Nitric oxide modulates renal sensory nerve fibers by mechanisms related to substance P receptor activation

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Kopp, Ulla C., Michael Z. Cicha, Lori A. Smith, and Tomas Hökfelt. Nitric oxide modulates renal sensory nerve fibers by mechanisms related to substance P receptor activation. Am J Physiol Regulatory Integrative Comp Physiol 281: R279–R290, 2001.—Nerve terminals containing neuronal nitric oxide synthase (nNOS) are localized in the renal pelvic wall where the sensory nerves containing substance P and calcitonin gene-related peptide (CGRP) are found. We examined whether nNOS is colocalized with substance P and CGRP. All renal pelvic nerve fibers that contained nNOS-like immunoreactivity (-LI) also contained substance P-LI and CGRP-LI. In anesthetized rats, renal pelvic perfusion with the nNOS inhibitor S-methyl-L-thiocitrulline (L-SMTC, 20 μM) prolonged the afferent renal nerve activity (ARNA) response to a 3-min period of increased renal pelvic pressure from 5 ± 0.4 to 21 ± 2 min (P < 0.01, n = 14). The magnitude of the ARNA response was unaffected by L-SMTC. Similar effects were produced by Nω-nitro-L-arginine methyl ester (L-NAME) but not D-NAME. Increasing renal pelvic pressure produced similar increases in renal pelvic release of substance P before and during L-SMTC, from 5.9 ± 1.4 to 13.6 ± 4.2 pg/min before and from 4.9 ± 12.6 ± 2.7 pg/min during L-SMTC. L-SMTC also prolonged the ARNA response to renal pelvic perfusion with substance P (3 μM) from 1.2 ± 0.2 to 5.6 ± 1.1 min (P < 0.01, n = 9) without affecting the magnitude of the ARNA response. In conclusion: activation of NO may function as an inhibitory neurotransmitter regulating the activation of renal mechanosensory nerve fibers by mechanisms related to activation of substance P receptors.

mechanosensory neurons; neuronal nitric oxide synthase; renal pelvis; afferent renal nerve activity; calcitonin gene-related peptide; dorsal root ganglion

ACTIVATION OF MECHANOSENSITIVE nerve fibers in the renal pelvic wall by increases in renal pelvic pressure of as little as 5 mmHg (39) increases ipsilateral afferent renal nerve activity (ARNA) (33–39), decreases contralateral efferent renal nerve activity, and increases contralateral urinary sodium excretion, known as a renorenal reflex response (35). The low threshold for activation of the renal pelvic mechanosensitive nerves suggests that changes in ARNA play a physiological role in the renal control of body water and sodium.

In the kidney, the majority of sensory nerve fibers containing substance P and calcitonin gene-related peptide (CGRP) are located in the renal pelvic wall (18, 41, 68). Substance P plays a crucial role in the activation of renal mechanosensitive nerves (33, 34, 36). Increasing renal pelvic pressure increases the release of bradykinin, which increases the renal pelvic release of PGE2 via activation of the phosphoinositide system (33). PGE2 causes a calcium-dependent release of substance P (32) that in turn increases ARNA by activation of substance P receptors in the renal pelvic area (33, 38). Regarding the role of CGRP, our studies suggest that CGRP potentiates the effect of substance P by retarding the metabolism of substance P (23).

Considerable evidence suggests that nitric oxide (NO) is not only of importance for control of vascular tone (19, 29, 49) but is, in fact, also a putative neuromodulator in the central and peripheral nervous system (8, 21). NO is formed by three different types of the enzyme NO synthase (NOS), referred to as neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS is localized to discrete populations of neurons in the central and peripheral nervous system (6, 7). A role for NO as a sensory transmitter was initially suggested from the localization of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase, a histochemical marker for NOS (15, 26, 58) in dorsal root ganglion (DRG) neurons (2). Interestingly, the highest numbers of DRG neurons positive for NADPH-diaphorase and NOS-like immunoreactivity (-LI) were found at caudal thoracic and rostral lumbar levels (2, 59), i.e., those DRG neurons that contain the cell bodies of the afferent renal nerves (9, 16, 68). In the periphery, there is much evidence for nNOS containing nerve fibers in the gastrointestinal tract and genitourinary tract (50, 67, 70). Many of the nNOS containing peripheral nerve fibers also contain substance P and/or CGRP (50, 69). In the kidney, nNOS-LI was found in the juxtaglomerular cells, renal medulla, and in nerves and neural somata in the renal pelvic wall (3, 42). Whether nNOS is colocalized with substance P and/or...
CGRP in the renal pelvic nerves was not determined in these studies. The interaction between NO and substance P is complex. Capsaicin, a known activator of substance P, increases NO release from nerve tissue in the urinary bladder (5). These studies, together with studies on DRG neurons demonstrating that the capsaicin-mediated increase in substance P is not altered by NOS inhibition (17), suggest that the capsaicin-induced NO production is due to mechanisms distal to substance P release. In support of this hypothesis are studies showing that heat-induced substance P release causes NO production via activation of neurokinin-1 receptors in the rat paw (63). Furthermore, substance P-induced hyperalgesia is blocked by NOS inhibition (52, 63). On the other hand, NO has been shown to modulate the release of substance P from central and peripheral sensory nerves (22, 25, 31, 40, 61).

