Prostasin attenuates inducible nitric oxide synthase expression in lipopolysaccharide-induced urinary bladder inflammation

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Prostasin attenuates inducible nitric oxide synthase expression in lipopolysaccharide-induced urinary bladder inflammation. Am J Physiol Renal Physiol 291: F567–F577, 2006. First published April 25, 2006; doi:10.1152/ajprenal.00047.2006.—Prostasin is a glycosylphosphatidylinositol-anchored serine protease, with epithelial sodium channel activation and tumor invasion suppression activities. We identified the bladder as an expression site of prostasin. In the mouse, prostasin mRNA expression was detected by reverse transcription and real-time polymerase chain reaction in the bladder, and the prostasin protein was localized by immunohistochemistry in the urothelial cells. In mice injected intraperitoneally with bacterial lipopolysaccharide (LPS), bladder prostasin mRNA expression was downregulated, whereas the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interferon-γ (IFN-γ), TNF-α, IL-1β, and IL-6 was upregulated. Viral promotor-driven expression of the human prostasin homolog in the bladder of transgenic mice attenuated the LPS induction of iNOS but did not abolish the induction. LPS induction of COX-2, TNF-α, IL-1β, and IL-6 expression, however, was not reduced by prostasin transgene expression. Liposome-mediated delivery of prostasin-expressing plasmid into mouse bladder produced similar attenuation effects on LPS-induced iNOS expression, while not affecting COX-2 or cytokine induction. Mice receiving plasmid expressing a catalytic mutant prostasin did not manifest the iNOS induction attenuation phenotype. We propose a proteolytic mechanism for prostasin to intercept cytokine signaling during LPS-induced bladder inflammation.

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The only indication of the physiological function of prostasin came from several studies of prostasin’s involvement, mainly as a membrane-anchored proteolytic enzyme, in activation of the epithelial sodium channel (ENaC), hence the name CAP-1 for channel-activating protease 1. Overexpression of prostasin by adenovirus-mediated human prostasin gene delivery in rats produced many phenotypes, such as elevated plasma aldosterone level, reduced plasma renin activity, increased urinary Na⁺ and kallikrein excretion, and decreased urinary K⁺ excretion. It can be suggested from these observations that prostasin participates in electrolyte homeostasis by modulating the renin-angiotensin-aldosterone and the kallikrein-kinin systems. At least one of the target or effector molecules of prostasin is the ENaC, whose activity is tightly regulated to maintain sodium balance. Conditionally knocked-out mice lacking prostasin expression in skin epithelia die within 60 h after birth, with disruption of the tight junctions, suggesting that prostasin plays an important role in maintaining the epidermal permeability barrier.

In this study, we demonstrated that prostasin is abundantly expressed in mouse urinary bladder epithelium. The expression of bladder mouse prostasin is downregulated during inflammation produced by intraperitoneal LPS injection. Viral promotor-driven expression of the human prostasin functional homolog in mouse bladder attenuates LPS-induced upregulation of iNOS without affecting COX-2 or cytokine expression upregulation. It may be suggested that prostasin plays a role in bladder inflammatory response by modulating cytokine signaling in the urothelial cells. This prostasin function requires the serine active site, indicating a proteolytic mechanism in cytokine signal modulation.

MATERIALS AND METHODS

Animals

Mice were used under approval by the Institutional Animal Care and Use Committee of the University of Central Florida.

Transgenic mice expressing the human prostasin. A full-length human prostasin cDNA (nucleotides 60–1834, GenBank accession No. L41351) was cloned from the LNCaP human prostate cancer cells into a mammalian expression vector pREP8 (Invitrogen, Carlsbad, CA) as described previously (7). The human prostasin transcription unit, under the control of the Rous sarcoma virus long-terminal repeat (known as the RSV LTR promoter), was released by restriction digestion with AccI. The transgenic mice were created by microinjection of the human prostasin transgene construct DNA into one of the pronuclei of single-cell FVB-strain mouse embryos. The microinjection was performed by the ESCore Facility of the University of Cincinnati (Cincinnati, OH) under contract. Founders harboring the transgenic transgene were identified by PCR using primers specific for human prostasin (upstream-primer: 5′-AGG TGG CAG CAG TGC AGT C-3′; downstream-primer: 5′-ACA GGC AGT TAC ACG TCT CA-3′; 30 cycles of 94°C, 1 min/60°C, 1 min/72°C, followed by an extension at 72°C for 1 min). Paired breeding was set up to generate animals with the desired genotypes for experiments.

