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Cyclooxygenase-2 protein and prostaglandin E_2 production are up-regulated in a rat bladder inflammation model

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Abstract

Cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins and prostaglandin E_2 production are evaluated in a rat model of inflammation in which *Escherichia coli* lipopolysaccharide is intraperitoneally injected or intravesically instilled into the bladder. While cyclooxygenase-1 mRNA and protein and cyclooxygenase-2 mRNA do not change in bladders treated with lipopolysaccharide, cyclooxygenase-2 protein is elevated in bladders from rats intravesically instilled with lipopolysaccharide or phosphate buffered saline (PBS) or intraperitoneally injected with lipopolysaccharide. Urinary prostaglandin E_2 levels and prostaglandin E_2 synthesis in bladder particulates are elevated by intravesical instillation and intraperitoneal injection of lipopolysaccharide. The nitric oxide donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine, increases prostaglandin E_2 synthesis in bladders from lipopolysaccharide intravesically instilled and intraperitoneally injected rats. Lipopolysaccharide increases prostaglandin E_2 synthesis by increasing cyclooxygenase-2 protein levels in rat bladder and prostaglandin E_2 synthesis may be further elevated by increases in nitric oxide caused by an up-regulation of inducible nitric oxide synthase (iNOS). © 2001 Published by Elsevier Science B.V.

Keywords: Prostaglandin; Cyclooxygenase; Nitric oxide (NO); Bladder infection; Bladder inflammation; Nitric oxide (NO) synthase, inducible

1. Introduction

Prostaglandins affect the micturition reflex and have a cytoprotective role in the bladder. Although urinary prostaglandins can originate from the kidney, it is well established that the bladder also is a site of prostaglandin synthesis (Abrams et al., 1979; Reyes and Klahr, 1990). Prostaglandin E_2 and prostaglandin I_2 are the predominant prostaglandins in the rat and human bladder. Prostaglandin synthesis in the bladder is modulated by a number of stimuli including bladder distension, inflammation, vesical mucosal trauma, nerve stimulation, muscarinic receptor stimulation and the chemotactic peptide, *N*-formylmethionyl-leucyl-phenylalanine (Mikhailidis et al., 1987). In addition, prostaglandin I_2, prostaglandin E_2 and thromboxane A_2 levels (Jeremy et al., 1984) as well as cyclooxygenase-2 expression (Park et al., 1997) are elevated in rat bladders during distention. Increased levels of urinary prostaglandin E_2 have been demonstrated during human urinary tract infections (Farkas et al., 1980, Wheeler et al., manuscript in preparation).

Two cyclooxygenase or prostaglandin H_2 synthase isoforms have been identified; cyclooxygenase-1 and cyclooxygenase-2 (Vane, 1998). Cyclooxygenase-1 and cyclooxygenase-2 are the key regulatory enzymes involved in the biosynthesis of prostaglandins from arachidonic acid. The cyclooxygenase-1 isoform is constitutively expressed in many cell lines, whereas the cyclooxygenase-2 isoform is induced in response to cytokines and other inflammatory factors. Cyclooxygenase-2 also can be expressed constitutively and constitutive cyclooxygenase-2 mRNA expression is greater in the kidney and urinary bladder than in other rabbit tissues (Guan et al., 1997).

In order to assess the role of cyclooxygenase-1 and cyclooxygenase-2 in inflammatory processes in the bladder, we used a previously described (Olsson et al., 1998; Stein et al., 1996) inflammation model in which protamine/*Escherichia coli* lipopolysaccharide is instilled into rat bladder. Four hours after the intravesical instillation of protamine/lipopolysaccharide, but not phosphate buffer saline (PBS), there is an influx of neutrophils, an up-regulation of interleukin-6, interleukin-10 and inducible nitric oxide synthase (iNOS) mRNA, and an increase in NOS

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activity in the bladder but not in the lung or kidney. When lipopolysaccharide is injected intraperitoneally, interleukin-6, interleukin-10 and iNOS mRNA and NOS activity are up-regulated, not only in the bladder but also in the lung and kidney. Urinary nitrate–nitrite (NO_x) and cyclic GMP levels are increased whether lipopolysaccharide is injected intraperitoneally or instilled into the bladder (Olsson et al., 1998). Up-regulation of cytokines, iNOS mRNA, protein and activity, along with urinary cyclic GMP levels is observed during human urinary tract infections (Wheeler et al., 1997, 1999; Smith et al., 1996a).

The effects of intraperitoneal lipopolysaccharide injection and instillation of lipopolysaccharide into rat bladder on the expression of cyclooxygenase isoforms are now evaluated by determining bladder cyclooxygenase-1 and cyclooxygenase-2 mRNA and protein levels. To determine if cyclooxygenase-2 up-regulation affects prostaglandin E_2 production, urinary prostaglandin E_2 levels are determined and the effect of indomethacin and the cyclooxygenase-2 selective inhibitor, nuflumic acid, on prostaglandin E_2 production in bladder particulates are determined.

Many of the cytokines, lipopolysaccharide, and signalling molecules that induce nitric oxide production during inflammatory reactions also increase prostaglandin synthesis (Goodwin et al., 1999). In order to assess whether NO is involved in the lipopolysaccharide-induced up-regulation of prostaglandin E_2, urinary prostaglandin E_2 levels are correlated with urinary nitrate production. In addition, the effects of the nitric oxide donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine and the product of the reaction of nitric oxide and superoxide, peroxynitrite, are evaluated on prostaglandin E_2 production in bladder membranes from control rats and from rats whose bladders are instilled with lipopolysaccharide or PBS or who are intraperitoneally injected with lipopolysaccharide.