The considerable evidence for NO being a messenger molecule of the central as well as of the peripheral sensory nerves in various visceral organs suggests that the nNOS-containing nerve fibers in the kidney (3, 42) may be of sensory origin. Therefore, we examined whether nNOS was colocalized with substance P and CGRP in renal pelvic nerves and DRG neurons at T9-L1. Because our findings demonstrated that nNOS was colocalized with substance P and CGRP in the renal pelvic sensory nerves, we then examined the functional role of NO in the activation of renal mechanosensory nerves by studying the effects of nNOS inhibition on the increase in ARNA and substance P release produced by increased renal pelvic pressure.

Because our studies showed that nNOS inhibition modified the ARNA response but not the increase in substance P release, we subsequently examined the effects of nNOS inhibition on the ARNA response to substance P receptor activation.

METHODS

Localization of Substance P, CGRP, and nNOS:
Immunohistochemical Procedures

Male Sprague-Dawley rats anesthetized with pentobarbital sodium (0.2 mmol/kg ip) were transfused cardially with warm (37°C) Ca²⁺-free Tyrode’s solution followed by cold (4°C) fixative containing 4% wt/vol paraformaldehyde and 0.2% wt/vol picric acid in 0.1 M PBS (pH 7.2) for 6 min (51, 65). The kidneys and DRGs at the T9-L1 level were fixed for double labeling (14, 60). The tissues were rehydrated according to the indirect immunofluorescence technique modified for double labeling (14, 60). The tissue sections were processed according to the indirect immunofluorescence technique modified for double labeling (14, 60). The tissues were rehydrated in 0.1 M PBS and incubated overnight in a humid chamber at 4°C with primary antiserum for CGRP [CGRP antiserum, mouse, 1:400; J. H. Walsh and H. C. Wong, (unpublished)], nNOS [sheep, 1:1,000 (24)], or a mixture of the two antisera diluted in PBS containing 1% bovine serum albumin, 0.3% Triton X-100, 0.01% sodium azide, and 0.02% bacitracin. The tissue-bound antibodies were detected by incubating the sections at 37°C for 30 min with Rhodamine Red-X (RRX)-conjugated donkey anti-mouse (1:80, Jackson Immuno Research, West Grove, PA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat (1:80, Jackson Immuno Research) antibodies in PBS containing 0.3% Triton X-100. After several washes in PBS, the sections were placed under a coverslip in glycerol-PBS with 0.1% p-phenylenediamine added to retard fading.

Substance P. The tyramide signal amplification (TSA) indirect technique [Ref. 1, New England Nuclear (NEN), Life Science Products, Boston, MA] was used to enhance the fluorescence signal binding. After preincubation with 0.03% H₂O₂ in PBS to minimize background and washes with PBS, the tissues were incubated with primary antiserum for substance P [rabbit, 1:4,000 (13)] according to the protocol above. The following day, anti-rabbit horseradish peroxidase antibodies (DAKO, Copenhagen, Denmark) were applied to the tissues followed by biotinylated tyramine using a modified protocol of a commercial TSA kit from NEN (62). The reactions were detected with streptavidin conjugated with fluorescein or Texas red (NEN).

Substance P + CGRP and substance P + nNOS. After completion of the protocol for indirect TSA for detection of substance P, the tissues were processed further by the indirect immunofluorescence technique for CGRP and nNOS, as described above.

The tissue sections were examined in a Nikon Microphot FX-fluorescence microscope. FITC- and RRX-induced fluorescence was studied with a Nikon B-1E filter cube (excitation at 480 ± 10 nm with a band-pass emission filter passing 520–550 nm and 546 ± 5 nm with a barrier filter at 590 nm, respectively).

