 mm² pieces) were transferred into 100 µl guanidine isothiocyanate (GITC) buffer (4 M GITC, 25 mM sodium acetate, 0.8%β-mercaptoethanol, pH 6.0), snap frozen in liquid N₂, and stored at −80°C. The whole bladders were identically manipulated and dissected but left unobstructed. Unoperated, normal mice served as additional negative controls (n=3). Bladder tissues were harvested after 3, 6, 12, and 24 h of obstruction (n=6 for 24-h obstruction and n=3 for other time points). To localize the site of COX variation, epithelial and stromal layers were separated by microscopic dissection. Whole bladders were also obtained for both normal (n=3) and obstruction as above (n=2 for each 3, 6, and 24 h time points) for histological analysis and Western immunoblot analysis.

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RNA isolation and cDNA preparation. Samples in GITC were thawed on ice and sonicated for 10–15 s. Twenty micrograms of ribosomal RNA from Escherichia coli (Boehringer-Mannheim, Indianapolis, IN) were added as a carrier, and 100 µl of sonicated samples were layered onto a discontinuous cesium chloride gradient (100 µl of 97% and 20 µl of 40% CsCl in 25 mM sodium acetate). Samples were centrifuged for 2 h at 300,000 g using Beckman TLA-100 ultracentrifuge (Beckman Instruments, Fullerton, CA). RNA pellets were resuspended in 0.3 M sodium acetate and precipitated with linear acrylamide, 4 M ammonium acetate, and 100% ethanol. The pellets were redissolved in 20–40 µl of Tris(hydroxymethyl)aminomethane-EDTA (Tris-EDTA) buffer.

Primer selection and PCR. Primers were selected based on previously published murine or rat COX-1 (8), COX-2 (15), and human β-actin (11) sequences found through GenBank database search. In initial studies, primer pairs were verified to yield a single product of expected size. The COX-1 primers used were as follows: sense, 5’-CTG CTG AGA GGG TGG TTC CAT 3’ (bp 602–621); antisense, 5’-GTC ACA CAC AGG GTT ATG CT 3’ (bp 981–1,000), amplifying an 398-bp product. A 584-bp COX-2 fragment was amplified using the following primers: sense, 5’-ACA CTC TAT CAC TGG CAT CC 3’ (bp 1,229–1,248); antisense, 5’-GAA GGG ACA CAC TCT CAT AC 3’ (bp 1,794–1,813). A 350-bp β-actin product was amplified using the following primers: sense, 5’-AAG CGC GGA ATG AGT ACC CAG ATC ATG TTG TTT 3’ (bp 703–733). PCR reactions were performed in the presence of 200 µM dNTP, 10 mM dithiothreitol, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 0.5 pmol of each primer, 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 1.5 µCi [32P]dCTP (Amersham, Arlington Heights, IL), and 1-5 µl of tissue cDNA (total volume 50 µl). After initial denaturation at 94°C for 3.5 min, PCR amplification was performed for 30–32 cycles at 94°C (denaturation), 56–58°C (anneal), and 72°C (extend) for 1 min each. An additional 8-min incubation at 72°C was done before completion.

PCR product confirmation and semiquantitative assessment. After amplification, PCR products were subjected to size separation by polyacrylamide gel electrophoresis. Product identity was further confirmed by restriction digest of PCR products using standard commercially available enzymes. For example, based on published murine COX-2 sequences, Pst I and Hinf I (both from Boehringer-Mannheim) were expected to cut our COX-2 RT-PCR products into 240/340 and 190/390 base pair fragments, respectively. COX-1 and β-actin products were confirmed in a similar manner. A limiting dilution method was used to make semiquantitative comparisons between cDNA samples, with PCR reactions performed on 1:1, 1:10, 1:100, and, if necessary, 1:1,000 dilutions. Product abundance was assessed in the limiting concentration range. All samples were normalized for β-actin expression. Band intensity was determined with Phosphor Analyst software on GS-250 Molecular Imager System (Bio-Rad, Hercules, CA). Positive controls for each COX-1 and COX-2 PCR assay were cDNAs obtained from adult mouse kidneys. Water and dissection medium blanks were run as controls for cDNA contamination.

