TRPV1 mediates the uterine capsaicin-induced NMDA NR2B-dependent cross-organ reflex sensitization in anesthetized rats

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1Department of Physiology, 2Department of Anatomy, College of Medicine and 6Department of Obstetrics and Gynecology, Chung-Shan Medical University Hospital, Chung-Shan Medical University, Taichung; 2Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung; 4Department of Physical Therapy and Graduate Institute of Rehabilitation Science, China Medical University, Taichung; 5Department of Obstetrics and Gynecology, Chang-Gung Memorial Hospital, Taoyuan; 7Medical Department, Saint Paul’s Hospital, Taoyuan; and 8Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan

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Peng H-Y, Chang H-M, Lee S-D, Huang P-C, Chen G-D, Lai C-H, Lai C-Y, Chiu C-H, Tung K-C, Lin T-B. TRPV1 mediates the uterine capsaicin-induced NMDA NR2B-dependent cross-organ reflex sensitization in anesthetized rats. Am J Physiol Renal Physiol 295: F1324–F1335, 2008. First published July 16, 2008; doi:10.1152/ajprenal.00126.2008.—Spinal cord-mediated cross-organ sensitization between the uterus and the lower urinary tract may underlie the high concurrence of obstetrical/gynecological inflammation and chronic pelvic pain syndrome characterized by urogenital pain. However, the neural pathway and the neurotransmitters involved are still unknown. We tested the hypothesis that the excitation of capsaicin-sensitive primary afferent fibers arising from the uterus through the stimulation of transient receptor potential vanilloid 1 (TRPV1) induces cross-organ sensitization on the pelvic-urethra reflex activity. Capsaicin (1–1,000 μM, 0.05 ml) was instilled into the uterus to induce cross-organ reflex sensitization. Activation of capsaicin-sensitive primary afferent fibers by capsaicin instillation into the uterine horn sensitized the pelvic-urethra reflex activity that was reversed by an intrauterine pretreatment with capsaizpine, a TRPV1-selective antagonist. Intrathecal injection of AP5, a glutamatergic N-methyl-D-aspartate (NMDA) antagonist, and Co-101244, an NMDA NR2B-selective antagonist, both abolished the cross-organ reflex sensitization caused by capsaicin instillation. These results demonstrated that TRPV1 plays a crucial role in contributing to the capsaicin-sensitive primary afferent fibers mediating the glutamatergic NMDA-dependent cross-organ sensitization between the uterus and the lower urinary tract when there is a tissue injury.

spinal cord; C-fiber; pain; desensitization

NEURAL-MEDIATED CROSS-ORGAN interaction within the pelvic cavity, which is resulted from the convergence of sensory pathways in the central nervous system, is necessary for the normal regulation of sexual, bladder, and bowel function (3, 5). Because the neural substrate for such cross-organ innervations exists under physiological conditions, alternations in these neural pathways by injury or inflammation may cause the development of overlapping pelvic disorders (47, 58).

The uterus is an important reproductive organ within the pelvic cavity and it is a major source of pelvic pain (4, 64).

Immunohistochemical studies demonstrated that there is an abundance of capsaicin-sensitive primary afferent fibers innervating the uterine cervix in rats (28, 65, 71a). It is well-established that injuries or inflammation, resulting from distention, infection, or malignancy, activate the capsaicin-sensitive primary fibers to induce obstetrical/gynecological neuropathic pain and postinflammatory hyperalgesia in the uterus itself by central sensitization (42). On the other hand, in clinical practice, chronic pelvic pain syndrome, which is characterized by high urinary frequency, urgency, and most notably, urogenital pain, often occurs in association with obstetrical/gynecological inflammation (1, 60, 75). The high concurrence rate of obstetrical/gynecological inflammatory pain and chronic pelvic pain syndrome suggests a possibility of cross-organ interaction between the uterus and the lower urinary tract (58). Therefore, whether injury or inflammation in the uterus may sensitize the activity of the lower urinary tract in a cross-organ manner via the overlapping neural pathways within the nervous system is an interesting question that requires further elucidation. Our laboratory recently demonstrated a windup-like spinal reflex potentiation (40, 41) that the strength of the pelvic-urethra reflex activity was dynamically potentiated by repetitive afferent inputs and it was presumed that this windup-like spinal reflex potentiation may be involved in postinflammatory hyperalgesia of the lower urinary tract (18, 22, 49, 50) or chronic pelvic pain syndrome characterized by urogenital pain (58). Thus, the first purpose of this study is to test whether there is a cross-sensitization between an irritated uterus and the spinal pelvic-urethra reflex activity as well as to see the response of lower urinary tract.