2. Materials and methods

2.1. Rat bladder inflammation model

All procedures were performed according to an institutionally approved Animal Use Protocol. Adult female Sprague–Dawley rats were sedated with xylazine (4 mg/kg) and ketamine (90 mg/kg). Rats were then injected intraperitoneally with lipopolysaccharide (10 mg/kg) in 500-μl sterile PBS or with 500-μl PBS alone (controls). Other rats were either instilled intravesically with protamine (10 mg/500 μl sterile PBS), which was removed and then lipopolysaccharide (1 mg/500 μl PBS) was instilled or were instilled with sterile PBS (500 μl) (Olsson et al., 1998). The animals were sacrificed 4 h after treatment unless otherwise noted and their bladders were removed and either their RNA was immediately extracted or they were immediately snap frozen in liquid nitrogen and stored at −80°C for Western blot analysis and measurement of prostaglandin E_2 synthesis. Urine was collected prior to and after intravesical PBS or lipopolysaccharide instillation or intraperitoneal PBS or lipopolysaccharide injection by applying gentle pressure on the abdomen.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) of cyclooxygenase cDNAs

Tissues were homogenized in TRizol (Life Technologies, Gaithersburg, MD) (100 mg tissue/ml TRizol) and total RNA was extracted. Single stranded cDNAs were synthesized from total RNA using oligo dT priming and Superscript II Reverse Transcriptase (Gibco-BRL, Life Technologies). Rat cyclooxygenase-1 specific primers for PCR analysis were sense: 5′-TTTGCACAACACTTCACCCACCAG-3′ and antisense 5′-AAACACC TCCTGGC/GCCACAGCCAT-3′. Rat cyclooxygenase-2 specific primers were: sense 5′-ACTTGCTCACTTTGTTGAGTCATTC-3′ and antisense 5′-TTTGATTAGTA CTGTAGGGTTAATG-3′ (Feng et al., 1993). As a control for cDNA synthesis, β-actin specific primers (sense 5′-AGCGGGAAATCGTGCGTG-3′ and antisense 5′-CAGGGT ACATGGTGGTTGCC-3′ were used. Polymerase chain reaction was performed with a Perkin Elmer 2400 Gene AMP PCR thermocycler. Initial denaturation was done at 94°C for 5 min followed by 20–30 cycles of amplification. Each cycle consisted of 40 s of denaturation at 94°C, 50 s of annealing at 56°C, and 60 s of enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 5 min to allow re-annealing of the amplified products.

2.3. Western blot analysis

Tissues were resuspended in an ice-cold buffer (1 ml/100 mg of tissue), which consisted of 20 mM HEPES (pH 7.2), 1 mM dithiothreitol, 0.05 mM phenylmethylsulfonyl fluoride, 0.2 mg/100 ml of leupeptin and 1 mg/100 ml each of pepstatin A, soybean trypsin inhibitor, antipain, and chymostatin. The resuspended samples were subsequently minced and homogenized with a Polytron (Brinkman, Westbury, NY) for 30 s at 4°C. After centrifugation ($20{,}000 \times g$, 20 min, 4°C), the supernatant was discarded and the pellet was resuspended in HEPES plus inhibitor buffer, and an aliquot removed for protein determination (Bradford, 1976). The pellet was diluted with sodium dodecyl sulfide (SDS) sample buffer containing 10% β-mercaptoethanol, and then heated (95°C, 10 min). Bladder membrane samples solubilized in SDS sample buffer were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–20% gradient gels (Biorad, Hercules, CA). The separated proteins were transferred to nitrocellulose membranes. Cyclooxygenase-1 and cyclooxygenase-2 were detected using murine cyclooxygenase-1 monoclonal antisera and murine cyclo-

oxygenase-2 polyclonal antisera raised to a synthetic peptide from the murine cyclooxygenase-2 sequence. Authentic cyclooxygenase-1 and cyclooxygenase-2 proteins did not cross-react with cyclooxygenase-2 and cyclooxygenase-1 antibodies, respectively. Immunoreactive proteins were detected with the enhanced chemiluminescence method (Amersham International, Buckinghamshire, UK).

2.4. Measurement of urinary prostaglandin E_2 and NOx levels

Prostaglandin E_2 levels were measured using a radioimmunoassay (NEN Life Science, Boston, MA) in urine supernatants diluted 1:50 in assay buffer. Urinary nitrate plus nitrite (NO_x) levels were measured after reduction of nitrate to nitrite with nitrate reductase. Rat urine diluted 10-fold in PBS or nitrate standards (1 to 40 μM) were incubated (30 min, 37°C) with 0.25 U/ml nitrate reductase, 250 μM NADPH, and 20 μM FAD. To oxidize NADPH, 10-mM pyruvate and 10-U lactate dehydrogenase were added. The mixture was incubated for an additional 10 min (37°C) and the nitrite concentration was determined. There was 100% conversion of nitrate to nitrite. Results were normalized with urinary creatinine (Smith et al., 1996b).

2.5. Prostaglandin E_2 synthesis

Bladder samples were minced, and homogenized in 100 mM K_2HPO_4 buffer (pH 7.8) containing 35 mU/ml aprotinin, 0.05 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). The homogenate was centrifuged for 5 min ($300 \times g$, 4°C) and the supernatant centrifuged ($50{,}000 \times g$, 4°C) for 60 min. The pellet was resuspended in homogenization buffer using a hand-held homogenizer. Aliquots of pellet (50–100 μg protein/100 μl) were incubated in duplicate with nitric oxide donors or with cyclooxygenase inhibitors for 10 min on ice and then a reaction mix (100 μl) consisting of 100 mM K_2HPO_4 buffer containing 2 mM glutathione (Mimata et al., 1988) and 30 μM arachidonic acid was added and the incubation continued at 37°C (Needleman et al., 1979). Production of prostaglandin E_2 was linear for 15 min. The reaction was stopped with 100 μM indomethacin (200 μl), the samples centrifuged and prostaglandin E_2 measured in a 1:50 dilution of the supernatant using a prostaglandin E_2 radioimmunoassay. prostaglandin E_2 production measured in the absence of arachidonic acid was subtracted from all prostaglandin E_2 values. Prostaglandin E_2 levels were normalized using protein levels (Bradford, 1976).

