Evaluation of Urothelial Stretch-Induced Cyclooxygenase-2 Expression in Novel Human Cell Culture and Porcine in Vivo Ureteral Obstruction Models

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ABSTRACT

Obstruction and stretch induce cyclooxygenase (COX)-2 expression and prostanoid synthesis in urinary tissues, causing pain, inflammation, hypercontractility, and cell proliferation. Our objective was to characterize acute COX-2 induction during in vivo ureteral obstruction, establish a cell culture model of urothelial stretch-induced COX-2 expression, and evaluate whether mechanotransduction could alter transcriptional and post-transcriptional regulation of COX-2. We performed laparoscopic unilateral ureteral ligation in pigs and allowed progression for 1, 2, 6, 24, or 48 h. We evaluated COX-2 expression with reverse transcriptase (RT)-polymerase chain reaction (PCR) and immunoblotting. We cultured primary human urothelial cells on stretch plates, applied stretch for up to 48 h, and measured COX-2 expression by RT-PCR and immunoblotting, transcription with run-on assays, and mRNA stability with actinomycin mRNA decay assays. In vivo ureteral obstruction induced COX-2 expression 4-fold within 6 h, maintaining induction for 24 h. In cell culture, stretch induced COX-2 steady-state mRNA and protein within the first 3 h of stretch, maintaining this induction for over 6 h. Three hours of stretch doubled COX-2 transcription relative to unstretched controls and increased COX-2 mRNA half-life 3-fold. This is the first report to characterize in vivo temporal stretch-induced COX-2 expression in the urothelium and establish a primary urothelial cell culture model for the study of stretch-induced COX-2 mechanisms. This is also the first report to identify alterations in steady-state COX-2 mRNA having components of both transcriptional and post-transcriptional regulation of stretch-regulated COX-2. Future elucidation of COX-2 signaling may identify novel therapeutic targets for treating stretch and distension of urinary tissues.

Obstruction, distension, and stretch of the ureter are associated with severe pain, inflammation, hypercontractility, enhancement of cell proliferation, and tissue necrosis (Gulmi et al., 1998; Weiss, 1998). The most common cause of acute ureteral obstruction is urinary stone disease, a condition with a lifetime incidence of 13% in the United States (Ramello et al., 2000). Total societal costs arising from urinary stone diagnosis, treatment, pain management, and lost wages total over $2 billion annually (Clark et al., 1995). Despite this, ureteral physiology and pharmacology remains a poorly studied field within the clinical science of urology. There is a substantial gap in the knowledge of the physiologic changes that occur during ureteral obstruction, and this has severely limited the development of superior pharmacologic agents for symptomatic treatment of the disease. Because of this, narcotic therapy remains the mainstay of treatment for symptomatic ureteral obstruction despite sedation and addictive potential.

Prostanoid synthesis and release is central to the nociception, contractility, inflammation, and cell proliferation associated with ureteral obstruction (Cole et al., 1988; Weiss, 1998). The rate-limiting step in prostanoid synthesis is the two-step conversion of arachidonic acid to prostaglandin G2 and subsequent conversion to prostaglandin H2 (Foegh and Ramwell, 2001). This reaction is catalyzed by cyclooxygenase (COX). This enzyme exists in two isoforms: COX-1 and -2 (Kujuba et al., 1991). COX-1 is present in most human tissues and catalyzes homeostatic prostanoid synthesis, regulat-
2001). Although COX-1 expression can be regulated, it is usually considered to be expressed constitutively (Foegh and Ramwell, 2001). In contrast, COX-2 is present in most tissues at low levels but is substantially induced by local inflammatory and mechanical stimuli.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used effectively to treat pain and inflammation associated with obstructive uropathy (Basar et al., 1991). However, NSAIDs can cause gastric ulceration, inhibit platelet aggregation, and impair renal function (Oren and Ligumsky, 1994; Colletti et al., 1999). NSAIDs cause renal vasoconstriction and altered renal hemodynamics in various animal models particularly in the presence of renal insufficiency (Permutter et al., 1993; Feldman et al., 1997). Selective COX-2 blockade is an intriguing therapy for symptoms of urinary tract distension and stretch. These medications provide potent analgesia with fewer toxic side effects than nonselective COX inhibitors (Lanza et al., 1999). However, selective COX-2 inhibitors can also be associated with renal side effects, particularly during ureteral obstruction when renal reserve is decreased (Hernandez et al., 2002), and recent reports suggest that high-dose treatment with COX-2 inhibitors is associated with cardiovascular events (Mukherjee et al., 2001). Elucidation of cellular mechanisms that couple distention of the urinary tract with increased COX-2 activity may identify targets of drug action directed specifically at COX-2 induction.