Neural sensitization, the responsiveness of the small-diameter C-fiber to noxious stimulation may be dynamically enhanced following injury or inflammation, is suggested to underlie secondary hyperalgesia and tactile alldynia (72, 78, 79, 81). Capsaicin-sensitive afferent fibers arising from viscera and peripheral tissue express the transient receptor potential vanilloid subfamily member 1 (TRPV1) (6, 8), which is now recognized as a molecular integrator of neural sensitization (12, 21). Neurophysiological investigations showed that activation of the TRPV1 receptor may dynamically upregulate the expression of glutamatergic N-methyl-D-aspartate (NMDA)
receptors on the spinal dorsal horn neurons (48, 59, 82) and therefore elicit injury- or inflammatory-induced neural sensitization (70, 71, 80, 81). Pharmacological studies using in vivo animal preparations demonstrated that the induction of spinal reflex potentiation is on spinal glutamatergic NMDA-mediated sensory inputs (14, 15, 37–39, 54–56), indicating a possible role of TRPV1 in the induction of cross-organ sensitization. Therefore, the second purpose of this study is to verify the role of TRPV1 and spinal glutamatergic NMDA receptors in the cross-organ sensitization between the uterus and the lower urinary tract.

MATERIALS AND METHODS

Animal Preparations

This study was reviewed and approved by the Institute Review Board of Chung-Shan Medical University, Taichung, Taiwan. Fifty-three female Sprague-Dawley rats weighing 200–300 g were anesthetized with urethane (1.2 g/kg ip). A PE-50 catheter (Portex, Hythe, Kent, UK) was placed in the left femoral vein for administration of anesthetics when needed. A midline abdominal incision was made to expose the pelvic viscera. Both ureters were ligated distally and cut proximally to the sites of ligation. The proximal ends of the ureters drained freely within the abdominal cavity. A bladder cannula was inserted into the lumen of the urinary bladder from a small incision made on the apex of the bladder dome and was secured with cotton thread. The open end of the cannula drained freely throughout the experiment so that the reflex activity would not be affected by bladder urine distension. Two wide-bore uterine cannulas were inserted into the lumen of the uterine horn through small incisions made on the top and the half of the right uterine horn and were secured with cotton thread. The uterine cannula inserted at the top of the uterus was connected to an infusion pump for capsaicin instillation, and the open end of the other cannula drained the uterus to avoid fluid accumulation within the uterus which could cause distension and activate the polymodal afferent fibers (Fig. 1A). The rats were monitored for a corneal reflex and a response to noxious stimulation to the paw throughout the course of the experiment. If responses were present, a supplementary dose (0.4 g/kg iv) of urethane was given through the venous catheter. When the experiments were completed, the animals were euthanized via an intravenous injection of potassium chloride saturation solution.

Intrathecal Catheter

The occipital crest of the skull was exposed and the atlanto-occipital membrane was incised at the midline with the tip of an 18-gauge needle. A PE-10 catheter was inserted through the slit and passed caudally to the L6-S1 level of the spinal cord. The volume of fluid within the cannula was kept constant at 10 μl in all experiments. A single 10-μl volume of drug solution was administered followed by a 10-μl flush of artificial cerebrospinal fluid.