2.6. Data analysis

Immunodetection band intensities were quantified with the Kodak Digital Science ID version 2.0.2 Image Analysis Application. All results were expressed as means ± S.E.M. and compared using analysis of variance followed by the Fisher *F*-test. The multi-comparison significance level was 95%.

2.7. Compounds

Lipopolysaccharide (prepared by trichloracetic acid extraction from *E. coli*, serotype O26:B6 or serotype O127:B8), protamine sulfate, lactate dehydrogenase and nitrate reductase (Sigma N7265 from Aspergillus), were purchased from Sigma, St. Louis, MO. *S*-nitroso-*N*-acetyl-D,L-penicillamine, peroxynitrite, indomethacin, nuflumic acid, and cyclooxygenase-1 and cyclooxygenase-2 antibodies and standards were purchased from Caymen Chemicals, Ann Arbor, MI. Peroxynitrite in 0.3 N NaOH was aliquoted and stored at −80°C. Just prior to use, the concentration of peroxynitrite was determined ($L = -1{,}670\ M^{-1}\ cm^{-1}$ at 302 nm) and a 1-mM solution prepared in 0.001 N NaOH, prior to final dilution into membranes. Indomethacin was prepared in dimethylsulfoxide and diluted with K_2HPO_4 buffer prior to use. Solutions of nuflumic acid and *S*-nitroso-*N*-acetyl-D,L-penicillamine were prepared in ethanol.

3. Results

3.1. RT-PCR analysis of cyclooxygenase-1 and cyclooxygenase-2 mRNA levels in rat bladder

Cyclooxygenase-1 and cyclooxygenase-2 mRNA are constitutively expressed in normal rat bladder. There was no apparent induction of either cyclooxygenase-1 or cyclooxygenase-2 mRNA 4 or 24 h ($n = 4$, for each time point) following intravesical instillation (Fig. 1) or intraperitoneal injection with lipopolysaccharide (data not shown).

3.2. Western blot analysis of cyclooxygenase-1 and cyclooxygenase-2 protein in rat bladder

Neither intraperitoneal injection of lipopolysaccharide, nor intravesical instillation of lipopolysaccharide or PBS altered cyclooxygenase-1 protein levels when measured 4 h after treatment (Fig. 2A and B). Furthermore, cyclooxygenase-1 levels were not altered 8 ($n = 3$) or 24 h ($n = 3$) after intravesical instillation of lipopolysaccharide (data not shown). Using a polyclonal cyclooxygenase-2 antibody to a murine cyclooxygenase-2 sequence, cyclooxygenase-2 protein was not detected in bladders 4 h after intraperitoneal injection of PBS ($n = 4$) or in bladders from non-treated rats ($n = 4$). Cyclooxygenase-2 protein was present 4 h after intraperitoneal injection of lipopolysaccharide ($n = 4$), 4 h after intravesical instillation of PBS ($n = 5$)

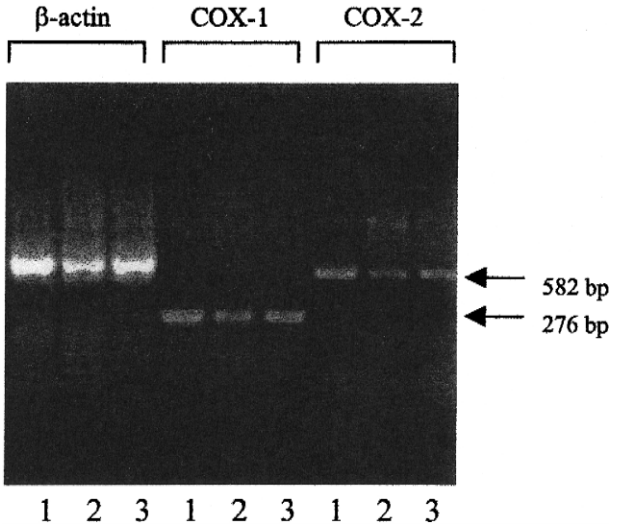

Fig. 1. RT-PCR analysis of cyclooxygenase-1 and cyclooxygenase-2 levels in rat bladder following intravesical instillation of protamine/lipopolysaccharide. β-actin cDNA are shown for band intensity comparison. Non-manipulated bladders are indicated by 1; bladders from rats instilled for 4 and 24 h with lipopolysaccharide and protamine are indicated by 2 and 3, respectively. Expression of cyclooxygenase-1 and cyclooxygenase-2 mRNA are not changed 4 ($n = 4$) and 24 ($n = 4$) h after protamine/lipopolysaccharide instillation. The predicted PCR products are 276 and 582 base pairs for cyclooxygenase-1 and cyclooxygenase-2, respectively.

and 4 h after intravesical instillation of lipopolysaccharide ($n = 5$) (Fig. 3A and B).

3.3. Prostaglandin E_2 and NO_x in rat urine after intravesical instillation and intraperitoneal injection of lipopolysaccharide

Four hours after intraperitoneal injection of lipopolysaccharide ($n = 10$) or intravesical instillation of lipopolysaccharide ($n = 8$) or of PBS ($n = 3$), urinary levels of prostaglandin E_2 were significantly elevated compared to controls ($n = 10$) (Fig. 4). Control urine was collected prior to the intravesical instillation or intraperitoneal injection of lipopolysaccharide or 4 h after the intraperitoneal injection of PBS. Prostaglandin E_2 levels in control rat urine (963 ± 131 pg prostaglandin E_2/ml) increased to 7224 ± 2645 pg prostaglandin E_2/ml after the intraperitoneal injection of lipopolysaccharide and to 6197 ± 1780 pg prostaglandin E_2/ml after intravesical lipopolysaccharide instillation. In rats who had PBS instilled into their bladder ($n = 3$), prostaglandin E_2 levels also increased significantly (3573 ± 1083 pg prostaglandin E_2/ml). NO_x levels in urine from control rats (373 ± 70 nmol/ml urine) and rats intravesically instilled with PBS (344 ± 173 nmol/ml urine) were not significantly different, but intraperitoneal injection and intravesical instillation of lipopolysaccharide significantly elevated NO_x levels over control values (955 ± 224 and 878 ± 188 nmol/ml urine, respectively). Urine creatinine levels did not differ between the groups.