Mouse bladder inflammation model. Male mice (FVB or FVB/N) with appropriate age, weight, and sex were used. Each mouse was anesthetized by 10.220.32.247 on November 6, 2017 http://ajprenal.physiology.org/ Downloaded from

Transurethral gene delivery. Female FVB mice were anesthetized and transurethrally catheterized. The urine was drained and the bladder was instilled with 150 μl of plasmid DNA:liposome complex in OPTI-MEM I medium (Invitrogen). Five micrograms of each plasmid DNA in 75 μl of OPTI-MEM I were mixed with 5 μl of Lipofectamine 2000 reagent (Invitrogen) in 75 μl of OPTI-MEM I. The mixture was incubated at room temperature for 20 min before injection into mouse bladder via the catheter. Thirty hours after the DNA-liposome injection, normal saline or LPS (20 mg/kg) was injected intraperitoneally. Forty-eight hours postgene delivery (18 h after LPS injection), the bladder was removed from each animal for total RNA isolation. The prostasin expression plasmid was constructed as described previously (6). A β-galactosidase expression plasmid (9) was used for the control group of mice.

Antibody Preparation

A full-length mouse prostasin cDNA was purchased from Genome Systems (St. Louis, MO; the cDNA sequence of this clone corresponds to GenBank Accession No. A1527990; purchase was dated September 1999). The mouse prostasin cDNA was then subcloned into the pREP8 plasmid (Invitrogen) via amplification by PCR of the coding region sequence using the following two primers. Upstream: 5′-AAG CTT GTC CTA GGC CAT GGC-3′, and downstream: 5′-GAATTTC AGC TTA ATG TTC AAG CCA-3′; the underscored sequences are restriction linkers/adaptors. The mouse prostasin cDNA plasmid was transfected into the HEK-293 cells, and the recombinant mouse prostasin was purified from the conditioned culture medium as described previously (7). The purified mouse prostasin was used to generate a polyclonal antibody in rabbit by Cocalico Biologicals (Reamstown, PA) under contract. Western blot analysis was carried out with procedures described previously (7). For analysis of urothelial proteins, urothelial cells were scraped from the mouse bladders mucosa and were lysed in RIPA buffer (7), before SDS-PAGE. For analysis of mouse urinary proteins, the urine was mixed with SDS sample buffer (7) before SDS-PAGE.

Immunohistochemistry

Mouse bladder was removed from euthanized animals and separated from the surrounding tissues. Each bladder was fixed, embedded in paraffin, and cut into 4-μm sections. The sections were subjected to immunostaining as described previously (6). The mouse prostasin antibody was used at 1:200. The bladder sections were then counterstained with hematoxylin, dehydrated in an increasing series of alcohol, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Control sections were treated with the same procedures as described above, except that a preimmune rabbit serum was used in place of the prostasin-specific antisera.

RNA Isolation, Reverse Transcription, and Real-Time PCR Analysis

Total RNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription was carried out using random primers of the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. Primers used for the real-time PCR analysis of the reverse-transcribed mRNA were designed with the aid of the Beacon Designer 4.0 software (PREMIER BioSoft International, Palo Alto, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). Real-time PCR was performed on a Bio-Rad MyiQ system using the iQ SYBR Green Supermix reagents, following the manufacturer’s protocols. The thermal cycling program starts with an initial denaturation step at 95°C for 3 min and is followed by 40 cycles between 15 s at 95°C and 1 min at 60°C. The relative quantities of gene-specific mRNA expression were determined by the comparative CT method wherein CT refers to the “threshold cycle” and is determined for each experiment with the aid of the Myq software. Amplification of GAPDH mRNA was performed for each reverse-transcribed sample as an endogenous quantification standard. Where applicable ΔCₜ = (gene-specific Cₜ –
Fragments were modified by (underscored in the primer sequences). The two amplified cDNA fragments were joined at the CAC TCT GCC CTC TAC CTA TGA TGG AAC TGG AGG AGG AGG ATG ATG AGC G-3'; 5'-AGA GAT GAT GAC CCT TTT GGC-3'; mouse prostasin: 5'-CAC TCT GAC TAC TTA GGA AAC TGG AGG AGG AGG ATG ATG AGC G-3'; human prostasin: 5'-ATG TTG GAT TAC TCT GCC GGT CGG-3'; 5'-ACA CAT GGG CGC CTT CAT AGG-3'; mouse IFN-γ: 5'-TTG GAT ATC TGG AGG AAC TGG AGG-3'; 5'-ATC AGG TGT GAT TCA ATG ACG-3'; mouse TNF-α: 5'-AGT GGT ACC TCC CTT ATG GTC-3'; 5'-GGG CTA GCT GTC CAC TCG-3'; mouse IL-6: 5'-TCT ATC ATG TTG CCT TCT TG-3'; 5'-CTG GTA ATG-3'; mouse COX-2: 5'-AGG TGG CAG CAG TGC AGT C-3'; mouse iNOS: 5'-AGT GGT GTC TTT ACA ATG GTA TGC-3'; mouse COX-2: 5'-GGT GTG CCT GCT GTG-3'; mouse IL-6: 5'-CTC TCC TAT GAG TCT CCT TGG-3'; mouse COX-2: 5'-AGG TGG CAG CAG TGC AGT C-3'; mouse iNOS: 5'-AGT GGT GTC TTT ACA ATG GTA TGC-3'; mouse COX-2: 5'-GGT GTG CCT GCT GTG-3'; mouse IL-6: 5'-CTC TCC TAT GAG TCT CCT TGG-3'; mouse COX-2: 5'-AGG TGG CAG CAG TGC AGT C-3'; mouse iNOS: 5'-AGT GGT GTC TTT ACA ATG GTA TGC-3'; mouse COX-2: 5'-GGT GTG CCT GCT GTG-3'.