Intraurethral Pressure Recording

In some experiments, to record the intraurethral pressure (IUP), two 4-0 nylon sutures were placed around the bladder trigone and ligated. A wide-bore intraurethra catheter was inserted through the opening of and into the urethra. Two nylon sutures were made above the opening of the urethra to immobilize the intraurethra catheter (see Fig. 3D). In these experiments, the intraurethral pressure was recorded continuously via the intraurethra catheter connected to a pressure transducer (P23 ID; Gould-Statham, Quincy, IL) that was connected to a computer system (Biopac, MP30, Santa Barbara, CA).

Fig. 1. Transient receptor potential vanilloid 1 (TRPV1)-positive capsaicin-sensitive afferent fibers. A: experimental arrangements showing the external urethra sphincter electromyogram activity (EUSE) recorded under the urinary bladder as it drains freely. B: left: micrographs showing the immunohistochemical location of the TRPV1-positive capsaicin-sensitive afferent fibers (marked by arrows) in the smooth muscle layer of the uterine horn. Right: double immunostaining micrographs showing colocalization of TRPV1 (green) and protein gene product 9.5 (PGP 9.5; red) occurs in the smooth muscle layer of the uterine horn.
Pelvic-Urethra Reflex Activity Recording

Epoxy-coated copper wire (50 μm; M.T. Giken, Tokyo, Japan) electromyogram electrodes were placed intra-abdominally into the area near the external urethra sphincter. The placement of the electrodes was performed using a 30-gauge needle with a hooked electromyogram electrode positioned at the tip (1.0-1.5 mm). The needle was inserted into the sphincter ~1–2 mm lateral to the urethra and then withdrawn, leaving the electromyogram wire embedded in the muscle. The external urethra sphincter electromyogram (EUSE) activities were amplified 20,000-fold by a preamplifier (Grass PS511AC, Cleveland, OH) and then continuously displayed on an oscilloscope (Tectronics TDS 3014, Wilsonville, OR) and a recording system with a sampling rate of 20,000 Hz (MP30, Biopac). The left pelvic nerve (Tectronics TDS 3014, Wilsonville, OR) and a recording system with activities were amplified 20,000-fold by a preamplifier (Grass P511AC, Wetzlar, Germany) on the following day and were placed first in a 0.01 M PBS (pH 7.4) containing 10% methanol and 3% hydrogen peroxide for 1 h to reduce endogenous peroxidase activity. Following this, sections were incubated in the blocking medium containing 0.1% Triton X-100, 3% normal goat serum, and 2% bovine serum albumin (all from Sigma, St. Louis, MO) for 1 h to block nonspecific binding. After several washes in PBS, the sections were incubated in goat polyclonal anti-TRPV1 antiserum (12498, Santa Cruz Biotechnology) at a dilution of 1:50 with the blocking medium for 48 h at 4°C. After this incubation in primary antibody, sections were further incubated with a rabbit-anti-goat biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) at room temperature for 2 h. The standard avidin-biotin complex (ABC) procedure (Vector Laboratories) with 3,3’-diaminobenzidine as a substrate of peroxidase revealed the immunoreaction product.

Colocalization of TRPV1 and PGP 9.5 Immunohistochemistry

For TRPV1 immunohistochemistry, all the euthanized rats were immediately perfused with 100 ml of Ringer solution and 300 ml of 4% paraformaldehyde in a 0.1 M phosphate buffer (PB), pH 7.4. After the perfusion, the uterus was quickly removed and kept in the same fixative for 2 h. The tissue block was then transferred to graded concentrations of sucrose buffer (10-30%) for cryoprotection at 4°C overnight. Serial 15-μm-thick sections of the uterine horn were cut transversely with a cryostat (Leica CM3050S, Leica Microsystems, Wetzlar, Germany) on the following day and were placed first in a 0.01 M PBS (pH 7.4) containing 10% methanol and 3% hydrogen peroxide for 1 h to reduce endogenous peroxidase activity. Following this, sections were incubated in the blocking medium containing 0.1% Triton X-100, 3% normal goat serum, and 2% bovine serum albumin (all from Sigma, St. Louis, MO) for 1 h to block nonspecific binding. After several washes in PBS, the sections were incubated in goat polyclonal anti-TRPV1 antiserum (12498, Santa Cruz Biotechnology) at a dilution of 1:50 with the blocking medium for 48 h at 4°C. After this incubation in primary antibody, sections were further incubated with a rabbit-anti-goat biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) at room temperature for 2 h. The standard avidin-biotin complex (ABC) procedure (Vector Laboratories) with 3,3’-diaminobenzidine as a substrate of peroxidase revealed the immunoreaction product.