Western immunoblot analysis. The whole bladders were thawed on ice and homogenized. The protein concentration of the bladder lysates was determined by spectrophotometric assays using commercial colorimetric reagents (Bio-Rad). The lysates were heated to 100°C for 10 min to cause denaturation. Proteins (75 µg total) were then subjected to electrophoresis under reducing conditions in 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad) using the LKB Multiphor II semidry electrophoresis apparatus (Pharmacia). The blot was initially blocked for 2 h in Tris-buffered saline (pH 7.5) containing 3% nonfat dry milk, followed by incubation for 30 min with the rabbit anti-murine polyclonal antibody to COX-2 (Cayman Chemical, Ann Arbor, MI) at 1:500 dilution. The second antibody was a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) at 1:20,000 dilution. Blots were developed using the ECL chemiluminescent reagent (Amersham) and subjected to autoradiography as directed by the manufacturer.
RESULTS

Confirmation of COX-1 and COX-2 mRNA in bladder. Both COX-1 and COX-2 mRNA were detectable at low levels in normal adult bladders by RT-PCR. cDNAs from bladder yielded products of the expected band size for β-actin (350 bp), COX-1 (398 bp), and COX-2 (584 bp), with minimal contaminating bands. Product identity was further confirmed by specific restriction digests, which yielded the expected fragment sizes. An example for COX-2 RT-PCR product confirmation is shown in Fig. 1.

COX-2 expression during fetal bladder development. Semiquantitative RT-PCR analysis for COX-2 mRNA expression during fetal bladder development revealed the highest level at E11.5 (~100-fold higher than adult, based on β-actin normalization). COX-2 expression levels remained above the adult levels throughout fetal development, although they declined progressively through subsequent gestational time points. Levels at birth (E20.5) were similar to that seen in adult bladder (Fig. 2).

COX-1 and COX-2 expression after bladder outlet obstruction. At all time points of obstruction, bladders were markedly distended on gross inspection compared with those from control animals. Sham-operated mice had bladders that were normal in appearance after 24 h. Histological examination of obstructed bladders by routine hemotoxylin and eosin staining after 3 and 6 h of obstruction did not reveal any inflammatory infiltrate, although occasional areas of inflammatory cells (mostly neutrophils) and tissue necrosis were detectable at 24 h of obstruction.

Semi quantitative comparison of COX-2 RT-PCR product abundance based on β-actin normalization revealed a median 30.2 ± 5.6-fold relative increase (n = 6, P < 0.01) in bladders obstructed for 24 h compared with controls (Fig. 3). COX-2 levels did not differ between sham-operated and normal bladders. COX-1 levels, in contrast, did not change with obstruction (Fig. 4). When epithelial and stromal layers were separated by microscopic dissection and analyzed by RT-PCR, COX-2

![Fig. 1. Confirmation of cyclooxygenase-2 (COX-2) reverse transcription-polymerase chain reaction (RT-PCR) products obtained from murine bladders. Product was of expected size (584 bp) and was completely digestable in a sequence-specific manner by restriction endonucleases Pst I and Hinfi I. Positions of restriction sites within COX-2 PCR product are shown by arrows. Identity of COX-1 RT-PCR product was confirmed in a similar manner.](http://ajprenal.physiology.org/)

![Fig. 2. RT-PCR analysis demonstrating the pattern of COX-2 mRNA expression during fetal bladder development. Day of conception is designated as embryonic day 0 (E0.0), and the litter is typically born at E20.5. At E11.5, bladder progenitor tissue, the cloaca, at the site of the nephric duct entry, was used for analysis. Comparably sized full-thickness bladder tissues were microdissected from embryos at each later gestational time point. Quantitative comparison of RT-PCR product abundance was made by normalizing for β-actin expression. COX-2 mRNA levels were highest at E11.5, progressively declining through subsequent gestational time points to low levels seen in adult bladders. Representative assay from 3 different experiments is shown.](http://ajprenal.physiology.org/)

![Fig. 3. RT-PCR analysis demonstrating COX-2 mRNA induction after 24 h of complete bladder outlet obstruction. Normal and sham-operated animal bladders served as negative controls. Based on normalization for β-actin expression, median 30.2 ± 5.6-fold induction (n = 6, P < 0.01; samples 1 and 2 are shown in this autoradiograph) of COX-2 mRNA was seen after 24 h of bladder obstruction.](http://ajprenal.physiology.org/)
mRNA was detectable in both at low levels under basal conditions. After obstruction, the induction of COX-2 occurred predominantly in the stromal layer (Fig. 5). In the time-course experiments ($n = 3$ for each time point), COX-2 mRNA levels reached the peak between 3 and 6 h of obstruction, and they gradually declined thereafter (Fig. 6). COX-2 induction was confirmed at the protein level by Western immunoblotting analysis. COX-2 protein (~72 kDa) was not detectable in any of the control bladders ($n = 3$), whereas progressively increasing levels were seen with increasing duration of bladder obstruction (Fig. 7).