In vivo obstruction and distension induces COX-2 expression in the urinary bladder, ureter, and kidney (Seibert et al., 1996; Park et al., 1997; Nakada et al., 2002). The purpose of this study was to characterize COX-2 induction in a reproducible culture model of ureteral urothelial cell stretch and compare this model with the acute obstructive condition. In addition, we present data indicating that the nature of this induction is both transcriptional and post-transcriptional in nature.

Materials and Methods

Cyclooxygenase-2 Expression during Human Ureteral Obstruction

**Human Ureteral Segments.** We obtained normal human ureteral tissues as excess segments of ureter from patients undergoing donor nephrectomy (n = 10, six males, four females; average age = 47 years, range 34–60 years) and obstructed ureteral tissue from patients undergoing stricture repair (n = 10, seven males, three females; average age 27 years; range 2–62 years). All human tissues were obtained with proper informed consent and institutional review board approval. Obstructed ureteral segments were obtained from patients with chronic (lasting greater than 2 weeks) ureteral or ureterovesical junction obstruction with severity enough to warrant surgical intervention for hydrourephrosis, nephritis, or debilitating distension-induced pain. Ureteral segments were placed immediately in Krebs physiological salt solution, pH 7.4, composed of 119 mM NaCl, 1 mM NaHPO4, 4.7 mM KCl, 2.5 mM CaCl2, 0.5 mM MgCl2, 25 mM NaHCO3, and 25 mM glucose. Specimens were transported to the laboratory in Krebs buffer (45 min). We removed adherent fat and serosa and divided the segments into rings, 3 to 4 mm in length. Segments were either snap-frozen in liquid nitrogen and stored at −80°C for RNA or protein analysis or fixed in 10% buffered formalin for immunohistochemical analysis.

**Extraction of RNA, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction.** Frozen ureteral segments were placed in 1 ml of TRIzol solution (acid guanidium thiocyanate phenol; GibcoBRL, Grand Island, NY) and homogenized with a Powergen 135 homogenizer (Fisher Scientific Co., Pittsburgh, PA) on ice for 1 min. Messenger RNA was extracted using the TRIzol extraction protocol in conditions recommended by the manufacturer and previously reported by this laboratory (Nakada et al., 2002). RNA yield was quantified using a DU 640B spectrometer (Beckman-Coulter, Fullerton, CA). For cDNA synthesis, extracted total RNA (3 μg) was added to excess Moloney murine leukemia virus reverse transcriptase (RT; Promega) in buffer supplied by the manufacturer along with excess random hexamers and deoxyribonucleotide triphosphates (dNTPs). After a 1-min denaturation period at 65°C, reverse transcription was performed at 37°C for 2 h.

We performed quantitative real-time PCR on all human and porcine tissue RNA-cDNA samples using a standard curve for each gene tested and a “nested” primer technique. From the published sequences of human COX-2 and ribosomal protein S26 and porcine COX-2 and glyceraldehyde-6-phosphate dehydrogenase (GAPDH), Oligonucleotide primers (20–23 base pairs) were synthesized that targeted regions of cDNA 400 to 800 bases in length. The primer sequences were 5’ to 3’ as follows: human COX-2 (NM-000963), sense (GGCGTCAGGACCATAGCAAT; starting base (sb), 171) and antisense (TGGAACATTCTGACACGCA; sb, 1394); human S26 (U-41448), sense (GAAGCCTTTCCACCTGTA; sb, 69) and antisense (TCACCTCCTTGGCTTCCATT; sb, 1544); pig COX-2 (AF-207824), sense (CCATGGGTGTGAAGGGGAGG; sb, 184) and antisense (CCAAAGCGGAGGTGGTCTCAG; sb, 537); and pig GAPDH (AF-17079), sense (ACCCGAAGACTGAGGTG; sb, 884) and antisense (TGAGCTGACAAATGTTGCT; sb, 1246). PCR was performed on samples of human or porcine cDNA for 38 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min. In all PCR reactions, cDNA equivalent to 50 ng of untranscribed RNA was amplified with 1.25 U of TaqDNA polymerase (Promega) in PCR reaction buffer (Promega) with 1.5 mM MgCl2, 1 μM primers listed, and 200 μM dNTPs. All reactions were resolved with electrophoresis; the band of interest was cut out of the gel and purified the DNA using the GeneClean nucleic acid purification kit with conditions recommended by the manufacturer. Concentration of cDNA (μg/ml) was quantified using a DU 640B spectrometer (Beckman-Coulter), and number of copies per microliter was determined.

