Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder

LORI A. BIRDER, GERARD APODACA, WILLIAM C. DE GROOT, AND ANTHONY J. KANAI

Department of Pharmacology and Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and Department of Biomedical Engineering, Duke University, Durham, North Carolina 27508

Birder, Lori A., Gerard Apodaca, William C. de Groat, and Anthony J. Kanai. Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. Am. J. Physiol. 275 (Renal Physiol. 44): F226–F229, 1998.—Nitric oxide (NO) has been implicated in the regulation of the lower urinary tract. However, the source(s) of NO production in the urinary bladder (UB) has not been determined. Accordingly, we used a porphyrinic microsensor placed on the surface of UB strips in vitro to directly measure endogenous NO production. The afferent neurotransmitter, capsaicin, and the mixed α/β-adrenergic agonist, norepinephrine (NE), both evoked transient (1–3 s) NO release (range 50 nM to 1.4 µM). Adrenergic-mediated release was not decreased following denervation of the UB but was abolished following selective removal of the mucosa. On the other hand, release evoked by capsaicin (range 50–900 nM) was significantly decreased after UB denervation. These data indicate that NE releases NO from UB epithelium, and capsaicin releases NO from epithelium as well as nerve tissue in the UB. In light of reports that NO may regulate epithelial integrity and function in other tissues, agonist regulation of a constitutive nitric oxide synthase activity in the UB may provide a novel mechanism for modulation of bladder and urothelial function.

Constitutive nitric oxide synthase; lower urinary tract; capsaicin-sensitive bladder afferents; epithelium

Nitric oxide (NO), a novel transmitter in the central and peripheral nervous systems (7, 19) may play a role in the neural control of lower urinary tract function. Nitric oxide synthase (NOS) immunoreactivity and NADPH-diaphorase (a marker for NOS) have been detected in afferent and efferent nerve fibers in the urinary bladder (UB) and urethra (2, 25). In addition, targeted disruption of the neuronal NOS (nNOS) gene results in voiding abnormalities, and inhibitors of NOS can alter micturition (9, 21). However, because of the difficulty of measuring the NO free radical, the life-half of which is less than 6 s, the stimuli that evoke NO production and the sources of NO in UB have not been previously determined. We have used a selective porphyrinic microsensor to measure NO concentrations on the surface of UB strips or cultured urothelial cells to determine the source(s) of NO in UB and the factors that evoke its release. This report describes the novel observation that NO can be released from the UB epithelium (urothelium) as well as from nerves. NO released from the urothelium may act to modulate urothelial integrity or may act as a chemical messenger in a signaling mechanism between the urothelium and adjacent structures such as sensory nerves. These findings are of potential clinical significance, since alterations in NO release and changes in urothelial function have been detected in pathological conditions such as interstitial cystitis (11, 15, 20, 26).

METHODS

The experiments were performed on 28 adult Wistar rats (15–250 g) of either sex in addition to adult rabbits used to prepare cultures of bladder epithelium. The Institutional Animal Care and Use Committee approved all procedures. Measurement of nitric oxide release from isolated bladder strips. Twenty-eight animals were deeply anesthetized using a combination of ketamine and xylazine (each 10 mg/kg IP). Isolated UB strips (2 × 5 mm) were removed, attached to glass coverslips, and placed in a chamber mounted on the stage of an inverted microscope for measurement of endogenous NO production. All recordings were performed in Ringer solution that contained (in mM) 140 NaCl, 5.2 KCl, 1.2 MgSO4, 1.8 CaCl2, 10 NaH2PO4/Na2HPO4, and 10 glucose, pH 7.4. Nafion-coated porphyrinic microsensors (NO detection limit, 1 nM; response time, 1 ms) were prepared and calibrated as previously described with some modifications (5, 17). The tip of a microsensor, which specifically detects NO, was placed directly on the bladder surface (5). Drugs were injected into the perfusate or applied locally as a bolus using a nanoejector. The currents generated by the oxidation of NO to NO+ at the porphyrinic interface (0.5 to 1.5 nA/cm2 for 1 µM NO under static conditions) were amplified and converted to voltages using a model 283 Potentiostat (EG & G Princeton Applied Research, Princeton, NJ), then digitized for viewing on a monitor and stored on a computer hard disk for later retrieval and analysis. The onset of release by a given agonist was rapid (within 1 s), with peak release within 2–3 s. As a control, all agents were applied without tissue present to assure that the electrode did not respond to the agonist. Alternatively, perfusate was applied to the tissue with the electrode in place to assure that flow did not cause cellular disruption or release of intracellular substances that might induce the synthesis of NO. A positive response was defined as greater than a 5% change in baseline.