In the experiments in which bladders were locally distended ($n = 4$), COX-2 was induced to a greater degree in the distal portion (distended) than in the proximal portion (nondistended) within the same bladder (Fig. 8). Similar analysis of COX-2 expression in both negative and positive control bladders did not reveal any regional differences between distal and proximal halves.

**DISCUSSION**

Our study clearly demonstrates that bladder wall stretch caused by complete outlet obstruction stimulates the expression of inducible cyclooxygenase, COX-2, at both mRNA and protein levels. In contrast, expression of the constitutive isoform, COX-1, was low (close to the detection limit by RT-PCR) under basal conditions, and it remained unaffected by obstruction. COX-2 mRNA induction was seen at the earliest time point that was examined (at 3 h of obstruction), and it reached the peak at 6 h. Thereafter, a gradual fall in COX-2 mRNA levels was seen with a longer duration of obstruction. This time course of bladder COX-2 induction after obstruction is consistent with an immediate early type of gene expression pattern. Immediate early genes are thought to be activated rapidly and transiently by extracellular stimulation to encode proteins that will participate in regulating transcription of other genes. COX-2 activation pattern in fibroblasts has also been described as an immediate early type, and there is evidence that COX-2 expression is regulated by both transcriptional activation and mRNA stabilization (7). The mechanism for gradual downregulation of COX-2 mRNA with longer obstruction is not known, but COX-2 mRNA is known to be short-lived (7). It is possible that some downstream COX-2 product may repress COX-2 transcription in a negative feedback fashion.

Although COX-2 message was detectable in both epithelial and stromal layers, its induction after obstruction occurred in the stromal layer. Our study does not identify the exact cell type responsible for COX-2 induction, but we believe that smooth muscle cells are a likely candidate for COX-2 activation in response to mechanical stretch. Previous studies have shown that mechanical stretch induces COX-2 expression in vascular smooth muscle cells (32) and in renal mesangial cells, mesenchymal cells of probably smooth muscle lineage (1). COX-1 levels did not change in renal mesangial cells after stretch stimulation (1), similar to our findings. Inflammatory cells (e.g., macrophages) may have contributed to COX-2 induction, but histological examination of the obstructed bladders did not reveal any inflammatory infiltrate after 3 and 6 h of obstruction, the time points at which COX-2 induction was the greatest. Increased wall tension due to elevated intravesical pressure may also have caused COX-2 induction. However, the difference in COX-2 levels...
between distended and nondistended portions within
the same bladder argues against the involvement of
either inflammation or elevated intravesical pressure
in COX-2 stimulation, since presumably, the intravesi-
cal pressure elevation should be equal within the same
bladder and the degree of inflammation similar even if
only a part of the bladder is distended. Thus it seems
reasonable to infer that local mechanical stretch plays
a key role in COX-2 activation in bladder smooth
muscle cells.

The COX-2 induction in bladder after obstruction
represents reactivation of a gene that is highly ex-
pressed during fetal development. COX-2 mRNA level
was nearly 100-fold higher for equal tissue mass at
E11.5 than in newborn or adult bladders. At this
gestational time point, the bladder progenitor doaca is
divided by the descending urorectal septum, and the
lower urinary tract begins to form (36). The function of
COX-2 in bladder development is not known, but its
involvement in the local regulation of proliferation
and/or apoptosis is possible. COX-2 expression can be
stimulated by growth factors and mitogens (7), and
COX-2 has been implicated in modulating apoptotic
pathways (18, 37).

Previous studies have established that bladder PG
synthesis is stimulated by distention. Bladder PG
synthesis was first reported by Gilmore and Vane (9) in
1971, who observed an elevation of circulating PGE2
after bladder distention. Distention of whole rat blad-
ders in vitro results in an intraluminal increase of
PGI2, PGE2, and thromboxane A2 (13). The type of PG
produced by the bladder varies somewhat with species,
but PGE2 and PGI2 seem to be the predominant prod-
ucts in human, rabbit, and rodents (12, 13, 16). In-
creased PG synthesis after local distention may be a
common response of hollow organs. Similar observa-
tions have been made for aorta (32) and gall bladder
(30). Our data suggest that such increase in bladder PG
synthesis after distention may occur, in part at least, by
induction of COX-2 gene expression.