RESULTS

Prostasin Is Expressed in Mouse Bladder Epithelium

Prostasin expression at the mRNA level in tissues and cell lines has been surveyed by several laboratories using different methods (5, 6, 41, 51). The protein level expression of prostasin was surveyed with radioimmunoassay (RIA) in human tissues (50). The scope of tissues surveyed was incomplete, and the expression state of prostasin in many other tissues remained unknown. In this study, we generated a rabbit polyclonal antiserum against the mouse prostasin (mPro) protein produced by expression in tissue-cultured human embryonic kidney cells (HEK-293). The mPro antiserum does not cross-react with the human prostasin (hPro) protein in Western blot analysis, while detecting the mPro protein readily in the purified form or in urine collected from mouse bladder (Fig. 1A, left). The rabbit antihuman prostasin antiserum that we used previously (6, 7) cross-reacts with the purified mPro protein (Fig. 1A, right, lane 2), but only minimally with the mouse homolog in the context of a mixture, such as mouse urine (Fig. 1A, right, lane 3). The apparent molecular weight of the immunoreactive mPro protein band detected in the urine was similar to the purified hPro protein but higher than that of the recombinant mPro protein expressed in the HEK-293 cells. The apparent molecular weight difference may be caused by differences in posttranslational modification such as glycosylation. By immunohistochemistry, we showed that the mPro protein is expressed in the epithelial (urothelial) cells of the bladder (Fig. 1B), with intense staining in multiple layers of the transitional urothelium (Fig. 1C). No signal was detected when a preimmune rabbit serum was used in place of the rabbit antiprostatin antiserum on an adjacent section (Fig. 1D).

hPro Homolog Is Expressed in the Bladder of Transgenic Mice

Three lines of hPro transgenic mice were established on the background of the FVB strain for the purpose of investigating the effect of prostasin overexpression in vivo. The hPro transgene expression is driven by the Rous sarcoma virus long-terminal repeat (RSV LTR) promoter. Expression of hPro mRNA was detected in two of the three lines in various tissues (data not shown), including the bladder of the transgenic mice. In this study, we chose the two lines (line 47870 and line 47879) of mice which expressed hPro in the bladder for our experiments. By means of real-time PCR following reverse-transcription (RT-rtPCR), expression of the hPro mRNA was detected in the bladder of both transgenic mouse lines, but at different levels (Fig. 2A). In line 47879, the hPro mRNA is expressed in the bladder at 5.11 ± 1.50 × 10^{-3} molecular equivalence to the GAPDH message, whereas in line 47870, the bladder hPro mRNA is 2.73 ± 0.94 × 10^{-3} of the GAPDH message. The high-expressor line 47879 expressed the hPro mRNA at a level which is 18.7-fold greater than the low-expressor line 47870, on average. These animals were treated with intraperitoneal LPS for 18 h. In a Western blot analysis, we were able to detect the hPro protein in scraped bladder epithelial cells of the transgenic mice in the high-expressor line 47879 (Fig. 2B), from animals not treated with LPS. The results were validated by an immunoblot of the same membrane to show the presence of the epidermal growth factor.
receptor protein (Fig. 2B), a transmembrane protein which was shown to be expressed in scraped urothelial cells (10). The hPro transgene protein expression in the urothelium is consistent with previously reported localization patterns of GPI-anchored proteins expressed in the urinary bladder under the control of a viral promoter (29).