Application of Drugs

The drugs used included the following: d-2-amino-5-phosphonovalerate (APS; a glutamatergic NMDA receptor antagonist, 10 μM, Sigma), 6-cyano-7-nitroquinoxaline-2,4-dione [CNQX; a glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, 10 μM, Sigma], glutamate (10 μM, Sigma), NMDA (10 μM, Sigma), 4-hydroxy-1-[2-(4-hydroxyphenyl)ethyl]-4-(4-methylbenzyl)piperidine (Co-101244; a selective NR2B antagonist, 3, 10, 30 μM, Tocris), 8-methyl-N-vanillyl-trans-6-nonenamide (capsaicin; a natural vanilloid compound, 1, 10, 100, 300, and 1,000 μM, Sigma), and N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-1-benzazepine-2-carbothioamide (capsazepine; a selective TRPV1, 300 μM, Sigma). Capsaicin, lidocaine, and capsazepine were instilled into uterine, and glutamate, NMDA, and Co-101244 were injected intracutaneously. All drugs were dissolved in artificial cerebrospinal fluid or DMSO and applied in a final DMSO concentration of less than 1%. A solution of identical volume to the tested agents was dispensed to serve as the vehicle.

Experimental Protocols

The schematic arrangement of EUSE recordings in response to an afferent pelvic nerve fiber stimulation in experiments, which spinal reflex potentiation was induced by repetitive electric shocks, is shown in Fig. 1A. After the urinary bladder had been drained free, the experimental protocol was as follows.

Protocol I. TEST STIMULATION. The intensity of stimulation was gradually increased from 0 to 30 V and a voltage that yielded a single spike action potential in reflex activities was chosen to standardize the baseline reflex activity. Single shocks with an intensity of this voltage were repeated at 30-s intervals and were given through a pair of stimulation electrodes to establish a baseline reflex activity for 10 min.

Protocol II. REPETITIVE STIMULATION. After a baseline period (usually 30 min), the repetitive stimulation (RS; 1 Hz, lasting for 10 min) with intensity identical to the test stimulation (TS) was applied to induce reflex potentiation.

Protocol III. GLUTAMATERGIC AGONIST/ANTAGONISTS ADMINISTRATION. Glutamatergic agonists including glutamate and NMDA were intrauterically injected 3 min before TS started, whereas glutamatergic NMDA receptor antagonists, APS and Co-101244, were administered intrauterically 3 min before the RS started to verify the role of glutamatergic neurotransmission.

Protocol IV. CAPSAICIN INSTILLATION. In experiments testing the capsaicin-induced cross-organ sensitization, capsaicin was infused (0.05 ml, 0.002 ml/s) into the uterine 85 s before TS started for it took 25 s to infuse the tested agent, i.e., electric stimulation started 1 min after capsaicin instillation had finished.

Protocol V. DOSE-RESPONSE RELATIONSHIP. In experiments where several capsaicin concentrations were tested intrauterine to establish the dose-response relationship, the next application of capsaicin was tested after a flush (0.5 ml, 0.002 ml/s for 25 s) followed by an equilibrium period of 30 min. In experiments that verified the reproducibility of capsaicin instillation, intrauterine capsaicin instillation with a concentration of 100 μM was also tested 30 min after the previous capsaicin instillation (100 μM) after a flush (0.5 ml, 0.002 ml/s for 25 s).