3.4. Prostaglandin E_2 production in bladder particulate fractions

In bladder particulate fractions, prostaglandin E_2 production was increased approximately fivefold by the addition of arachidonic acid (30 μM). Indomethacin inhibitable conversion of arachidonic acid to prostaglandin E_2 was increased approximately threefold in bladder membranes from rats intraperitoneally injected or intravesically instilled with lipopolysaccharide compared to control rats. Intravesical instillation of PBS did not increase prostaglandin E_2 conversion significantly in bladder membranes (Fig. 5). Indomethacin (30 μM) inhibited prostaglandin E_2 production in membranes from control, intraperitoneally lipopolysaccharide injected, and intravesically lipopolysaccharide and PBS instilled bladders by approximately 75%. The IC_{50} for the cyclooxygenase-2 selective inhibitor, nuflumic acid, on prostaglandin E_2 production was 0.59 ± 0.18 μM in lipopolysaccharide intravesically instilled and lipopolysaccharide intraperitoneally injected bladder particulates ($n = 5$) compared to 15.2 ± 6.9 μM for PBS instilled bladder particulates ($n = 3$). The effect of nuflumic acid on prostaglandin E_2 production in PBS and

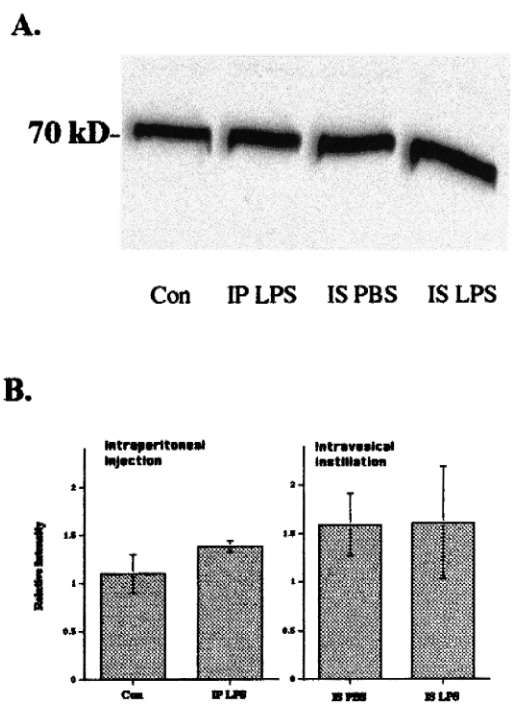

Fig. 2. (A) Immunological detection of cyclooxygenase-1 protein (15 μg total protein/lane) in rat bladder 4 h following intravesical instillation of protamine/lipopolysaccharide (IS LPS, $n = 6$) or PBS (IS PBS, $n = 6$) or intraperitoneal injection of lipopolysaccharide (IP LPS, $n = 3$). Bladders from non-manipulated rats or rats who had been intraperitoneally injected with PBS ($n = 8$) served as controls. A representative blot is shown. (B) Band intensity measurements of the 70-kDa protein correlating to cyclooxygenase-1. Western blot demonstrates no induction of cyclooxygenase-1 when LPS is injected intraperitoneally or instilled intravesically. Data are expressed as means ± S.E.M.