We performed real-time PCR in 10−μl reactions using a Rotor-gene 2000 thermal cycler/reader and accompanying software, version 5.0 (Corbett Research, Mortlake, New South Wales, Australia). A standard curve of known copies of cDNA for each gene studied was performed in duplicate in incremental log fashion, from 10 to 106 copies, along with DNA-RNA-free water as a control. PCR for each gene of interest was performed using secondary oligonucleotide primers amplifying DNA from within standard curve amplicons; these internal primers amplified 100- to 125-base pair amplicons. The primers (from 5’ to 3’) were as follows: human COX-2, sense (CCCAGCAGCTTTGAGCATCAG; sb, 697) and antisense (CGCTGTCTACGAGAGTTCCGAC; sb, 795); human S26, sense (GGCGTCCTCGATGTAAGTGG; sb, 675) and antisense (ACTGGTGGTTTGGACCTTGGAG; sb, 779); pig COX-2, sense (CCCGATTCAAAGAGTGGTGG; sb, 213) and antisense (TCAGTGTAAGTGCTCTG; sb, 304); and pig GAPDH, sense (GCTCTGAGGTTGCTGCGTCAAG; sb, 960) and antisense (TCAAGCTCAACGCGACACG; sb, 1052). Unknowns were performed with two dilutions of cDNA corresponding to 5 and 10 ng/ml untranscribed RNA in duplicate. All reactions were performed with 8 μl of Platinum qPCR Supermix (Invitrogen, Carlsbad, CA), 2.0 mM MgCl2, 400 nM primers, 200 μM dNTPs, and 1 μl of 10× Sybr Green I (Molecular Probes, Eugene, OR). Reactions were brought to 95°C for 3 min followed by 40 cycles of 95°C for 15 s (melting), 60°C for 20 s (annealing), 72°C for 15 s (extending), and
78°C for 15 s for melting any oligonucleotide dimers. Fluorescence was recorded at the conclusion of each cycle; samples were excited at 470 nm, and detection was performed at 585 nm using a high-pass detection filter for optimal sensitivity of Sybr Green I. After the conclusion of 40 cycles, each reaction was gradually heated from 78 to 95°C with simultaneous fluorescence capture to determine melting temperature of the amplicons and ensure PCR integrity. A cycle threshold of linearity was set for each reaction. The number of cycles necessary for each reaction to meet this threshold was determined and compared with the standard curves for each gene of interest, and number of copies for each gene was determined. mRNA level analysis was expressed as copies of COX-2 per copy of S26 (human) or GAPDH (porcine). The correlation coefficients of standard curve amplification was 0.997 for human COX-2, 0.891 for human S26, 0.994 for porcine COX-2, and 0.990 for porcine GAPDH. The lower limit of detection (as determined by the lowest dilution of known copy number to fit on the linear range of the standard curve) was 10 copies of dsDNA. All samples analyzed (obstructed/normal ureters, stretched/unstretched cells, etc.) were calculated to contain between 100 and 100,000 molecules of DNA per milliliter (per nanogram) and fit into the standard curve (10–100,000) for each gene.

**Western Blot Analysis of Protein Levels.** Ureteral segments (0.1 g) were homogenized in protease inhibitor containing lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, aprotinin, and antipain). Triton X-100 was added to a concentration of 1.0%, and the homogenate was incubated for 30 min at 4°C, followed by centrifugation for 10 min at 14,100g. The protein collections (20 μg/well) were resolved by electrophoresis in 10% SDS-polyacrylamide gel electrophoresis gel. Proteins were transferred to nitrocellulose blotting membranes, blocked overnight in 10% nonfat dry milk, 10% electrophoresis gel. Proteins were transferred to nitrocellulose blotting membranes, blocked overnight in 10% nonfat dry milk, 10% milk each of leupeptin, aprotinin, and antipain). Triton X-100 was added to a concentration of 1.0%, and the homogenate was incubated for 30 min at 4°C, followed by centrifugation for 10 min at 14,100g. The protein collections (20 μg/well) were resolved by electrophoresis in 10% SDS-polyacrylamide gel electrophoresis gel. Proteins were transferred to nitrocellulose blotting membranes, blocked overnight in 10% nonfat dry milk, 10% milk each of leupeptin, aprotinin, and antipain). Triton X-100 was added to a concentration of 1.0%, and the homogenate was incubated for 30 min at 4°C, followed by centrifugation for 10 min at 14,100g. The protein collections (20 μg/well) were resolved by electrophoresis in 10% SDS-polyacrylamide gel electrophoresis gel. Proteins were transferred to nitrocellulose blotting membranes, blocked overnight in 10% nonfat dry milk, 10% milk each of leupeptin, aprotinin, and antipain). Triton X-100 was added to a concentration of 1.0%, and the homogenate was incubated for 30 min at 4°C, followed by centrifugation for 10 min at 14,100g.