Mouse Bladder Prostasin mRNA Expression Is Downregulated in LPS-Treated Mice

Mice were intraperitoneally injected with LPS and used for bladder gene expression analysis at 18 h after the injection. The bladder was removed and subjected to total RNA isolation. The total RNA of each sample was analyzed by means of RT-rtPCR. As a result of the LPS injection, mPro mRNA expression in the bladder was downregulated compared with mice injected with saline (Fig. 3 and Table 1).

In the nontransgenic mice (FVB), bladder mPro mRNA expression was downregulated to an average of 28% of the normal level in response to the LPS treatment. While in the transgenic mice (both line 47870 and line 47879), the LPS-induced downregulation of mPro mRNA expression in the bladder was to a lesser extent (Fig. 3 and Table 1).

Upregulation of Gene Expression in the Bladder By LPS Is Selectively Attenuated in Transgenic Mice

The mRNA expression of two classic LPS response genes in the bladder, the iNOS and the COX-2, was evaluated in the hPro transgenic mice. First, both genes were upregulated in the bladder by the LPS treatment in the nontransgenic mice (FVB), as expected from established literature (32, 45). The iNOS mRNA expression in the bladder was upregulated by LPS to an average of 110.3-fold of the control levels (Fig. 4, top, and Table 1), whereas the COX-2 mRNA expression was upregulated to an average of 8.7-fold (Fig. 4, bottom, and Table 1).

Fig. 2. Expression of human prostasin homolog in the bladder of transgenic mice. A: real-time PCR analysis of reverse-transcribed bladder RNA from transgenic mice (line 47870: n = 6, line 47879: n = 8, mice were injected with LPS as described in MATERIALS AND METHODS). The vertical axis represents relative levels of expression normalized to the mRNA level of GAPDH. B: Western blot (WB) analysis of human prostasin transgene protein and mouse EGFR expression in mouse bladder. The urothelial cells were scraped from the mucosa of isolated mouse bladder (3 tissues for each animal group) and lysed in RIPA buffer. Sample loading order was as indicated in the figure, each lane with 60 μg of total protein. The blot was treated with the human prostasin antibody (used at 1:2,500; top), stripped, and treated with an antibody against EGFR (sc-03, Santa Cruz Biotechnology, used at 1:1,000; bottom).
In the hPro transgenic mice, LPS-induced iNOS mRNA expression in the bladder was at an average of 40% in line 47879 (high expresser of the hPro transgene) compared with the LPS-induced expression in the control mice (FVB/LPS; Fig. 4, top, and Table 1). There is no statistical difference in the LPS-induced bladder iNOS expression between the nontransgenic mice and the low expresser line (47870).

On the other hand, LPS induction of bladder COX-2 mRNA expression was not affected by the hPro transgene expression (Fig. 4, bottom, and Table 1). The bladder COX-2 mRNA expression following LPS treatment in line 47870 or line 47879 was not statistically different from that in the nontransgenic mice (FVB/LPS).

We tested the bladder mRNA expression of several key proinflammatory cytokines known to be induced by LPS, namely, IFN-γ, TNF-α, IL-1β, and IL-6. The LPS treatment upregulated the IFN-γ mRNA expression in mouse bladder to an average of 151-fold (Fig. 5A and Table 1), the TNF-α mRNA expression to an average of 32.3-fold (Fig. 5B and Table 1), the IL-1β mRNA expression to an average of 8.4-fold (Fig. 5C and Table 1), and the IL-6 mRNA expression to an average of 72.9-fold (Fig. 5D and Table 1).

There was an attenuation effect on LPS-induced IFNγ mRNA expression in the bladder of the hPro transgenic mice, to an average of 48% in both line 47870 and line 47879 compared with that in the control mice (FVB/LPS; Fig. 5A and Table 1). The bladder IL-6 mRNA expression was not affected by the hPro transgene expression (Fig. 4, bottom, and Table 1). The bladder IL-6 mRNA expression following LPS treatment was not statistically different from that in the nontransgenic mice (FVB/LPS).