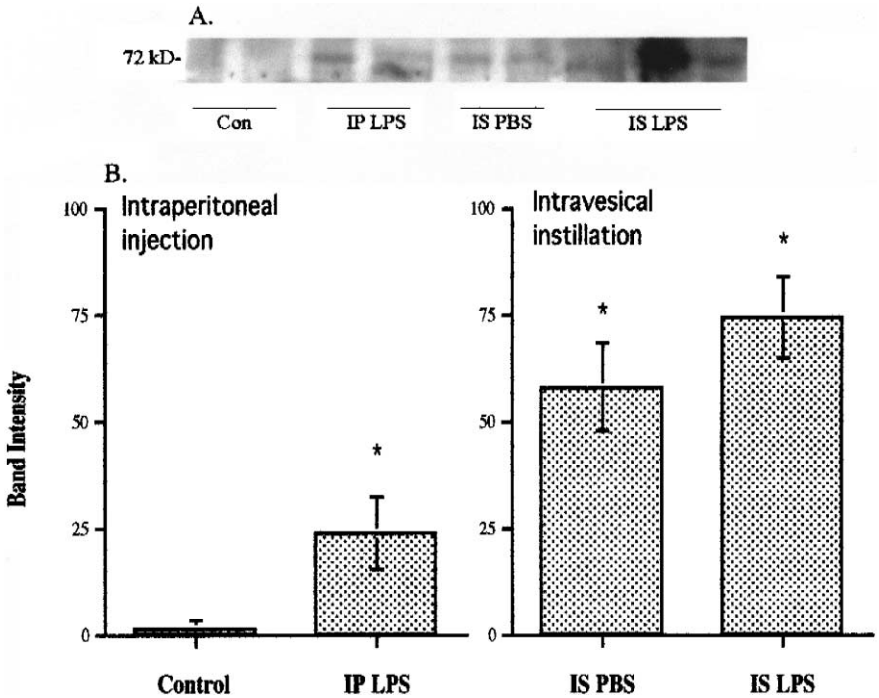

Fig. 3. (A) Immunological detection of cyclooxygenase-2 protein following intravesical instillation of protamine/lipopolysaccharide (IS LPS, $n = 7$) and PBS (IS PBS, $n = 5$) or intraperitoneal injection of lipopolysaccharide (IP LPS, $n = 6$). Bladders from non-manipulated rats or rats who had been intraperitoneally injected with PBS ($n = 7$) served as controls. A representative blot is shown. (B) Band intensity measurements of the 72-kDa protein correlating to cyclooxygenase-2. Western blot analysis demonstrates induction of cyclooxygenase-2, 4 h after intraperitoneal injection of lipopolysaccharide ($p < 0.05$). There is no significant difference in band intensity 4 h following bladder instillation of either lipopolysaccharide or PBS, however, induction with both treatments is increased over controls. Data are expressed as means ± S.E.M. and values significantly different from control are shown with an asterisk.

lipopolysaccharide instilled bladder particulates is shown in Fig. 6. Peroxynitrite, at low concentrations (0.001–0.01 μM) had no effect on prostaglandin E_2 production, but at higher concentrations, peroxynitrite (0.1 M–50 μM) inhib-

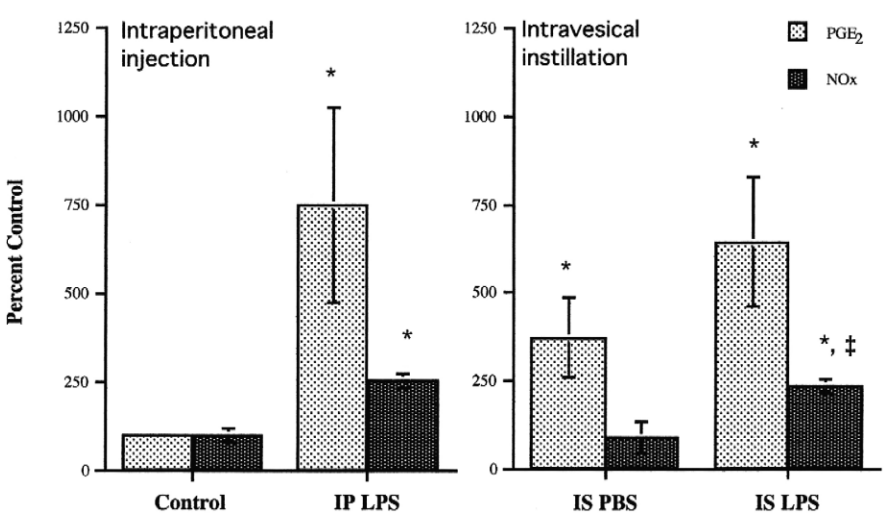

Fig. 4. Urinary prostaglandin E_2 and NO_x levels after intraperitoneal injection of lipopolysaccharide (IP LPS, $n = 10$) or bladder instillation of lipopolysaccharide/ protamine (IS LPS, $n = 8$) or PBS (IS PBS, $n = 3$). Control urines ($n = 10$) were collected prior to the instillation or injection of lipopolysaccharide or 4 h after intraperitoneal injection of PBS. There were no significant difference in prostaglandin E_2 and NO_x levels among the groups of control rats. Results are expressed as means ± S.E.M. and values significantly different from controls are shown with an asterisk. ‡Indicates values significantly different from IS PBS.

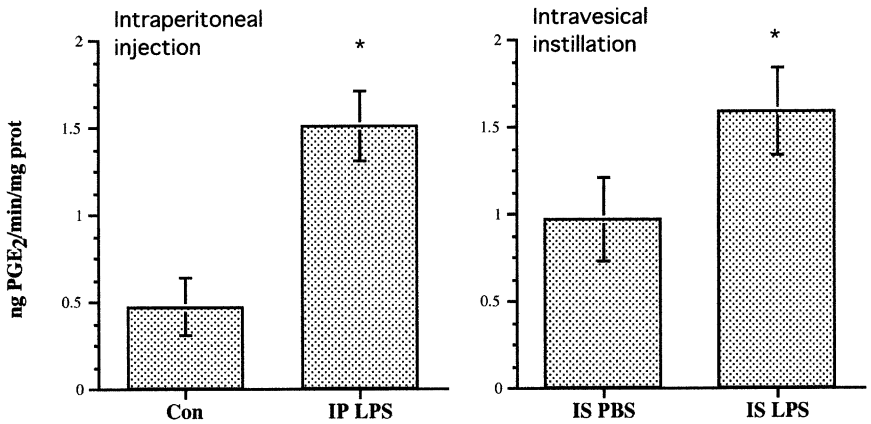

Fig. 5. Prostaglandin E_2 production in rat bladder particulates following intraperitoneal injection of lipopolysaccharide (IP LPS, $n = 12$) or intravesical instillation of PBS (IS PBS, $n = 10$) or protamine/lipopolysaccharide (IS LPS, $n = 8$). Prostaglandin E_2 production in the presence of indomethacin (30 μM) and arachidonic acid is subtracted from prostaglandin E_2 production in the presence of arachidonic acid alone. Data are expressed as means ± S.E.M. and values significantly different from controls ($n = 5$) are shown with an asterisk. Controls were intraperitoneally injected with PBS.

ited prostaglandin E_2 production in bladder particulates. The decrease in prostaglandin E_2 production by peroxynitrite was not different when bladder particulates from control ($n = 3$), PBS ($n = 3$) or lipopolysaccharide intravesically instilled (Fig. 7, $n = 3$) or lipopolysaccharide intraperitoneally injected ($n = 4$) rats were compared. *S-*