Effects of NOS Inhibition: In Vivo Studies

The study was performed on male Sprague-Dawley rats weighing 182–351 g (mean 277 ± 6 g). Anesthesia was induced with pentobarbital sodium (0.2 mmol/kg ip) and maintained with an infusion of pentobarbital sodium (0.04 mmol·kg⁻¹·h⁻¹ iv) in isotonic saline at 50 µl/min into the femoral vein. Arterial pressure was recorded from a catheter in the femoral artery. The procedures for stimulating and recording ARNA have been previously described in detail (33–39). In short, the left kidney was approached by a flank incision, a PE-10 catheter was placed in the right ureter for collection of urine, and a PE-60 catheter was placed in the left ureter with its tip in the pelvis. The left renal pelvis was perfused, via a PE-10 catheter placed inside the PE-60 catheter, throughout the experiment at 20 µl/min with vehicle or various renal perfusate administered in the different experimental protocols. ARNA was stimulated by increasing renal pelvic pressure or administering substance P (see below) into the renal pelvis via the PE-10 catheter. Renal pelvic pressure was increased by elevating the catheter above the level of the kidney. ARNA was recorded from the peripheral portion of the cut end of one renal nerve branch placed on a bipolar silver-wire electrode. ARNA was integrated over 1-s intervals, the unit of measure being microvolts per second per second. Postmortem renal nerve activity, which was assessed by crushing the decentralized renal nerve bundle peripheral to the recording electrode, was subtracted from all values of renal nerve activity. ARNA was expressed as a percentage of its baseline value during the control period (33–39).
Throughout the experiment, except during the two experimental periods when substance P was added to the pelvic perfusate.

**Drugs.** All agents, except otherwise stated, were from Sigma (St. Louis, MO). L-NAME, D-NAME, and L-SMTC (n = 4) were dissolved in 0.15 M NaCl. Thiorphan was dissolved in 100% ethanol and further diluted in 0.15 M NaCl; the final ethanol concentration being 0.1%. L-SMTC (n = 10), and substance P were dissolved in 0.1% thiorphan in 0.15 M NaCl to minimize metabolism of substance P.

**Analytic procedure.** Contralateral right urinary sodium concentrations were determined with a flame photometer. Right urinary sodium excretion was expressed per gram kidney weight.

Substance P in the renal pelvic effluent was measured by ELISA as previously described in detail (32). The rabbit substance P antibody (IHC 7451, Peninsula, San Carlos, CA) demonstrated 100% cross-reactivity with fragments 2–11, 3–11, 4–11, and 5–11; less than 5% with 6–11; and less than 1% with fragment 7–11, neurokinins B and A, endothelin-1, somatostatin, and vasoactive intestinal polypeptide.

**Experimental Protocols**

Approximately 1.5 h elapsed between the end of surgery and the start of the experiment to allow the rat to stabilize; stabilization was determined by 30 min of steady-state urine collections and ARNA recordings.

**Effects of NOS inhibition on the ARNA response to increased renal pelvic pressure.** The experiment consisted of two parts separated by a 10-min interval. Each part consisted of a 20-min control, a 3-min experimental, and a 30-min recovery period. Renal pelvic pressure was increased during each experimental period. Two groups were studied. In the first group (n = 11), the renal pelvis was perfused with vehicle (0.15 M NaCl) throughout the first part and the nonselective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) at 5 mM throughout the second part of the experiment. Thiorphan (0.1%) was added to the renal pelvic perfusate during each experimental period. Substance P (3 μM) was added to the renal pelvic perfusate in 10 of the 14 rats in which urine was collected for substance P assay.

**Effects of nNOS inhibition on the ARNA response to increased renal pelvic pressure.** One group was studied (n = 14). The experimental protocol was identical to that of previous groups except the renal pelvis was perfused with the selective nNOS inhibitor S-methyl-L-thiocitrulline (L-SMTC, 20) at 20 μM during the second part of the experiment. Thiorphan (0.1%) was added to the renal pelvic perfusate in 10 of the 14 rats in which urine was collected for substance P assay.

**Effects of nNOS inhibition on the ARNA response to substance P.** The experiment consisted of two parts separated by a 10-min interval. Each part consisted of a 10-min control, a 5-min experimental, and a 25-min recovery period. Two groups were studied. In the first group (n = 9), the renal pelvis was perfused with vehicle (0.1% thiorphan) in 0.15 M NaCl throughout the first part and L-SMTC (20 μM) throughout the second part of the experiment. Substance P (3 μM) was added to the renal pelvic perfusate during each experimental period. In the second group (n = 5), which served as time controls, the experimental protocol was the same, except the renal pelvis was perfused with vehicle throughout the experiment, except during the two experimental periods when substance P was added to the pelvic perfusate.