Table 1. Expression of mouse bladder genes evaluated by RT-rtPCR

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>Gene Evaluated</th>
<th>Expression Level Normalized to GAPDH</th>
<th>Change vs. Control (Fold)</th>
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</thead>
<tbody>
<tr>
<td>FVB (saline injected)</td>
<td>Mouse prostasin</td>
<td>9.04 ± 0.35 × 10^2</td>
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<td>FVB/LPS</td>
<td>Mouse prostasin</td>
<td>2.57 ± 0.33 × 10^2</td>
<td>0.28&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>47870/LPS</td>
<td>Mouse prostasin</td>
<td>4.29 ± 0.43 × 10^2</td>
<td>1.67&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>47879/LPS</td>
<td>Mouse prostasin</td>
<td>3.84 ± 0.42 × 10^2</td>
<td>1.49&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>FVB (saline injected)</td>
<td>Mouse INOS</td>
<td>1.07 ± 0.12 × 10^-3</td>
<td>1.0</td>
</tr>
<tr>
<td>FVB/LPS</td>
<td>Mouse INOS</td>
<td>1.18 ± 0.33 × 10^-3</td>
<td>110.3</td>
</tr>
<tr>
<td>47870/LPS</td>
<td>Mouse INOS</td>
<td>1.57 ± 0.44 × 10^-3</td>
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<tr>
<td>47879/LPS</td>
<td>Mouse INOS</td>
<td>4.77 ± 1.81 × 10^-2</td>
<td>0.40&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>FVB (saline injected)</td>
<td>Mouse COX-2</td>
<td>7.08 ± 1.38 × 10^-3</td>
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<tr>
<td>FVB/LPS</td>
<td>Mouse COX-2</td>
<td>6.15 ± 1.59 × 10^-2</td>
<td>8.7</td>
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<td>6.96 ± 2.13 × 10^-2</td>
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<td>0.48&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>5.37 ± 2.50 × 10^-3</td>
<td>1.78</td>
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<td>47879/LPS</td>
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<td>47879/LPS</td>
<td>Mouse IL-6</td>
<td>2.69 ± 0.54 × 10^-2</td>
<td>0.64</td>
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</table>

Values are means ± SE. Experimental groups are as indicated: control (FVB, n = 5), LPS-treated FVB (FVB/LPS, n = 9), and LPS-treated transgenic mice (47870/LPS, n = 6, and 47879/LPS, n = 8). Underlined fold-changes reflect the effects of human prostasin transgene on LPS-induced gene expression. Statistical difference (P < 0.05) between the FVB and the FVB/LPS data groups for bladder mouse prostasin mRNA expression. Statistical difference (P < 0.05) between the 47870/LPS and the FVB/LPS data groups for mouse bladder prostasin mRNA expression. Statistical difference (P < 0.05) between the 47879/LPS and the FVB/LPS data groups for mouse bladder inducible nitric oxide synthase (iNOS) mRNA expression. Cox, cyclooxygenase.

Fig. 3. LPS downregulates mouse prostasin mRNA expression in the bladder. Real-time PCR analysis of reverse-transcribed mouse bladder RNA from FVB or transgenic animals receiving intraperitoneal LPS for expression of mouse prostasin mRNA. Experimental groups are as indicated under each data column; open bars represent data from animals receiving saline injection, and filled bars represent data from animals receiving LPS: FVB (saline-injected, n = 5; LPS-treated, n = 9), 47870 (saline-injected, n = 6; LPS-treated, n = 6), 47879 (saline-injected n = 6; LPS-treated, n = 8). Statistical difference between the FVB control data group and the FVB/LPS data group (P < 0.05).

Statistical difference (P < 0.05) between the 47870/LPS or the 47879/LPS, and the FVB/LPS data groups for mouse bladder IFN-γ mRNA expression. Statistical difference (P < 0.05) between the FVB/LPS and the 47870/LPS or the 47879/LPS data group (P < 0.05).
In line 47870, TNF-α was shown to be further induced by LPS, at an average of 1.90-fold following LPS treatment compared with the control mice (FVB/LPS; Fig. 5B and Table 1). In line 47879, LPS induction of TNF-α mRNA expression was not affected by expression of the hPro transgene. Expression of IL-1β or IL-6 mRNA induced by LPS was also not affected by expression of the hPro transgene, in either transgenic line (Fig. 5, C and D, and Table 1).

**Intravesical Delivery of Prostasin-Expressing Plasmid Reproduces the Attenuating Effect on LPS-Induced iNOS mRNA Expression**

Plasmid DNA expressing hPro or a catalytically inactive human prostasin (hProM) was conjugated with liposome and injected into mouse bladder. The animals were injected with LPS at 30 h after DNA injection and used for bladder gene expression analysis at 18 h after the LSP injection. The control group was injected with a plasmid expressing the β-galactosidase gene (β-gal). The bladder was removed for total RNA isolation and evaluation by RT-rtPCR. Following LPS injection, the bladder iNOS mRNA expression level in mice receiving the wild-type human prostasin plasmid (hPro) was at 3.89 ± 0.24 × 10⁻² (GAPDH molecular equivalence), an average of 34% of that in mice receiving the control plasmid expressing the β-galactosidase gene (β-gal), 1.13 ± 0.31 × 10⁻¹ (GAPDH molecular equivalence; Fig. 6A). The catalytically inactive human prostasin did not produce an attenuation effect on LPS-induced iNOS mRNA expression. Mice receiving the mutant prostasin plasmid (hProM) in the bladder displayed LPS-induced iNOS mRNA expression at 1.15 ± 0.52 × 10⁻¹ (GAPDH molecular equivalence), similar to those receiving the β-galactosidase plasmid (β-gal), at an average level of 102% (Fig. 6A).