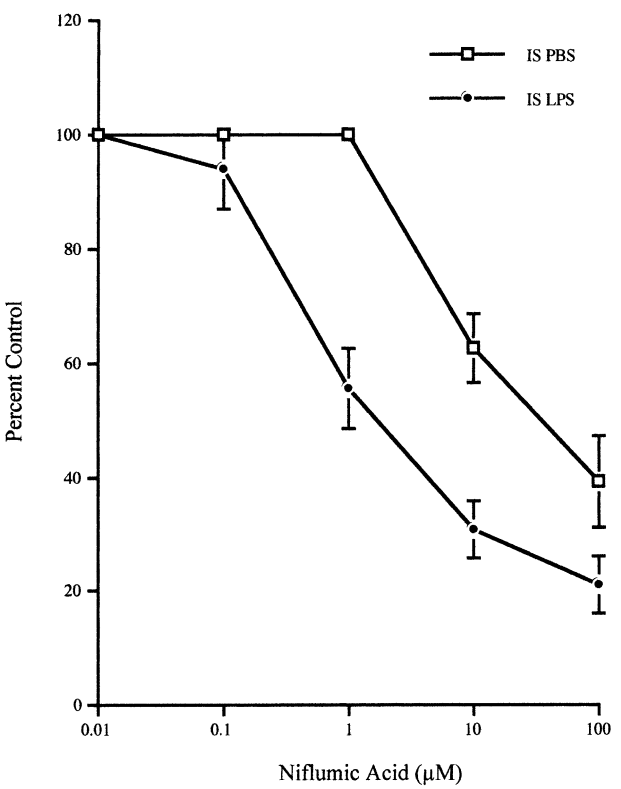

Fig. 6. The effect of the cyclooxygenase-2 selective inhibitor, nuflumic acid, on prostaglandin E_2 production in bladder particulates from bladders intravesically instilled with lipopolysaccharide (IS LPS) or PBS (IS PBS). Nuflumic acid was added to bladder particulates and incubated (4°C, 10 min) before addition of reaction mix. Prostaglandin E_2 production was measured for 10 min (30°C). Data are expressed as percent inhibition from control values (no cyclooxygenase inhibitor present). Control values (means ± S.E.M.) for bladder particulates intravesically instilled with PBS or lipopolysaccharide were 1.26 ± 0.07 ng prostaglandin E_2/min/mg protein ($n = 3$) and 1.93 ± 0.28 ng prostaglandin E_2/min/mg protein ($n = 4$), respectively.

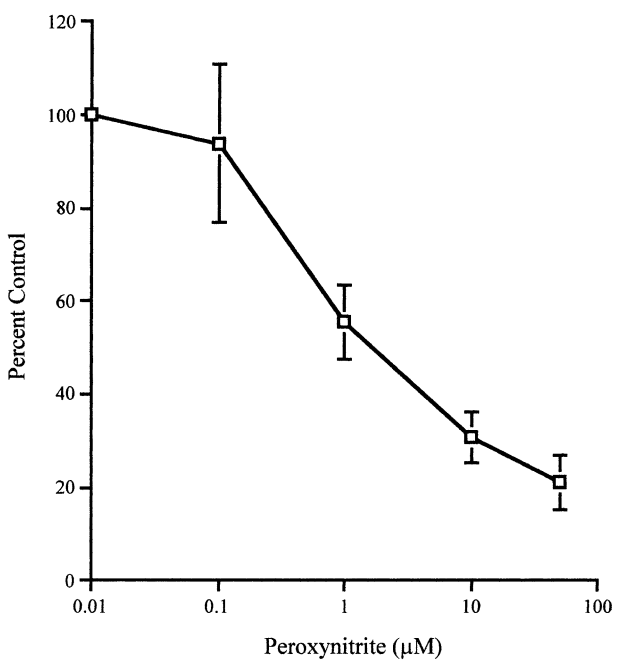

Fig. 7. The effect of peroxynitrite on prostaglandin E_2 production in bladder particulates from lipopolysaccharide intravesically instilled rats. Prostaglandin E_2 production in the presence of peroxynitrite or vehicle (0.001 N NaOH) was measured for 10 min (30°C), after a 10-min preincubation (4°C). Prostaglandin E_2 production in the presence of indomethacin (30 μM) and arachidonic acid is subtracted from prostaglandin E_2 production in the presence of arachidonic acid alone. Prostaglandin E_2 production in the presence of 0.001 N NaOH (vehicle) was not significantly different than H_2O. Results are from three experiments done in duplicate (means ± S.E.M.) and are expressed as percent of prostaglandin E_2 production by vehicle alone. The control values for bladder particulates intravesically instilled with lipopolysaccharide was 1.59 ± 0.25 ng prostaglandin E_2/min/mg protein.