Preparation of urothelial cultures. Establishment and characterization of urothelial cultures has been described in a preliminary report (32). A complete characterization of this culture system will be provided in a subsequent communication. Briefly, female New Zealand White rabbits were anesthetized with phenobarbitol, then their bladders were excised, cut open, and gently stretched, epithelium side down, on a rack. The muscle was dissected away, and the epithelium (with attached submucosa) was incubated overnight in minimal essential medium (Cellgro) containing penicillin/streptomycin/fungizone (MEM medium) and 2.5 mg/ml dispase (GIBCO). The epithelium was then gently scraped from the underlying tissue and treated with 0.25% (wt/vol) trypsin to generate a single cell suspension. The single cell suspension was then washed several times with MEM medium, and the cells were resuspended in defined, serum-free keratinocyte medium (GIBCO) and plated on collagen-coated dishes or
Transwell filters. Cells were used between 2–7 days in culture. All of the cells in these cultures were cytokeratin positive and, therefore, were presumably of epithelial origin.

Denervation experiments. In a separate group of deeply anesthetized animals (n = 8), the major pelvic ganglia (MPG) were removed (bilaterally), and animals survived an additional 4 days prior to death (3). UB tissue strips were removed as described above and used to measure endogenous NO production.

Removal of the mucosa. To study the effect of agents on the isolated UB mucosa (8 animals), the mucosa was selectively removed from the UB smooth muscle by pinning the isolated UB strips on a Sylgard-coated plate, then gently peeling the muscle layer away from the underlying mucosal layer (10). Both mucosal and the underlying smooth muscle layers were used to measure endogenous NO production. Removal of the mucosal layer from underlying smooth muscle was verified histologically using hematoxylin-eosin staining to evaluate tissue morphology.

Statistics. Data represent the means ± SE for determinations in at least 10 different preparations. Analysis of variance and the Student-Newman-Keuls test were used for multigroup comparisons. Values of \( P < 0.05 \) were considered statistically significant.

RESULTS

Agonist-evoked release of NO from UB. After intracellular synthesis, the lipophilic NO free radical readily diffuses into the cell membrane, where the highest concentrations are measured (31). Thus agonist-evoked NO release was measured by placing the porphyrinic microelectrode sensor directly on the mucosal surface of the UB (Fig. 1A). Basal release of NO could not be detected in UB strips from normal rats (n = 12). However, application of the Ca\(^{2+}\) ionophore, A-23187 (10 µM), increased NO production in the UB (mean of 300 nM, range of 80–500 nM), demonstrating activation of a calcium-dependent constitutive NOS. Nitric oxide production was also increased by application of the mixed \( \alpha/\beta \)-adrenergic agonist, norepinephrine (NE), at 1 µM (mean of 740 ± 92 nM NO; range of 50 nM to 1.4 µM NO release), or the neurotoxin, capsaicin, at 500 nM (410 ± 159 nM NO; range of 50–900 nM NO release). Release was not evoked by carbachol (1 µM; Fig. 1B) nor by application of the \( \alpha_2 \)-adrenergic agonist clonidine (1 µM). Adrenergic-evoked NO release was reduced (80% ± 6.2%, \( P < 0.05 \)) by the nonselective \( \beta_1/\beta_2 \)-receptor antagonist propranolol (20 µM) and by the \( \alpha_2 \)-adrenergic antagonist phentolamine (20 µM) (20% ± 5.1%, \( P < 0.05 \)).

Regional specificity of adrenergic-evoked NO release. The peak NO concentration produced in response to adrenergic agonists was not significantly different in the body or neck of the UB. In contrast, the neurotoxin capsaicin, in a concentration (500 nM) that selectively activates C-type sensory nerves (4), elicited greater NO release from the bladder body (420 ± 130 nM NO) than

---

Fig. 1. A: endogenous NO release was measured by placing the tip of a Nafion-coated porphyrinic microsensor directly on the surface of the isolated urinary bladder (UB) strip. A three-electrode recording system was used consisting of a working electrode (porphyrinic microsensor), saturated calomel reference electrode, and platinum counter electrode. B: transient NO release due to constitutive nitric oxide synthase (NOS) measured from the mucosal surface of isolated UB strips following the application of norepinephrine (NE, range 0.25 to 1 µM). Carbachol (10 µM) did not release NO. Arrows indicate the start of drug application. Control application of drug vehicle does not release NO. C: concentration of NO released from isolated strips of the UB by NE (1 µM) under different conditions. Mucosal surface, NE-evoked NO release measured with the microsensor on the mucosal surface of UB strips. Denervated bladder, NE-evoked NO release from the mucosal surface of the UB following bilateral removal of major pelvic ganglia 4 days prior to the experiment. Serosal surface, NE-evoked NO release from the serosal surface of the UB. UB minus mucosa, NO release from underlying UB smooth muscle following removal of the mucosal layer. The concentrations of NO released from serosal surface and UB minus mucosa preparations are significantly (\( P < 0.05 \)) different from mucosal surface or denervated bladder preparations; n = 10 observations for each graph.
from the bladder neck (55 ± 18 nM NO). The NOS inhibitor, N α-monomethyl-L-arginine (L-NMMA, 50 µM), completely blocked NE and partially blocked (70% ± 6.3%) capsaicin-evoked release of NO from isolated UB strips.