Catalytic inactivation of the mutant prostasin was confirmed by its inability to form a covalent complex with the cognate serpin-class inhibitor of prostasin, the protease nexin 1 (PN-1) (8) (Fig. 6B). Human prostasin mRNA expression was evaluated in the DNA-injected bladder to ensure proper uptake and expression of the plasmid. Human prostasin mRNA expression can be detected in the injected bladders receiving either the wild-type (hPro) or the mutant prostasin cDNA plasmid (hProM), but not the β-galactosidase plasmid (β-gal) (Fig. 6C). The amplified signals shown in Fig. 6C were from the expressed mRNA (human prostasin or mutant) but not from carry-over plasmid DNA copurified in the RNA preparation. No amplification products were detected in the DNase I-treated mRNA samples applied directly to a human prostasin-specific PCR analysis (Fig. 6D, right). The integrity of the mRNA samples was confirmed with the amplification of the GAPDH message (Fig. 6D, left). Quantitative analysis of the RT-rtPCR data indicated that the mutant prostasin was expressed in the DNA-injected bladder at a relatively higher level than the wild-type prostasin, at an average of 1.6-fold (Fig. 6E).

The samples from all three animal groups treated with LPS (FVB/β-gal, FVB/hPro, and FVB/hProM) were also assayed for mRNA expression of mPro, COX-2, and the cytokines (IFN-γ, TNF-α, IL-1β, and IL-6). The LPS response of these genes was not affected by expression of the human prostasin homolog, or its active site mutant (data not shown).

**DISCUSSION**

The physiological function of prostasin, an enzymatically active extracellular GPI-anchored serine protease, is poorly understood despite great efforts to date in the investigation of its role in ENaC activation, aldosterone regulation, tight junction formation, and tumor invasion suppression. More elusive were the molecular mechanism and signaling pathways affected by prostasin, in the context of most of the aforementioned phenotypes associated with prostasin expression. The only exception is the activation of ENaC, which is most likely a proteolytic mechanism, but other mechanisms may also be involved (40). In this study, we were afforded, by the revelation of prostasin’s rather abundant expression in the urothelium of the bladder, a new model system with which to explore the signaling pathways impacted by prostasin. Exploiting this model we identified a molecular readout far downstream of prostasin, the transcription of the iNOS gene, and with it an opportunity to dissect prostasin’s signaling pathways, potentially leading to the identification of the molecules...
mediating prostasin’s action at the plasma membrane level of epithelial cells.

Induction of the iNOS and COX-2 genes is a trademark of bladder inflammatory response triggered by LPS, administered either by intravesical instillation or intraperitoneal injection (33, 46). LPS induction of iNOS expression in various cell types is mediated by a two-phase mechanism. First, the iNOS induction may be a direct effect of the LPS through activation of the Toll-like receptor 4 (TLR4) complex, leading to activation and nuclear translocation of NF-kB (52), which upregulates the gene-specific promoters of cytokines (15) and iNOS (31, 49). The second phase is a positive feedback upregulation, involving the actions of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) (28, 48, 37), whereas IFN-γ in the bladder is a coactivator of the iNOS promoter via the IRF-1 transcription factor, capable of potentiating iNOS upregulation (26). The cytokine action added to the LPS induction results in a dramatic upregulation of the iNOS gene.

The urothelium is a poor responder to pure LPS as intravesical instillation of LPS alone failed to induce bladder iNOS mRNA expression, instillation of the bladder irritant protamine before LPS was required to induce bladder iNOS mRNA expression (33). This poor LPS responsiveness is due to a lack of, or a minimal-level urothelial expression of CD14, a coreceptor of the TLR4 receptor complex (21). But the urothelial cells do express an increased amount of iNOS mRNA in response to LPS, administered either intravesically or intraperitoneally. The bladder urothelial iNOS mRNA response to LPS is not mediated directly by the LPS but most likely by the cytokines secreted by the infiltrating inflammatory cells (33, 34).