**Localization of Substance P, CGRP, and nNOS**

Substance P- and CGRP-LI containing nerve terminals were found distributed in the renal pelvic wall (Fig. 1). The highest density of substance P- and CGRP-LI-containing processes was found in the muscular layer with few fibers penetrating the uroepithelial layer of the pelvic wall. Double labeling showed that all terminals in the renal pelvic wall containing substance P also contained CGRP.
substance P-LI contained CGRP-LI and vice versa. Substance P- and CGRP-LI-containing fibers were also found in the wall of the arteries and veins in the renal pelvic area. The density of the substance P- and CGRP-LI-containing fibers was lower in the vessel wall than in the renal pelvic wall. Labeling kidney sections with an antibody to nNOS showed a high density of nNOS-LI-containing nerve terminals in the renal pelvic wall (Fig. 2A), similar to that of substance P- (Fig. 2B) and CGRP-LI-containing nerve terminals. Because the distribution of nNOS-LI-containing terminals was similar to that of the substance P- and CGRP-LI-containing terminals, we double-labeled kidney sections with antibodies to nNOS and substance P and CGRP, respectively. These studies showed that all nNOS-LI-containing nerves in the renal pelvic wall contained substance P- and CGRP-LI (Fig. 3). To further examine whether the nNOS-LI-containing nerves were of sensory origin, we double labeled DRGs at the T9-L1 level with antibodies to nNOS, substance P, and CGRP, respectively; DRGs at T9-L1 contain the majority of the cell bodies of the afferent renal nerves (9, 16, 68). Numerous neuronal cell bodies in the L1 DRGs were labeled with the antibodies to substance P and CGRP (Fig. 4, A and B). Although all CGRP-LI-containing cell bodies contained substance P-LI, there were some cell bodies that contained CGRP-LI but not substance P-LI. Double labeling of adjacent sections of T11 DRGs with antibodies to substance P and nNOS (Fig. 4, C and D) and to CGRP and nNOS (Fig. 4, E and F) showed that all cell bodies containing nNOS-LI also contained substance P- and CGRP-LI. However, there were cell bodies containing substance P- and CGRP-LI but not nNOS-LI. A similar distribution of nNOS-, substance P-, and CGRP-LI was found in DRGs from all levels (T9-L1) examined.

Effects of NOS Inhibition on the ARNA Response to Increased Renal Pelvic Pressure

Because our immunohistochemical studies showed the presence of nNOS in renal sensory nerve terminals, we hypothesized that NO may be involved in the activation of renal mechanosensitive nerve fibers. We tested this hypothesis by examining the effects of renal pelvic perfusion with the nonselective NOS inhibitor L-NAME on the ARNA response to increased renal pelvic pressure. During renal pelvic perfusion with vehicle, increasing renal pelvic pressure of 20 ± 1 mmHg for 3 min elicited a prompt increase in ARNA (Fig. 5). Renal pelvic perfusion with L-NAME did not alter baseline ARNA, being 1,657 ± 121 μV·s⁻¹·s⁻¹.
before and $1,587 \pm 127 \mu \text{V} \cdot \text{s}^{-1} \cdot \text{s}^{-1}$ during the first control period after the start of the l-NAME perfusion. l-NAME did not alter the magnitude of the ARNA response to increased renal pelvic pressure but produced a marked prolongation of the ARNA response (Fig. 5). Although during vehicle perfusion, the ARNA response was returned toward control values within 2 min after reduction of renal pelvic pressure to zero, ARNA remained elevated for over 20 min after renal pelvic pressure had returned to zero in the presence of l-NAME. Calculating the AUC of ARNA vs. time showed that l-NAME produced a significant enhancement of the ARNA response to increased renal pelvic pressure (Fig. 5, $P < 0.01$). The duration of the ARNA response to increased renal pelvic pressure was 5 ± 1 min before and 28 ± 3 min during l-NAME. Renal pelvic perfusion with l-NAME did not alter the increase in contralateral urinary sodium excretion produced by increased renal pelvic pressure from $2.3 \pm 0.5$ to $3.2 \pm 0.7 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (36 ± 7%) during vehicle and from $2.9 \pm 0.5$ to $3.7 \pm 0.8 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ [24 ± 10%, not significant (NS) vs. vehicle] during l-NAME. Basal mean arterial pressure (110 ± 2 mmHg) and heart rate (331 ± 14 beats/min) remained unaltered throughout the experiment.