Urinary PGs may be involved in the modulation of
micturition reflexes. Early experiments documented
ability of prostaglandins to induce a slow, tonic con-
traction of bladder smooth muscle cells in vitro (2). Topical
application of PGE2 onto quiescent rat bladders in vivo
was shown to induce a series of reflex bladder contrac-
tions (19). Similarly, in human subjects, intravesical
instillation of PGs into the bladder lowers the volume

Fig. 7. Western immunoblot analysis of COX-2 induc-
tion after variable duration of complete bladder outlet
obstruction. After whole bladder tissue lysates were
separated (total 75 µg protein/lane) using sodium do-
decyl sulfate-polyacrylamide gel electrophoresis, pro-
tiens were transferred onto a nitrocellulose membrane.
They were then hybridized with 1:500 rabbit anti-
murine COX-2 polyclonal antibody. Blots were devel-
oped using ECL chemiluminescent reagent. Lanes C
1–C 3, 3 normal bladders as negative controls. Rest are
bladders after 3, 6, 12, and 24 h of obstruction.

Fig. 8. Effect of local bladder disten-
tion on COX-2 expression. Polyethyl-
ene ring (4 × 8 mm) was placed over
proximal half of bladder, and urethral
obstruction was performed as before,
resulting in distended distal (D) half
and nondistended proximal (P) half
within same bladder. Bladder tissue
from each region was analyzed for
COX-2 expression by RT-PCR. Similar
regional analysis was performed in
negative control (normal) and positive
control (total distention) bladders. Ten-
fold dilutions were performed for each
sample to make appropriate quantita-
tive comparisons.

COX-2

β-actin

Negative control Positive control
Regional distention #1 Regional distention #2
threshold for bladder contractions, thus lowering the capacity (2, 3). Pharmacological inhibition of prostaglandin synthesis using COX inhibitors (e.g., indomethacin) has been found to lower the bladder tone in vitro (2) and to increase the bladder capacity and compliance in vivo (23). The effects of COX inhibitors can be reproduced by selective PG receptor antagonists (22). One of the potential local target of PGs may be the capsaicin-sensitive primary afferent nerve fibers in the bladder (22, 24). There is evidence that prostaglandins can sensitize capsaicin-sensitive nociceptive pathways by directly acting on nerve terminals (10). PG ability to trigger reflex bladder contractions is abolished when animals are pretreated systemically with capsaicin, an agent that selectively destroys a category of nonmyelinated afferent nerve fibers (24, 34). In human studies, the prerequisite requirement for PG effects in the bladder seemed to be the presence of intact neural pathways, further suggesting its role in activation of neural target cells (2, 3).

COX-2 induction in the bladder in response to obstruction may have significant clinical implications. It is a well-documented clinical phenomenon that the bladder responds to obstruction by developing hyperactivity of micturition reflexes (27). The precise molecular mechanism by which this phenomenon occurs is not known. The current study demonstrating COX-2 induction in the bladder after obstruction suggests an attractive hypothesis. That is, increased local prostaglandin synthesis, secondary to COX-2 induction, triggers reflex bladder contractions in the obstructed bladder. This hypothesis may be applicable to various syndromes of bladder outlet obstruction such as benign prostatic hyperplasia.

Cellular proliferation and hypertrophy is another adaptive response of the bladder to outlet obstruction (14, 17). A similar proliferative response is seen in the heart when it is subjected to increased workload and mechanical stretch (41). Various immediate early genes have been shown to be activated, including genes primarily expressed in the fetal period (14, 33, 41). Our data demonstrate that COX-2 is highly expressed during the period of active proliferation and differentiation in fetal bladder development. Thus COX-2 induction, shown to be associated with various settings of cell proliferation, may also play a role in the development of pathological bladder hypertrophy and hyperplasia in chronic partial obstruction.

Several studies have shown significant improvements in patients with idiopathic detrusor instability using COX inhibitors (e.g., indomethacin), but most patients could not sustain the treatment due to high incidence of side effects, particularly gastric discomfort (3). All the COX inhibitors currently in clinical use are isoform nonspecific, inhibiting both COX-1 and COX-2 (4, 38). Gastric mucosal cytoprotection is thought to be regulated by COX-1, and COX-2 specific inhibitors may be able to provide desired COX inhibition in the inflammatory and proliferative conditions without unwanted gastrointestinal side effects (26). COX-2 specific inhibitors, currently a focus of intense research in major pharmaceutical industries (38), might also be efficacious in reducing pathological changes associated with bladder outlet obstruction.

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