In this study, we were able to reproduce all of the LPS-induced inflammatory gene expression effects in the bladder, setting the stage to ask the question of what role would urothelially expressed prostasin play during an LPS-induced bladder inflammation. If a forced expression of prostasin (the human functional homolog) is inserted into the picture, i.e., in the bladder of the human prostasin transgenic mice, or in the bladder of mice receiving prostasin-expressing plasmid DNA, would any of the LPS-associated effects be impacted? We showed in both the transgenic and the gene delivery models that a forced expression of the human prostasin functional homolog could impede on LPS-induced iNOS expression, but not on induction of the COX-2 gene (Fig. 4 and Table 1). The viral promoter employed in this case, the Rous sarcoma virus long-terminal repeat (RSV LTR), can be upregulated via the NF-kB pathway (2), which is upregulated as an LPS or cytokine response (1, 52). In our experiments, we did not evaluate for a potential upregulation of the hPro transgene expression in the bladder, but the LPS treatment and the induced inflammation should not have a suppressive effect on hPro transgene expression. We could detect hPro protein expression in scraped urothelial cells from animals of the high-expressor line (47879), but not the low-expressor line (47870). The level of hPro transgene mRNA expression in line 47879, at the point of 18 h after the LPS challenge, was at ~13% of the level of mPro mRNA expression, a significant boost to the overall presence of prostasin serine protease. We performed gene expression analysis on both lines but will only consider the high-expressor line (47879) for data interpretation in this study.

More important was the observation that induction of the cytokine mRNA expression locally in the bladder (i.e., TNF-α,
IL-1β, and IL-6 mRNA) by LPS was not impeded by the forced hPro expression, suggesting that the LPS-TLR4 signaling events are not affected in these animals. These events trigger the cytokine expression responses necessary for urothelial iNOS induction. This speculation is consistent with the observation that hPro attenuated LPS-induced iNOS expression in the bladder but did not abolish the induction. The remaining upregulated iNOS mRNA expression may be attributed to the infiltrating inflammatory cells, which respond directly to LPS for iNOS induction (31, 49). We challenged an hPro-transfected murine macrophage cell line RAW 264.7 with LPS, and the iNOS induction response was not affected by this ectopic prostasin expression (data not shown), suggesting that the prostasin-associated phenotype may be cell type specific. The unimpeded portion of bladder iNOS expression may also be attributed to the smooth muscle cells, which do not express appreciable amounts of native prostasin (mPro) (Fig. 1) but have been shown to express iNOS in response to TNF-α and IL-1β (25). The fact that COX-2 induction by the ip-LPS was not affected by hPro expression helps pin-point the iNOS expression attenuation phenotype associated with hPro to the urothelial cells, because these cells do not express COX-2 in response to inflammation caused by bacterial infection, but respond by expressing iNOS (34).

The highly specific manner of prostasin’s attenuating effect on the iNOS induction in the bladder urothelial cells in response to LPS may be used as the basis of placing prostasin’s action at the cytokine receptor level. Prostasin did not affect the bladder proinflammatory cytokine production at the transcription level, suggesting that prostasin could affect iNOS expression through a protein-protein interaction at the level of cytokine signaling. This protein-protein interaction mechanism is most likely occurring at the plasma membrane because of prostasin’s membrane localization, indicating a point of impact in the extracellular portion of the cytokine receptors or the cytokines themselves. The presence of forced prostasin expression intercepted the upregulation of iNOS gene expression mediated through the proinflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6). The interception of cytokine signaling by prostasin is apparently dependent on its protease activity because the catalytically inactive mutant prostasin did not produce the attenuating effect as the wild-type prostasin serine protease. It is possible that prostasin could cleave the extracellular domains of the cytokine receptors, rendering the receptors inactive. Alternatively, prostasin could also proteolytically process the cytokines themselves, reducing their signal strengths required for maximal iNOS induction. These potential mechanisms of action will be the subject of continued research investigation.

![Figure 6](http://ajprenal.physiology.org/)
Two phenotypes observed in both lines of the transgenic mice, mPro expression rescue and IFN-γ induction attenuation following an LPS challenge, were not reproduced in the gene delivery model. LPS-induced IFN-γ expression was attenuated to similar levels (~48%) in both lines of the transgenic mice, but the iNOS phenotype is only associated with the higher level hPro transgene expression in line 47879. It appears that this level of attenuation of LPS-induced IFN-γ expression was not a mechanism for iNOS attenuation in this animal model; the remaining IFN-γ may still be sufficient to serve its potentiator role for iNOS induction (26).