To test the specificity of l-NAME to block NOS, we examined the effects of d-NAME, the inactive enantiomer of l-NAME, on the responses to increased renal pelvic pressure. Renal pelvic perfusion with d-NAME had no effect on either the magnitude or duration of the ARNA response to increasing renal pelvic pressure 22 ± 0 mmHg (Table 1). Likewise d-NAME did not affect the increases in contralateral urinary sodium excretion produced by increasing renal pelvic pressure from $2.1 \pm 0.4$ to $2.7 \pm 0.5 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (38 ± 7%) during vehicle and from $3.0 \pm 0.6$ to $3.8 \pm 0.8 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (24 ± 11%, NS vs. vehicle) during d-NAME. Mean arterial pressure (113 ± 2 mmHg) and heart rate (393 ± 10 beats/min) remained unchanged throughout the experiment.

**Effects of nNOS Inhibition on the ARNA Response to Increased Renal Pelvic Pressure**

Our findings showing the presence of nNOS in renal sensory nerve fibers, together with the prolongation of the ARNA response to increased renal pelvic pressure produced by l-NAME, suggested that NO may modulate the ARNA responses to activation of renal mechanosensitive nerves by activation of nNOS. To examine
this issue, we studied the effects of L-SMTC, an inhibitor of NOS that has a 17-fold higher affinity for rat nNOS than eNOS (20). During renal pelvic perfusion with vehicle, increasing renal pelvic pressure 17 ± 1 mmHg resulted in a similar increase in ARNA (Fig. 6) as that produced in the previous two groups of rats (Fig. 5, Table 1). L-SMTC did not alter baseline ARNA being 1,294 ± 46 μV·s⁻¹·s⁻¹ before and 1,275 ± 50 μV·s⁻¹·s⁻¹ during the first control period after the start of the L-SMTC perfusion. L-SMTC did not alter the magnitude of the ARNA response to increased renal pelvic pressure but produced marked prolongation of the ARNA response, the duration of the ARNA response being 5.2 ± 0.4 min before and 21 ± 2 min during L-SMTC (P < 0.01). Calculating the AUC of ARNA vs. time showed that L-SMTC markedly enhanced the ARNA response to increased renal pelvic pressure (P < 0.01, Fig. 6). This is an effect similar to that produced by L-NAME. Renal pelvic perfusion with L-SMTC did not alter the increase in contralateral urinary sodium excretion produced by increased renal pelvic pressure, from 2.6 ± 0.8 to 3.8 ± 1.4 μmol·min⁻¹·g⁻¹ (33 ± 12%) during vehicle and from 3.3 ± 0.9 to 4.1 ± 1.0 μmol·min⁻¹·g⁻¹ (33 ± 15%, NS vs. vehicle) during L-SMTC. Basal mean arterial pressure (115 ± 2 mmHg) and heart rate (361 ± 23 beats/min) remained unaltered throughout the experiment.

To examine whether the prolonged ARNA response during L-SMTC was related to increased release of substance P, we measured renal pelvic release of substance P in 10 of the 14 rats being perfused with L-SMTC. Before L-SMTC, increasing renal pelvic pressure resulted in a significant increase in renal pelvic release of substance P that was returned toward control values during the first 5-min recovery period after the renal pelvic pressure was reduced to zero (Fig. 7). Renal pelvic perfusion with L-SMTC did not alter basal renal pelvic release of substance P or the magnitude and duration of the increase in substance P release produced by increasing renal pelvic pressure.

**Effects of nNOS Inhibition on the ARNA Response to Substance P**

The lack of an effect of L-SMTC on the increase in substance P release produced by increased renal pelvic pressure suggested that NO may modulate the ARNA response to increased renal pelvic pressure by mechanisms distal to the release of substance P. We tested this idea by examining whether renal pelvic perfusion with L-SMTC altered the ARNA response to renal pelvic administration with substance P. During renal pelvic perfusion with vehicle, a 5-min renal pelvic perfusion with substance P resulted in a significant but transient increase in ARNA (Fig. 8) that lasted 70 ± 10 s. Renal pelvic perfusion with L-SMTC did not alter the magnitude of the ARNA response to substance P but produced marked prolongation of the ARNA response, the duration of the ARNA response being 336 ± 114 s during L-SMTC (P < 0.01 vs. vehicle). Calculation of the AUC of ARNA vs. time showed that L-SMTC significantly enhanced the ARNA response to substance P (P < 0.01, Fig. 8). Basal mean arterial pressure (113 ± 3 mmHg) and heart rate (357 ± 13

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**Table 1. The ARNA responses to increasing renal pelvic pressure 22 mmHg for 3 min in the presence of renal pelvic perfusion with vehicle and 5 mM d-NAME**

<table>
<thead>
<tr>
<th>Renal Pelvic Perfusion</th>
<th>AUC (%/s)</th>
<th>Duration of ARNA response (min)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>d-NAME</td>
<td>Vehicle</td>
</tr>
<tr>
<td>6,225 ± 631</td>
<td>6,384 ± 511</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
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Values are means ± SE. n = 9; ARNA, afferent renal nerve activity; AUC, area under the curve of ARNA vs. time; d-NAME, N-nitro-d-arginine methyl ester.
beats/min) remained unaltered throughout the experiment.