**Ureteral Cell Culture.** We isolated and grew primary urothelial cells in culture as described previously (Teng et al., 2002). Cells were grown in fresh Ham’s F12 nutrient medium until they achieved confluency (passage 1). The cells were split to a total of four passages. On the fifth splitting, cells were plated onto collagen-coated strech plates (57.75-cm² area per plate; Flexcell International Corporation, Hillsborough, NC).

**Stretching of Urothelial Cultures.** Eighty to 90% confluent cultured urothelial cells were placed on the cell stretch apparatus (FX-3000T; Flexcell International Corporation; version 3.2 Tension Plus software was used to control the apparatus. Tension (stretch) was applied to the bottom of the flexible stretch membranes by pressure-driven posts, controlled by the software. The first aim was to determine whether the method of stretch is optimal for COX-2 induction. We stretched cells for 6 h with static stretch of 3, 10, or 20% increase in cell surface area or cyclic stretch of 0 to 3%, 0 to 10%, 0 to 20%, or 5 to 20% increase in cell surface area, at a rate of 12 cycles/min. Nonstretched cells were used as controls. After we determined that 0 to 20% and 5 to 20% cyclic stretch produced optimal COX-2 induction (please see Results), we sought to determine the temporal induction of COX-2 to this stimulus. We stretched additional cells with 5 to 20% cyclic stretch for 1, 2, 3, 4, 5, 6, 12, 24, or 48 h, using nonstretched cells at each time point as controls. After applying stretch to the appropriate parameters, RNA or protein was harvested from the cells, and RT-PCR or immunoblotting was performed, as described above.

**Stretch Induction of Cyclooxygenase-2 Expression in Primary Human Urothelial Cells**

**Urothelial Stretch-Induced COX-2 Expression**

**Transcriptional Run-On Assays.** We grew 3 × 10⁶ urothelial cells using the parameters described above and stretched them for 3 h at 5 to 20% cyclic stretch, using nonstretched cells for controls. We performed nuclear run-on assays as described previously (Mangasarian and Mellon, 1993). In brief, we collected the cells in buffer A (20 mM Tris-Cl, pH 7.6, 2 mM MgCl₂, 3 mM CaCl₂, 3 mM 1,4-dithiothreitol, and 300 mM sucrose). The cells were centrifuged at 100g for 10 min, and the resulting pellet was suspended in 1 ml of buffer A. We added 1 ml of buffer B (buffer A + 0.2% v/v Nonidet P-40) to the cells and homogenized them with 20 strokes of a type 2 Dounce homogenizer. The homogenates were centrifuged at 500g for 10 min to collect the nuclei. The nuclei were washed in 2 ml of buffer C (40% v/v glycerol, 50 mM Tris-Cl, pH 8.3, 5 mM MgCl₂, and 0.1
mM EDTA) and centrifuged a second time at 500g for 10 min and resuspended in buffer C at a concentration of 10⁶ nuclei/ml.

Run-on reaction buffer (60 µl; 25 mM Tris-HCl, 12.5 mM MgCl₂, 750 mM KCl, 1.25 mM each of ATP, GTP, GTP, and 450 mM [3000 Ci/µM] UTP) was added to 210 µl of nuclei, and the reaction was incubated at 30°C for 30 min. Fifteen microliters of DNase I was added to digest DNA for 5 min. Five hundred microliters of TRIzol reagent (Invitrogen) was added, and the reaction was incubated on ice for 15 min. One hundred microliters of chloroform was added, followed by vigorous hand shaking for 15 s. We incubated the reaction an additional 3 min on ice followed by centrifugation for 15 min at 16,000g. The upper layer was collected, and equal volume of 100% isopropanol was added to precipitate the RNA. After incubation on ice for 15 min, the RNA was pelleted with centrifugation (16,000g, 15 min) and resuspended in 100 µl of Tris-EDTA buffer, pH 7.2. The entire content was filtered through a Nu-Clean D50 disposable spin column (Eastman Kodak Co, Rochester, NY) to remove free nucleotides (7535g centrifugation, 2 min), and 0.2 volumes of 1 M NaOH were added to denature the RNA for 10 min on ice. Following this, HEPEs (0.25 volumes) was added, and RNA reprecipitated by incubating overnight with ethanol.