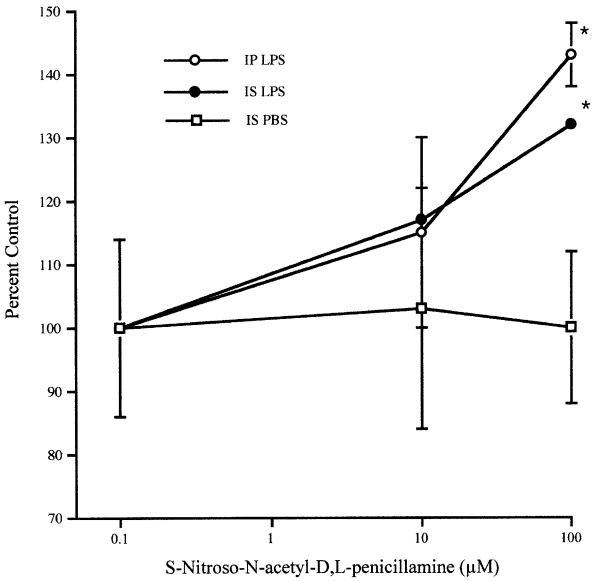

Fig. 8. The effect of *S*-nitroso-*N*-acetyl-D,L-penicillamine on prostaglandin E_2 production in bladder particulates from lipopolysaccharide intraperitoneally injected or lipopolysaccharide or PBS intravesically instilled rats. Prostaglandin E_2 production in the presence of *S*-nitroso-*N*-acetyl-D,L-penicillamine was measured for 10 min (30°C), after a 10-min preincubation (4°C). Results (means ± S.E.M.) are expressed as percent stimulation over vehicle. *S*-nitroso-*N*-acetyl-D,L-penicillamine significantly increases prostaglandin E_2 production in lipopolysaccharide intravesically instilled and intraperitoneally injected bladder particulates. Control values for bladder particulates intraperitoneally injected with lipopolysaccharide or intravesically instilled with lipopolysaccharide or PBS were 1.51 ± 0.20 ng prostaglandin E_2/min/mg protein ($n = 4$), 1.79 ± 0.29 ng prostaglandin E_2/min/mg protein ($n = 3$), and 0.97 ± 0.25 ng prostaglandin E_2/min/mg protein ($n = 3$), respectively.

nitroso-*N*-acetyl-D,L-penicillamine (100 μM) increased prostaglandin E_2 production $43 \pm 5\%$ and $32 \pm 12\%$, respectively, in bladder membranes intraperitoneally injected or intravesically instilled with lipopolysaccharide, but did not increase prostaglandin E_2 production in PBS intravesically instilled bladders (Fig. 8).

4. Discussion

During the course of a urinary tract infection, attachment of *E. coli* to bladder urothelium stimulates epithelial hyperplasia (Uchida et al., 1989), increases cytokine production (Wheeler et al., 1999) and neutrophil infiltration (Uchida et al., 1989), and induces iNOS (Wheeler et al., 1997). The inflammation model used here, in which lipopolysaccharide is instilled intravesically into rat bladders, results in epithelial shedding and an influx of neutrophils into the epithelium (Stein et al., 1996) in addition to the induction of the cytokines interleukin-6 and interleukin-10, and of iNOS. Furthermore, there is an increase in bladder NOS activity, and nitrate and cyclic GMP levels (Olsson et al., 1998). Since prostaglandin E_2 levels are increased during urinary tract infections (Farkas et al., 1980; Wheeler et al., manuscript in preparation), we examined cyclooxygenase-1 and cyclooxygenase-2 expression in the rat bladder and changes in urinary prostaglandin E_2 levels after intraperitoneal lipopolysaccharide injection and intravesical instillation of protamine/lipopolysaccharide or of PBS.

Nested RT-PCR analysis of cyclooxygenase-1 and cyclooxygenase-2 expression, indicates that both messages are present constitutively in the rat bladder, and that their expression is not demonstrated to be further induced 4 or 24 h after the intraperitoneal injection or intravesical instillation of lipopolysaccharide. Previous studies also indicate that cyclooxygenase-2 mRNA is constitutively present in rat (Park et al., 1997) and human (Wheeler et al., manuscript in preparation) bladder. Since cyclooxygenase-2 is regulated at the transcriptional and translational level (Evett et al., 1993; Pouliot et al., 1998; Zaitsu et al., 1999), we used a cyclooxygenase-2 antibody to a specific cyclooxygenase-2 peptide to observe changes in cyclooxygenase-2 translation. Cyclooxygenase-2 protein is not detected in control rats but is present 4 h after intravesical instillation of PBS or lipopolysaccharide/protamine. Cyclooxygenase-2 protein is detected in bladders from rats intraperitoneally injected with lipopolysaccharide, but not with PBS. Cyclooxygenase-1 protein is constitutively expressed in normal non-manipulated rat bladders and its expression is not changed by intraperitoneal injection of lipopolysaccharide or intravesical instillation of lipopolysaccharide/protamine or of PBS. In addition, urinary prostaglandin E_2 levels are elevated after intravesical instillation and intraperitoneal injection of lipopolysaccharide. In summary, while no increase in cyclooxygenase-1 or cyclooxygenase-2 transcription is detected after lipopolysaccharide or PBS intravesical instillation or lipopolysaccharide intraperitoneal injection, cyclooxygenase-2, but not cyclooxygenase-1 protein expression is increased by lipopolysaccharide and PBS intravesical instillation and by intraperitoneal injection of lipopolysaccharide. The increase in cyclooxygenase-2 protein after PBS instillation may occur because of vesical mucosal trauma, which increases prostaglandins (Mikhailidis et al., 1987) or because of bladder distention (Park et al., 1997). Implantation of a bladder catheter using sterile technique results in a 30-fold increase in prostaglandin E_2 content, which can remain elevated for at least 1 week (Morikawa et al., 1989). This indicates that in addition to vesicular trauma and/or stretch (Park et al., 1999), bacterial products like lipopolysaccharide, which are increased in urinary tract infections and sepsis, increase cyclooxygenase-2 protein and urinary prostaglandins.

The effect of lipopolysaccharide on cyclooxygenase-2 induction was further examined by measuring prostaglandin E_2 production in bladder particulates. Prostaglandin E_2 production is elevated in bladder particulates from rats intravesically instilled and intraperitoneally injected with lipopolysaccharide. The IC_{50} values of nuflu-