In agreement with our previous studies (37), repeated administration of substance P produced reproducible increases in ARNA in the time control experiments (Table 2).

DISCUSSION

The results of these experiments show that numerous nNOS-LI-containing nerve terminals are located in the renal pelvic wall. Furthermore, our studies show that these fibers virtually always contain substance P- and CGRP-LI. Since these three markers also to a large extent were colocalized in T9-L1 DRG neurons, it is highly likely that nNOS in the renal pelvic wall is present in sensory nerve terminals. Thus our data suggest that at least three messenger molecules, NO, substance P, and CGRP, can be released from these terminals. Our functional studies showed that the ARNA response to increased renal pelvic pressure was markedly prolonged by renal pelvic perfusion with L-NAME and L-SMTC. L-SMTC did not alter the increased release of substance P produced by increased renal pelvic pressure but prolonged the ARNA response to renal pelvic administration of substance P. These studies suggest that NO may function as an inhibitory neurotransmitter regulating the activation of renal mechanosensory nerve fibers by a mechanism related to activation of substance P receptors.

Localization of Substance P, CGRP, and nNOS in Renal Pelvic Nerves and DRG

In the kidney, the majority of nerve fibers containing substance P- and CGRP-LI were found in the renal pelvic wall. The greatest number was seen in the muscle layer with some fibers penetrating into the uroepithelium. These findings are in accordance with previous studies (41, 68). Our findings further showed that there was a complete colocalization of substance P- and CGRP-LI in all renal pelvic nerve terminals, i.e., all nerve fibers containing substance P-LI also contained CGRP-LI and vice versa. These findings differ to some extent from previous studies that showed the CGRP-containing nerves outnumbered substance P-containing nerves in the renal pelvic wall (18, 68). Apart from the various studies’ using different antibodies to substance P that may have different affinities to

Fig. 6. Effects of increasing renal pelvic pressure for 3 min during renal pelvic perfusion with vehicle and L-SMTC (20 μM) on ARNA (paired experimental design). **P < 0.01 vs. 0, ‡P < 0.01 ARNA response during L-SMTC vs. vehicle.

Fig. 7. Effects of increasing renal pelvic pressure for 3 min during renal pelvic perfusion with vehicle (dashed line) and L-SMTC (20 μM, solid line) on renal pelvic release of substance P (paired experimental design). ↑, increased; **P < 0.01 vs. the average value of substance P release during control and recovery periods.
the peptide, our study used indirect TSA (1) to enhance the fluorescent signal of the substance P antibody/antigen complex. Furthermore, in contrast to previous studies, we examined colocalization of substance P and CGRP by double staining the tissue sections with antibodies to substance P and CGRP. Of interest in this context is a previous study that used a double-labeling technique to show colocalization of substance P and CGRP in all sensory nerve fibers in guinea pig ureters (27).

Our studies also showed that the majority of the nNOS-LI-containing nerve fibers were located in the renal pelvic wall, in agreement with studies by Liu et al. (42). Our studies further showed that nNOS-LI was colocalized with substance P- and CGRP-LI in the renal pelvic nerves. All nerve terminals containing nNOS-LI also contained substance P- and CGRP-LI. To further examine the origin of the renal pelvic nerve fibers containing nNOS-LI, DRGs were examined for possible colocalization of nNOS with substance P and CGRP. The DRGs examined were from the T9-L1 level, because these DRGs contain the majority of the cell bodies of the afferent renal nerves (9, 16, 68). CGRP-LI was found in numerous neurons in DRG at T9-L1, the fluorescent intensity varying somewhat, in agreement with previous studies (11). Many, but not all, of these neurons contained substance P-LI. Conversely, all neurons containing substance P-LI also contained CGRP-LI. The lack of complete colocalization of substance P-LI and CGRP-LI in DRG at T9-L1 would appear to be in conflict with the virtually total coexpression of the two neuropeptides in the peripheral nerve endings in the renal pelvic wall. However, these findings may be explained by the fact that these DRGs contain neurons that project to the other visceral organs, including the lower ureteral tract (11, 56), uterus (50), and intestine (2), in addition to those projecting to the kidney. Alternatively, the levels of substance P-LI in some neurons may be too low to be detected with the present sensitivity of our immunohistochemical method. The present study shows that nNOS-LI is localized in many DRG neurons at the T9-L1 level, in agreement with previous studies (2, 11). Furthermore, our study showed that all nNOS-LI-containing neurons also express substance P- and/or CGRP-LI. Taken together, these studies strongly suggest that the nNOS-containing renal pelvic nerves are of sensory origin.