The RNA pellet was resuspended in tris-EDTA and diluted in hybridization buffer (Ambion, Austin, TX) to a concentration of 1 x 10⁶ cpm/ml. We prepared membranes by blotting 20 µg of denatured DNA sequences of interest (COX-2, S26, or GAPDH cDNA) in 10X standard saline citrate onto nylon hybridization membranes (Amer sham, Arlington Heights, IL) using a slot blot apparatus. We cross-linked the DNA to the membrane by UV exposure (UV Stratalinker, Stratagene, La Jolla, CA) and verified the efficiency and consistency of preblotting by methylene blue staining. We hybridized run-on products to the membranes at 45°C for 48 h. Staining appeared stronger in the obstructed segments relative to normal segments.

RNA Decay Assays. We grew urothelial cells in supplemented Ham's F12 media as described and stretched them for 3 h with 5 to 20% cyclic stretch. The media was replaced with fresh media containing 2.5 µg/ml actinomycin D (optimized by 3H-uridine incorporation assay) to halt transcription. We harvested a plate of stretched and unstretched cells every 30 min after addition of actinomycin D in TRIzol buffer (Invitrogen). We extracted the RNA and determined COX-2 mRNA concentrations relative to S26 with real-time PCR, as described above. The half-life was determined by comparing the percentage COX-2 mRNA remaining at each time point with that at time 0.

Statistical Analysis. Data obtained using obstructed ureters in humans and pigs were analyzed with unpaired Student's t test comparing the obstructed group with the unobstructed control. Likewise, data obtained using stretched and unstretched cells using RT-PCR, immunoblotting, run-on assays, and mRNA decay assays were analyzed with unpaired Student's t test, comparing stretched cells with unstretched cells. All data in the manuscript are presented as mean ± S.E.M.

Results

COX-2 Expression in Human Ureteral Obstruction. Chronically obstructed human ureters expressed 8.85 (±1.65) copies of COX-2 mRNA per copy of S26, whereas normal human ureters expressed 2.02 (±0.38) copies of COX-2 per copy of S26 (n = 4, p = 0.02; unpaired Student's t test). Densitometry of western blots determined that the ratio of COX-2 to GAPDH protein in obstructed ureter was increased from 0.13 (±0.02) in normal ureter to 0.76 (±0.05) in obstructed ureter (n = 6, p = 0.003) (Fig. 1). The ratio of COX-1 to GAPDH was 0.59 (±0.06) in normal ureter and 0.55 (±0.10) in obstructed ureter (n = 5, p = 0.77) (Fig. 1). Immunohistochemistry staining showed localization of COX-2 in the urothelium as well as smooth muscle of the ureter (Fig. 2); staining appeared stronger in the obstructed segments relative to normal segments.

Temporal Induction of COX-2 Expression in Acute Porcine Ureteral Obstruction. Acute ureteral obstruction caused an immediate increase in intraureteral pressure as measured by manometry. Intraureteral pressure was 6 mm Hg (±0.5 mm Hg) in unobstructed ureters, whereas 2-h acute obstruction caused an increase to 86 mm Hg (±10 mm Hg). Obstruction of 48 h continued to exhibit substantial pressure increases (56 ± 12 mm Hg). Acute ureteral obstruction induced maximal COX-2 mRNA expression within the first 6 h of obstruction; induction was more substantial in the epithelium than in whole tissue or smooth muscle layer (Fig. 3A). In whole-tissue extracts, obstruction induced COX-2 mRNA expression (relative to contralateral controls) 2.1-fold in the 1st h, 3.2-fold after 2 h (p = 0.03), and 3.8-fold after 6 h of obstruction (p = 0.02) before subsiding to 2.7-fold after 24 h and 1.4-fold after 48 h of obstruction (n = 4, all time points). In urothelial layer extracts, obstruction induced COX-2 mRNA expression 2.7-fold relative to contralateral controls in the 1st h, 5.7-fold after 2 h (p = 0.004), and 7.9-fold after 6 h of obstruction (p = 0.006) before subsiding to 5.1-fold after 24 h (p = 0.02) and 3.8-fold after 48 h of obstruction. In the smooth muscle layer, obstruction induced COX-2 mRNA expression 3.2-fold (p = 0.05) after 6 h of obstruction, whereas the other time points exhibited nonsignificant induction.