mic acid for human recombinant cyclooxygenase-1 and cyclooxygenase-2 are 16 and 0.1 μM, respectively (Barnett et al., 1994). When IC_{50} levels are measured in bladder particulates, the IC_{50} for nuflumic acid in lipopolysaccharide instilled bladders (0.59 μM) is closer to that for human recombinant cyclooxygenase-2 than that for human recombinant cyclooxygenase-1. The IC_{50} for nuflumic acid observed in PBS instilled bladders (15.2 μM) is closer to that for human recombinant cyclooxygenase-1. Similar differences in the effects of selective cyclooxygenase-2 and non-selective cyclooxygenase inhibitors were obtained when urodynamic function was measured in rat bladders following lipopolysaccharide treatment (Lecci et al., 2000). Prior to lipopolysaccharide treatment, application of arachidonic acid caused bladder contractions, which were blocked by a cyclooxygenase-1/cyclooxygenase-2 nonselective inhibitor, but not a cyclooxygenase-2 selective inhibitor. After lipopolysaccharide was either instilled intravesically or injected intraperitoneally, both cyclooxygenase-1/cyclooxygenase-2 non-selective and cyclooxygenase-2 selective inhibitors blocked the lipopolysaccharide induced-increases in micturition frequency and the pressure threshold for micturition. Control animals instilled with vehicle alone, also had an increase in micturition frequency and an increase in the pressure threshold for micturition that was blocked by cyclooxygenase-2 selective inhibitors. The greater prostaglandin E_2 production and the lower IC_{50} values for nuflumic acid in bladder particulates intravesically instilled with lipopolysaccharide than with PBS are consistent with a larger induction of cyclooxygenase-2 in lipopolysaccharide than in PBS-treated bladders.

iNOS and cyclooxygenase-2 are co-induced during many inflammatory processes and many studies suggest an interrelationship between NOS and cyclooxygenase (Salvemini et al., 1993; Lianos, 1998; Goodwin et al., 1999). Cyclooxygenase contains a heme moiety that can react with NO (Goodwin et al., 1998), and data suggest that peroxynitrite, the highly reactive species formed from NO and superoxide, also reacts directly with cyclooxygenase to activate prostaglandin synthesis (Goodwin et al., 1999). NO inhibitors decrease cytokine-induced release of prostaglandin E_2 in human astroglial cells (Mollace et al., 1998) and prostaglandin E_2 production by recombinant cyclooxygenase-1 and cyclooxygenase-2 enzymes can be potentiated by NO donors (Salvemini et al., 1993). NO donors also potentiate prostaglandin E_2 production in rat mesangial cells that have been treated with interleukin-1 (Tetsuka et al., 1996). In addition, urinary prostaglandin E_2 levels in iNOS deficient mice are decreased 78% compared with levels in control mice (Marnett et al., 2000). In the rat bladder, there are significant differences between the induction of cyclooxygenase-2 and iNOS. Cyclooxygenase-2 mRNA is constitutively expressed in rat bladder while iNOS mRNA is detected only after treatment with lipopolysaccharide (Olsson et al., 1998). While intravesical instillation of lipopolysaccharide induces cyclooxygenase-2 protein and increases prostaglandin E_2 production as well as iNOS mRNA, bladder instillation of PBS increases cyclooxygenase-2 protein but not iNOS mRNA or its products.

The NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine, increases prostaglandin E_2 production in bladder particulates from rats intraperitoneally injected with lipopolysaccharide or intravesically instilled with lipopolysaccharide/protamine but not in bladder membranes from rats intravesically instilled with PBS,. This suggests that *S*-nitroso-*N*-acetyl-D,L-penicillamine has a more profound effect on cyclooxygenase-2 than on cyclooxygenase-1. Peroxynitrite reacts with cyclooxygenase to increase prostaglandin E_2 production (Goodwin et al., 1999) and can also cause nitration of cyclooxygenase tyrosine residues with resultant decreased prostaglandin H_2 production (Boulos et al., 2000). Peroxynitrite decreases prostaglandin E_2 production in bladder membranes from control, PBS or lipopolysaccharide intravesically instilled or lipopolysaccharide intraperitoneally injected rats. Differences in the amount of superoxide present or in the sensitivity of cyclooxygenase to peroxynitrite relative to other enzymes, which may non-specifically decrease prostaglandin production, may explain the differences in regulation of prostaglandin synthesis by NO and peroxynitrite in the bladder.

The elevated levels of urinary prostaglandin E_2 measured during a urinary tract infection may occur when iNOS and cyclooxygenase-2 pathways operate to amplify a physiological or pathological host response. Findings suggesting that an impaired prostaglandin response to infection is a factor responsible for recurrent cystitis (Khan et al., 1998), supports a role for cyclooxygenase-2 in the host defense against infection. Prostaglandins also are involved in the modulation of micturition reflexes (Mikhailidis et al., 1987). Bladder inflammation increases prostaglandin E_2 production as well as bladder tone, micturition frequency and the pressure threshold for micturition (Meini et al., 1998; Lecci et al., 2000). The symptoms of lower urinary tract infections, which include frequency, urgency, and voiding in small amounts, may be explained, at least in part, by increases in prostaglandin production.

In summary, our study suggests that intravesical instillation or the intraperitoneal injection of lipopolysaccharide in rat bladder increases the amount of cyclooxygenase-2 protein and prostaglandin E_2 production independent of bladder distension or trauma, which also increase cyclooxygenase-2 protein and/or prostaglandin E_2 production. Differences in the induction of cyclooxygenase-2 by lipopolysaccharide intraperitoneal injection or lipopolysaccharide or PBS intravesical instillation may be due to differences in the cellular location where cyclooxygenase-2 is up-regulated and to whether iNOS or other pro-inflammatory substances are induced when cyclooxygenase-2 is up-regulated. Stretch or PBS instillation may induce cyclooxygenase-2 expression in muscle cells (Park et al., 1999)

with no concomitant increase in the levels of NO or reactive oxygen species and no increase in inflammatory cell numbers (Olsson et al., 1998). On the other hand, intraperitoneal injection or intravesical instillation of lipopolysaccharide causes an influx of neutrophils (Olsson et al., 1998; Stein et al., 1996), which are known to express cyclooxygenase-2 (Minghetti et al., 1999) along with the induction of iNOS. Immunohistochemical localization of cyclooxygenase-2 and other inflammatory mediators after PBS or LPS intravesical instillation may provide insight into whether prostaglandin production is deleterious or protective in the inflammatory process.

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