Increases in NO production in the bladder during urinary tract infections (45) have previously been linked to iNOS activation, which occurs at 4 h after LPS administration (33, 34, 36). Recently, another isoform of the nitric oxide synthase, the endothelial NOS (eNOS), was shown to be an early bladder responder to intraperitoneal administered LPS, at 1 h following the LPS treatment, before infiltration of inflammatory cells (27). The eNOS-related early-phase NO production is believed to have a beneficial effect, probably in killing bacteria (12). But the late-phase NO production seen in bacterial infection of the bladder, mediated via iNOS after the majority of the invading bacteria is cleared (34), may play different roles such as promoting urothelial cell apoptosis/shedding and interfering with urothelial cell differentiation (14). These roles or events are potentially intensified by a downregulation of in situ urothelial prostasin expression. There is an attenuating effect on the LPS downregulation of the native mouse prostasin gene (Fig. 3 and Table 1) in the transgenic animals, for both the high- and the low-expresser lines. But this mPro mRNA expression rescue phenotype was not reproduced in the gene delivery model after LPS injection. The mPro expression rescue phenotype in the transgenic animals is probably mediated by a mechanism unrelated to hPro transgene expression because it did not correlate with the hPro expression level in the bladder.

Prostasin may be viewed as a protective agent during the bladder inflammatory response to LPS, while the inflammatory response itself is a sufficient event to downregulate prostasin iNOS gene upregulation and potentially the deleterious effects associated with the late-phase NO production. This putative protective role of prostasin serine protease via downmodulating cytokine signaling contrasts the proinflammatory role of classic, secreted neutrophil serine proteases, such as cathepsin G, elastase, and proteinase 3, which are proteolytic activators of the proinflammatory cytokines (47).

By current understanding, the bladder’s defense against bacterial infection is orchestrated through the following key events: engulfment of the invading pathogen by the urothelial umbrella cells, activation of innate immunity and secretion of cytokines, and recruitment of proinflammatory cells with further production of cytokines (32). In response to these events and molecular mediators, the urothelium undergoes a renewal process, shedding off the urothelial umbrella cells via an apoptotic mechanism and taking away with them the majority of the invading pathogen. The renewal process is completed by differentiation of the underlying uroepithelial cells. NO may be a key factor in the apoptotic process (11), but its long-term presence, produced by the iNOS, could interfere with the healing of the urothelium. Our hypothesis is that prostasin regulates the long-term NO production by reducing iNOS expression in the urothelial cells, promoting differentiation. Uroplakins are markers of uroepithelial differentiation and are expressed in an inverse pattern against iNOS in the urinary bladder urothelium of patients with bladder outlet obstruction caused by benign prostatic hyperplasia (35). This observation is consistent with the current view that prolonged iNOS presence in the bladder urothelium and/or its microenvironment inhibits urothelial differentiation. We can draw further support for our hypothesis from the observation that absence of prostasin by conditional knockout in the skin results in lack of occludin expression and disruption of tight junctions (30), structures that are as well essential for bladder to function as a barrier organ. Tight junction disruption and tight junction protein expression downregulation are also events induced by LPS and mediated by iNOS-dependent NO production in the intestinal, liver, and lung epithelia (18, 19, 20). These events may all be stringed together by the theme of reduction or lack of prostasin expression and its modulator role on iNOS expression.

In summary, we showed in this study that the GPI-anchored extracellular active serine protease prostasin is a new player in the bladder urothelium during inflammation caused by LPS. Induction of the iNOS gene in the urothelium by the proinflammatory cytokines is regulated by active prostasin but not its catalytic mutant. The urinary bladder is a good candidate for gene therapy owing to its easy anatomic accessibility (4), and prostasin serine protease may be exploited for use to treat bladder disorders stemming from the state of sustained inflammation or iNOS expression.

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REFERENCES

Prostaglandin E2 (PGE2) is a major product of prostanoid synthesis and plays a crucial role in various physiological processes. It is produced by the cyclooxygenase (COX) enzymes, and its synthesis is regulated by prostanoid transporters and enzymes. In the present study, we investigated the regulation of PGE2 synthesis in human airways and its potential role in asthma.

**Materials and Methods**

We used human bronchial epithelial cells (HBECs) and airway smooth muscle cells (ASMCs) as models to study PGE2 synthesis. We measured PGE2 levels using an enzyme-linked immunosorbent assay (ELISA) and quantified COX enzyme activity using a spectrophotometric assay. We also studied the effect of various pharmacological agents on PGE2 synthesis.

**Results**

Our results showed that PGE2 synthesis in HBECs and ASMCs was significantly increased upon stimulation with histamine, a known mediator of airway inflammation. Furthermore, we observed that PGE2 synthesis was upregulated by the phosphorylation of COX enzymes, which was mediated by the mitogen-activated protein kinase (MAPK) pathway.

**Conclusions**

Our findings provide new insights into the regulation of PGE2 synthesis in human airways and suggest potential therapeutic targets for the treatment of asthma and related airway diseases. Further studies are needed to validate these findings and to explore the clinical implications of our observations.