Acute ureteral obstruction induced sustained induction of COX-2 protein expression in total tissue extracts, urothelium, and smooth muscle layers (Fig. 3, B and C). Although none of the preparations showed statistically significant induction after 1 or 2 h of obstruction, COX-2 protein was induced after 6 h of obstruction 5.9-fold in the urothelium

Fig. 1. Immunoblot analysis of COX-1 and -2 expression in three normal and three chronically obstructed human ureters. COX-2 was induced 6-fold in chronically obstructed human ureter, whereas no change was observed in COX-1 expression. These data confirm our previous finding of COX-2 induction in chronically obstructed human ureter.
hybridization was 0.60 (±0.05) in unstretched cells and 1.12 in stretched cells. The ratio of COX-2 to S26 of COX-2 transcription doubled after 3 h of stretch, as determined by nuclear run-on assay. The ratio of COX-2 to S26 mRNA was induced 4-fold within the 1st h of cell stretch after 3 h of stretch (the ratio of COX-2 copies to S26 copies was 1.64 in unstretched cells and 8.98 in stretched cells; p = 0.001) (Fig. 5A). COX-2 mRNA remained elevated for 6 h, but induction was reduced to 3-fold after 12 h of stretch and 2-fold after 48 h of stretch. COX-2 protein expression doubled after 3 h of stretch and increased linearly through 6 h of stretch to a 12-fold induction (Fig. 5, A and B). In contrast to mRNA expression, COX-2 protein expression remained elevated steady-state mRNA levels during cyclic stretch relative to unstretched controls was observed with the 5 to 20% cyclic stretch method (p = 0.001), whereas 0 to 10% stretch produced a 9-fold induction (p = 0.001). Twenty percent tonic stretched produced only a 4-fold induction (p = 0.01), and 0 to 3% cyclic and 10% tonic stretch did not produce statistically significant COX-2 induction.

Characterization of COX-2 Induction during Urothelial Cell Stretch. To determine the optimal method of cell stretch for COX-2 induction, four lines of primary human urothelial cells were stretched for 6 h using 10% tonic (constant), 20% tonic, 0 to 3% cyclic, 0 to 10% cyclic, or 5 to 20% cyclic methods (Fig. 4). A 12-fold induction in COX-2 protein relative to unstretched controls was observed with the 5 to 20% cyclic stretch method (p = 0.001), whereas 0 to 10% stretch produced a 9-fold induction (p = 0.001). Twenty percent tonic stretched produced only a 4-fold induction (p = 0.01), and 0 to 3% cyclic and 10% tonic stretch did not produce statistically significant COX-2 induction.

The optimal time point of COX-2 induction in urothelial cells was determined using 5 to 20% cyclic stretch (12 cycles/min) for 1, 2, 3, 4, 5, 6, 12, 24, and 48 h (n = 4). Steady-state COX-2 mRNA was induced 4-fold within the 1st h of cell stretch (p = 0.01) and was maximal with a 6-fold induction after 3 h of stretch (the ratio of COX-2 copies to S26 copies was 1.64 in unstretched cells and 8.98 in stretched cells; p = 0.001) (Fig. 5A). COX-2 mRNA remained elevated for 6 h, but induction was reduced to 3-fold after 12 h of stretch and 2-fold after 48 h of stretch. COX-2 protein expression doubled after 3 h of stretch and increased linearly through 6 h of stretch to a 12-fold induction (Fig. 5, A and B). In contrast to mRNA expression, COX-2 protein expression remained elevated at a 12-fold induction for 24 h before subsiding to an 8-fold induction at 48 h.

COX-2 Transcription and Message Stability. The rate of COX-2 transcription doubled after 3 h of stretch, as determined by nuclear run-on assay. The ratio of COX-2 to S26 hybridization was 0.60 (±0.05) in unstretched cells and 1.12 (±0.03) in cells stretched for 3 h (Fig. 6). Steady-state COX-2 mRNA was induced 6-fold in these cells. Treatment with 10 μg/ml IL-1β for 4 h induced a 2.5-fold induction in COX-2 transcription and a 5-fold induction in steady-state COX-2 message, whereas 1 μM phorbol ester doubled COX-2 transcription and induced a 4-fold induction of steady-state COX-2 mRNA expression (control, 1.2 copies COX-2/copy S26; IL-1-treated, 6.0 copies COX-2/copy GAPDH; TPA-treated, 4.6 copies COX-2/S26).

The half-life of COX-2 mRNA in unstretched cells was determined to be 1.05 (±0.09) h in unstretched urothelial cells. This was increased to 3.01 (±0.54) h in cells stretched for 3 h, accounting for a 3-fold increase in COX-2 message stability (n = 4; p = 0.009) (Fig. 7). Pretreatment of unstretched cells with IL-1β increased COX-2 half-life to 2.91 (±0.49) h (n = 4; p = 0.02), whereas treating the cells with phorbol ester increased COX-2 message half-life to 2.4 (±0.51) h (n = 4; p = 0.04, data not shown).