Agonist-evoked NO release effect of denervation. The contribution of nervous tissue to NO release from the UB was examined 4 days following bilateral removal of the MPG. In denervated UB strips, the NO release induced by capsaicin was significantly decreased (60% ± 7.2% decrease, P < 0.05, 246 ± 30 nM NO). However, there was not a significant change in the release induced by NE (P > 0.05, Fig. 1C).

Capsaicin-evoked NO release from urothelial cells. Because denervation did not completely block capsaicin-induced NO release, it is likely that capsaicin also releases NO from a nonneural source; thus we examined the effect of capsaicin on primary cultures of urothelial cells. In these cells, capsaicin (500 nM) elicited a significant NO release (245 ± 58 nM NO; n = 10 cells).

NE-evoked NO release role of the UB mucosa. The mixed α/β-adrenergic agonist, NE, evoked NO release from both the bladder mucosal and serosal surfaces (Fig. 1, B and C). However, the maximal concentration of NO was greater at the mucosal surface (Fig. 1C). To further investigate this disparity, we sought to determine whether NO is produced in the mucosal layer and then diffuses to the serosal surface. This was accomplished by selectively removing the mucosal layer. Histological sections of isolated UB strips in which the mucosa was removed showed a significant denudation of the epithelial layer (not shown). In addition, the peak NO concentration released by isolated mucosa was not significantly different (710 ± 120 nM NO, P > 0.05) from that released by isolated bladder strips, whereas the NE-evoked NO release from the underlying smooth muscle after removal of the mucosa was significantly less (90 ± 48 nM NO, P < 0.05; Fig. 1C). These data suggest that adrenergic-evoked NO release from UB is dependent in large part on the presence of the mucosa.

DISCUSSION

The present study, which used a porphyrinic microsensor to detect NO, revealed that agonist-induced NO release from UB is dependent on both neural and nonneural tissues.

Capsaicin-induced NO release from UB is due, in part, to activation of a population of small diameter afferent nerves (24), since release was markedly reduced by denervation. Capsaicin may release NO directly from these nerves or evoke release indirectly from other cells via the action of neuropeptides (calcitonin gene-related peptide, substance P) released from afferent terminals (16). However, denervation did not completely block capsaicin-induced NO release, suggesting that NO may also be released from other sources. The bladder epithelium is a possible source of NO, since primary cell cultures consisting of urothelium but lacking neurons release NO following application of capsaicin. Capsaicin-induced release of NO and neuropeptides in the UB could contribute to the acute biphasic effect of capsaicin to first excite and then inhibit voiding function (16).

Although adrenergic agonists can release NO from isolated afferent neurons (6), the present data demonstrates that NO release from bladder strips is not mediated by nerves, but seems to depend in large part upon nonneural mechanisms in the mucosa. The adrenergic-evoked release of NO in bladder strips was reduced by 85% after removal of the mucosal layer. It seems likely that NO arises, in part, from the urothelial cells, since NOS immunoreactivity has been detected in epithelial cells in bladder, gut, and lung (1, 12, 21, 23). However, release in the mucosal layer might also originate from other cells such as endothelial cells of blood vessels.

NO release from bladder mucosa could have multiple functions and play a role in pathological mechanisms as noted in the gut (1, 8, 22). For example, chronic inflammation in the intestine is associated with changes in NO metabolism that seems to influence the barosensitivity of the epithelium (13, 18, 27). UB disorders such as interstitial cystitis, which can be accompanied by inflammation, are also associated with abnormalities in NO production and changes in urothelial permeability (11, 15, 20, 26). Chronic bladder inflammation also increases the expression of NOS in bladder afferent neurons (28). Since NO has minimal direct effects on the rat detrusor muscle (21) but does have effects on sensory neurons (29, 30), NO released in the bladder may modulate micturition by influencing the excitability of bladder afferents (14, 21). It is also possible that excess NO production leads to tissue injury and urothelial dysfunction. Further studies are needed to determine whether the elevated NO levels that follow chronic injury or inflammation contribute to urothelial abnormalities.

In summary, this study indicates that NO release in the UB arises from both nerves and nonneural tissue. NO release from bladder mucosa evoked by NE suggests a novel mechanism for this signaling molecule in maintenance of urothelial function.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-51402 and DK-49430.

Address for reprint requests: L. A. Birder, Univ. of Pittsburgh School of Medicine, E 1304 BST Dept. of Pharmacology, Pittsburgh, PA 15261.

Received 28 October 1997; accepted in final form 23 April 1998.

REFERENCES