Table 2. ARNA responses to repeated administration of 3 μM substance P into the renal pelvis in the absence of l-SMTC (time control)

<table>
<thead>
<tr>
<th>AUC (%/s)</th>
<th>Duration of ARNA response (s)</th>
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<tr>
<td>802 ± 174</td>
<td>991 ± 361</td>
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<tr>
<td>74 ± 17</td>
<td>53 ± 17</td>
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</table>

Values are means ± SE, n = 5; AUC, AUC of ARNA vs. time; l-SMTC, S-methyl-L-thiocitrulline.

Role of NO in the Activation of Renal Pelvic Mechanosensory Nerves

NO is not only a regulator of smooth muscle tone but also a neuromodulator (8, 21, 66). NOS inhibition increases the activity of carotid chemo- and mechanosensory nerves, suggesting an inhibitory role of NO in the carotid chemo- and baroreceptor reflexes. In the present studies, renal pelvic perfusion with l-NAME produced a stereospecific enhancement of the ARNA response to increased renal pelvic pressure in the presence of unchanged arterial pressure. The increased ARNA response was due to a marked prolongation of the ARNA response. Similar effects were produced by l-SMTC administered at a concentration in the range of that shown to produce selective inhibition of nNOS activity in the renal microvasculature (28). These studies suggest that nNOS inhibition increases the duration of the activation of afferent renal nerves. Similar results were demonstrated in a study in cultured DRG...
neurons that examined the role of NO in the PGE$_2$-mediated enhancement of capsaicin-evoked inward current (43). l-NAME was found to markedly prolong the PGE$_2$-induced enhancement of the capsaicin response by inhibiting the NO-cGMP pathway (43). The potential relevance of these findings to the current study relates to PGE$_2$ being a crucial mediator in the activation of renal mechanosensory nerves (33, 34) and to capsaicin being a known activator of sensory neurons, including the afferent renal nerves (36). Taken together, these studies suggest that NO plays a role in the deactivation rather than the activation of the mechanosensory nerve fibers in the pelvic wall.

**Mechanisms Involved in the Enhanced ARNA Response Produced by nNOS**

Our previous studies showed that the increase in ARNA produced by increased renal pelvic pressure is related to renal pelvic release of substance P (33, 34, 38). Thus we reasoned that the prolonged ARNA response to increased renal pelvic pressure during nNOS inhibition was due to enhanced release of substance P into the renal pelvis. Previous studies lend support for this hypothesis. NOS inhibition increased electrically induced ileal contractions, an effect blocked by substance P receptor antagonists. Yet, NOS inhibition did not increase the contractile response to substance P, suggesting that endogenous NO inhibits substance P release via a presynaptic effect (61, 64). Likewise, studies on spinal cord synaptosomes showed that NO decreased KCl-induced release of substance P (31). However, the results of the present study do not support the notion that the enhanced ARNA response to stimulation of renal pelvic mechanosensory nerves is due to increased and/or prolonged release of substance P. Renal pelvic perfusion with L-SMTC did not alter basal or stimulated renal pelvic release of substance P. Increasing renal pelvic pressure in the absence and presence of L-SMTC resulted in an increase in renal pelvic release of substance P that returned to control values within 5 min after renal pelvic pressure was lowered to zero. Likewise, the increase in renal pelvic release of substance P was unaltered by l-NAME; substance P release during control, experimental, and recovery periods was 9 ± 1, 23 ± 4, and 10 ± 2 pg/min before and 9 ± 1, 18 ± 4, and 9 ± 1 pg/min during l-NAME, respectively (n = 4). These studies demonstrate that nNOS inhibition does not alter the release of substance P from the renal pelvic sensory nerves. Our findings are in accordance with studies in cultured DRG neurons that showed that NO donors do not alter the release of substance P produced by bradykinin or KCl. Furthermore, NOS inhibition had no effect on capsaicin-induced release of substance P (17).