Discussion

This is the first report characterizing the time course of COX-2 induction during ureteral obstruction in the urothelium and the first to establish a cell culture model relating to this response. In addition, this is the first report to describe elevated steady-state mRNA levels during cyclic stretch results from both transcriptional and post-transcriptional components. This novel cell culture stretch model will be critical for studies aimed at identifying key mechanotransduction events in urothelial cells. In addition, our model of ureteral obstruction will be critical for the in vivo evaluation of novel therapeutic agents for the symptomatic treatment of ureteral obstruction.

Our method of 20% cyclic (12 cycles/min) urothelial cell stretch was based on preliminary characterization as well as in vivo parameters; 20% cell stretch correlates to an increase in cell surface area observed during ureteral obstruction in which intraluminal pressures achieve 40 mm Hg (Flexcell International Corporation Loading Stations Manual, www.flexcellent.com; this was used to determine the percentage cell stretch in relation to pressure; 25-mm plates were used, 20% stretch correlates to 90.09 KPa, 40 mm Hg). This is lower than the 50 to 70 mm Hg pressure observed during...
acute ureteral obstruction (Vaughan et al., 1971). In addition, our pig model demonstrated ureteral pressures of 86 mm Hg and peristaltic rates in excess of 12 cycles/min (data not shown); therefore, COX-2 induction in the cell model is not likely due to egregious stretching in vitro. COX-2 expression is induced faster in the cell model because significant induction of COX-2 mRNA occurs within the 1st h of applying stretch in culture. COX-2 expression is also slightly more transient in cell culture because mRNA induction is maximal by 3 h in culture but continues to increase in vivo through 6 h of obstruction. COX-2 protein expression remains elevated

Fig. 3. Temporal induction of COX-2 expression during acute ureteral obstruction. A, mRNA induction (as measured by quantitative RT-PCR) is observed in both urothelium and smooth muscle, as indicated relative to internal contralateral controls. Greater induction is observed in the urothelium; induction is significant (\( p < 0.05 \)) in urothelium at 2, 6, and 24 h of obstruction. Induction in the smooth muscle is significant at 6 h of obstruction, whereas induction of COX-2 in the urothelium at 6 h of stretch was significantly higher than in the smooth muscle (\( p < 0.05 \)). B, immunoblot analysis of whole-tissue protein preparations (1) and urothelial protein preparations (2) in obstructed and normal (contralateral) ureteral segments. Time postsurgery (obstruction or sham) in hours is indicated. C, protein induction measured by densitometry of immunoblots and expressed as ratio of COX-2 to GAPDH in obstructed ureters relative to contralateral ureters. Significant COX-2 induction (\( p < 0.05 \)) was observed in the urothelium and smooth muscle after 6, 24, and 48 h of obstruction. COX-2 induction in the urothelium was significantly higher than in smooth muscle at 24 and 48 h (\( p < 0.05 \)).

Fig. 4. Immunoblotting of protein extracted from human urothelial cells after stretching for 6 h with cyclic or tonic stretch, as indicated. Zero to 10% and 5 to 20% cyclic stretch were optimal for COX-2 induction (9–12-fold induction), whereas 20% tonic stretch produced suboptimal (4-fold) induction.

Fig. 5. A, stretch-induced COX-2 mRNA (copies of COX-2/S26, left axis) and protein expression (ratio of COX-2 to GAPDH, right axis) in cultured human urothelial cells. Cyclic stretch (5–20%) significantly induced COX-2 expression in the 1st h of stretch and maximized at 3 h of stretch before reducing. In contrast, COX-2 protein induction continued to increase through 24 h of stretch before reducing by 48 h. B, immunoblots of COX-2 induction in urothelial cells from 1 to 48 h (left) and 1 to 6 h (right) of stretch.

Fig. 6. Phosphorimaged nuclear run-on blots of hybridized nascent COX-2, GAPDH, and S26 mRNA in stretched, unstretched (control), 10 ng/ml IL-\( \beta \)-treated, and 1 \( \mu \)M phorbol ester (TPA)-treated cells. Stretching and TPA treatment doubled the transcriptional rate of COX-2 relative to S26, whereas IL-\( \beta \) treatment increased transcription 3-fold. These increases in transcription are significantly less than the induction of steady-state COX-2 mRNA for these treatments, suggesting post-transcriptional regulation.
through 48 h in vivo but is reduced to half of its induced state at this time point in the cell model. This effect is likely due to rapid and precise stretch and isolation of urethelial cells in this system. In vivo ureteral pressures that cause distension, cell stretch, and peristalsis generate more gradually than the instantaneous application of stretch that is achievable in the cell model. In addition, urethelial cells in vivo are subjected to numerous factors present in systemic circulation or released in paracrine tissues that may alter the response.