Because our studies suggest that NO modulates the activation of renal mechanosensory nerves by mechanisms distal to the release of substance P, we examined whether nNOS inhibition altered the ARNA response to activation of substance P receptors. Substance P receptors have been localized to the renal pelvic area by autoradiography (12). Similar to our previous studies (37, 38), the current study showed that renal pelvic administration of substance P increased ARNA. The duration of the ARNA response to substance P (1.2 ± 0.2 min) was markedly shorter than the duration of the renal pelvic perfusion (5 min). The transient nature of the ARNA response to substance P was most likely not related to metabolism of substance P, because the renal pelvis was perfused with the endopeptidase inhibitor thiopan throughout the experiment. Renal pelvic perfusion with L-SMTC markedly prolonged the duration of the ARNA response to substance P. In the presence of L-SMTC, the duration of the ARNA response was 5.6 ± 1.9 min. The mechanisms by which NO could modulate the activation of substance P receptors are not known. Evidence is emerging that NO, by cGMP activation, is involved in the desensitization, rather than the activation, of sensory neurons (43). NO enhances and NOS inhibition reduces the desensitization of the bradykinin-induced inward cation current in DRG neurons (48). Interestingly, preventing receptor internalization blocked the NO-elicited reduction of the bradykinin-induced activation of peripheral sensory nerves in an in vitro neonatal spinal cord preparation (54). Other contributing mechanisms to the persistent activation of the afferent renal nerves during NOS inhibition may be related to NOS inhibition modifying depolarization of the cell membrane. NO has been shown to decrease sodium current through voltage-sensitive sodium channels in DRG and nodose ganglia (4, 53), thus serving as a negative feedback regulator. Consequently, in the presence of NOS inhibition, stimulation of the sensory (renal) nerves would lead to prolonged depolarization. Taken together, these studies suggest that NO generation may lead to prolonged depolarization.
be an important regulatory mechanism in determining sensory neuron sensitivity.

Where in the chain of events leading to increased ARNA after increased renal pelvic pressure (Ref. 33 and Fig. 9) nNOS is activated cannot be deduced from the current studies. However, there is evidence for substance P increasing NO production in sensory nerves in the bladder wall (5), as well as spinal cord (63). The mechanisms involved in substance P-induced NO production may be related to substance P increasing intracellular calcium via activation of phosphoinositidase C. The increased intracellular calcium together with calmodulin would lead to activation of NO and production of NO (44).

In summary, the present study shows that nNOS-LI is colocalized with substance P- and CGRP-LI in renal pelvic sensory nerves. Renal pelvic perfusion with the NOS inhibitor L-NAME or the NOS-selective inhibitor L-SMTC enhanced the ARNA response to increased renal pelvic pressure in the absence of changes in arterial pressure. The enhanced ARNA was due to a marked prolongation of the ARNA response, the ARNA response lasting much beyond the duration of the stimuli. Our studies further showed that L-SMTC did not alter the increase in substance P release produced by increased renal pelvic pressure but markedly prolonged the activation of substance P receptors. Taken together, these data demonstrate that nNOS is located in the sensory nerve terminals in the renal pelvic wall. Our data suggest that NO does not play a role in the activation but rather deactivation of the renal pelvic mechanosensory nerves. Our data further suggest that NO modulates the sensitivity of these nerves by a mechanism related to activation of the substance P receptors (Fig. 9).

Perspectives

The persistent increase in ARNA after removal of the stimuli (increased pelvic pressure) during nNOS inhibition may suggest a role for NO as a negative feedback mechanism in the activation of the afferent renal nerves. Substance P increases NO production in the bladder wall (5). Thus we speculate that the increased release of substance P produced by increased renal pelvic pressure may lead to an increased NO production that may in turn result in desensitization of substance P receptors via increased cGMP production (44). In addition, NO may play a role in the coordination of renal pelvic peristalsis. Substance P and CGRP released from the sensory nerve terminals not only have an effect on the afferent renal nerves, but they also have an efferent effect on renal pelvic smooth muscle cells. In vitro studies have shown that substance P contracts and CGRP relaxes the renal pelvic muscle wall (45). Although there is limited information on the role of NO modifying renal pelvic function, there is evidence for NO being the major neural inhibitory regulator of urethral muscular tone (30). nNOS-knockout mice have enlarged urinary bladders and increased irregular micturition frequency, indicative of increased outflow resistance (10). Likewise in the gastrointestinal system, nNOS-containing nerves contribute to the relaxation of smooth muscle associated with peristalsis (70).

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