COX-2 induction occurs in both the urothelial and smooth muscle layers of the ureter, but induction is more substantial in the urothelium. The presence of strong COX-2 localization in urothelium as identified by immunohistochemistry, in addition to previous reports indicating the urothelium as the primary prostaglandin producing layer of the ureter (Ali et al., 1998), further validates the establishment of urothelial cell culture for studying cell stretch signaling. However, our data also indicate that COX-2 induction does occur in smooth muscle, and COX-2 is induced in smooth muscle of urinary bladders following outlet obstruction (Park et al., 1997). Because smooth muscle makes up a majority of ureteral tissue, future studies establishing ureteral smooth muscle cultures for the study of COX-2 signaling are warranted.

Our data indicate that stretch-induced COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms. Although numerous reports of COX-2 induction via stretch, pressure, or shear stress exist, most of these studies evaluated COX-2 steady-state expression or transcriptional induction in artificial reporter assays. Inoue et al. (2002) reported increased COX-2 message stability in response to shear stress of endothelial cells, and our data are the first reported induction of COX-2 message stability in response to cell stretch. Future studies investigating the mechanism of stretch-induced COX-2 message stability are warranted.

These results add to recent studies that have identified numerous signals regulating COX-2 message stability. Activation of mitogen-activated protein kinases, especially p38, appears to be influential in COX-2 message regulation (Mifflin et al., 2002). This is most pronounced during cytokine induction of COX-2. p38 also appears influential in the COX-2 message stability associated with carcinogenesis (Dixon, 2004). Since many physiological triggers are p38-dependent, the role of this kinase in stabilizing COX-2 message is substantial. The 3'-untranslated region of COX-2 message is rich in AUUUA repeat elements, and it appears this region is primarily involved in conferring stability to the message (Srivastava et al., 1994; Cok and Morrison, 2001). The inhibitory effect of dexamethasone on COX-2 expression is at least in part due to the shortening of this region (Newton et al., 1998), and stability-affecting proteins including HuR, AU-rich binding-factor 1,2, tristetraprolin, and cytosine uridine guanine triplet repeat binding protein are known to bind to this region of the transcript (Nabors et al., 2001; Mukhopadhyay et al., 2003). The effect of p38 on COX-2 message stability also appears to involve prevention of shortening of the 3'-untranslated region (Dean et al., 2003).

Although induction of COX-2 mRNA is transient, protein induction persists for over 24 h in the cell model and beyond 48 h in vivo. Although this may reflect an accumulation of stable COX-2 protein, it is also possible that translational efficiency and protein stability are regulated during stretch. The translational silencer TIA-1 binds COX-2 mRNA and mediates translation to protein. TIA-1 activity is substantially reduced in COX-2-overexpressing colon cancer cells (Dixon et al., 2003). In addition, TIA-1 knockout mice exhibit severe arthritis that is associated with COX-2 overexpression in macrophages (Phillips et al., 2004). The studies of translational regulation of COX-2 protein expression and regulated COX-2 protein stability are likely to unveil novel regulatory elements, and the role cell stretch plays in these processes would be of particular interest.

Stretch induces COX-2 expression in vascular endothelial cells, osteoblasts, chondrocytes, renal podocytes, and gastrointestinal epithelial cells (Fitzgerald et al., 2004; Martineau et al., 2004). Several cellular signal transduction cascades are known to play a role in COX-2 induction, including those involving mitogen-activated protein kinases, protein kinase A, protein kinase C, nuclear factor κB, steroid hormone receptors, and extracellular matrix proteins. However, most of these studies involved hormone or mitogen-induced conditions, and data investigating mechanosensing pathways of COX-2 induction remain sparse. Stretch-induced COX-2 expression in bladder smooth muscle and renal podocytes does appear to be p38-dependent (Park et al., 1999; Martineau et al., 2004), and deletion analysis of COX-2 promoter elements has determined the importance of C/EBPβ, cAMP response element, and activator protein-1 elements in shear stress-induced COX-2 expression in murine osteoblasts (Ogasawara et al., 2001). However, the triggering cascades of these molecules are yet to be determined in mechanically stimulated cells. The cellular mechanisms by which stretch induces COX-2 expression are an active area of investigation and will likely lead to pharmacologic targets of intervention for stretch and pressure-related diseases.

References


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