Western Blotting

Animals were decapitated after the experimental procedures were finished. The dorsal half of the spinal cord segments from L6-S1 ipsilateral to the stimulation site was dissected and the amount of protein was quantitated. Protein samples (20 μg) were separated on SDS-PAGE (8%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% nonfat milk and probed sequentially with antibodies against phosphorylated NR2A (1:1,000, Tocris Bioscience), phosphorylated NR2B (1:1,000, Tocris Bioscience), and

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antibody against β-actin (1:500, Millipore). The blots were incubated with horseradish peroxidase-conjugated antibody (1:2,000, Santa Cruz) for 1 h at room temperature and visualized with ECL solution (5 min) followed by film exposure (2 min). Densitometric analysis of the WB membranes was done with Science Lab 2003 (Fujifilm). Results were normalized against β-actin and are presented as means ± SD.

Data Analysis

The electromyogram activity was recorded using a sampling rate of 5,000 samples/s with a conventional band-pass filter setting (30–3,000 Hz). The spike number elicited by shocks of stimulation was averaged using the mean spike numbers evoked by the last three stimulations (18th, 19th, and 20th pulses). Comparisons across different stimulation paradigms as well as all drug- and vehicle-treated groups were determined using one-way, repeated-measures ANOVA, followed by a post hoc test (SigmaStat 2.0; Systat Software, San Jose, CA). In all cases, a difference of P < 0.05 was considered as a statistically significant difference.

RESULTS

Immunohistochemical Investigations

Several studies have suggested that the visceral capsaicin-sensitive afferent fibers, which express TRPV1, contribute to visceral nociception (8, 33, 63). Initial experiments were performed in an attempt to test whether the capsaicin-sensitive fibers innervate the uterine horn. As shown in Fig. 1B, left, immunohistochemistry reveals that the TRPV1-positive capsaicin-sensitive fibers innervate the smooth muscle in the uterine specimens (marked by arrows). Using double staining technique, we further verify the TRPV1 immunostaining depicts staining of nerve bundle and the micrograph in Fig. 1B, right, showed colocalization of TRPV1 with PGP 9.5 (marked by arrows).

Baseline Reflex Activity and Windup-Like Reflex Potentiation

We then tested the hypothesis that excitation of capsaicin-sensitive primary afferent fibers arising from the uterus may sensitize the pelvic-urethra reflex activity in a cross-organ manner. The first attempt was to establish a stable baseline reflex activity and the RS-induced windup-like reflex potentiation (14, 39, 40). As shown in Fig. 2A, single pulses of TS (1/30 Hz) on the afferent nerve evoked a baseline reflex activity with single action potentials with a mean reflex latency of 54.39 ± 12.11 ms (n = 35), whereas a RS (at the same intensity as the TS, delivered at 1 Hz) induced reflex potentiation, which was characterized by an elongated firing that subsided quickly (Fig. 2B). The summarized data in Fig. 2C show that the mean spike number, counted 10 min following the stimulation onset, evoked by the RS was significantly higher than the TS (**P < 0.01 to TS, n = 35).

Glutamatergic Agonists and Antagonists

Next, we reexamined the role of glutamatergic neurotransmission in the induction of the RS-induced windup-like reflex potentiation (14, 37, 38, 41). As shown in Fig. 2A, TS on the afferent nerve evoked a baseline reflex activity with single action potentials. Intrathecal administration of glutamate (TS+GLU, 10 μM, 10 μl) and NMDA (TS+NMDA, 10 μM, 10 μl) both induced a longer-lasting reflex potentiation. Intrathecal injection of NMDA NR2B-selective an-
tagonist, Co-101244, attenuated the glutamate-induced reflex potentiation (TS+GLU+Co). On the other hand, as shown in Fig. 2B, RS produced a long-lasting potentiation in the reflex activity. Intrathecal administration of CNQX (RS+CQX, 10 μM, 10 μl), AP5 (RS+AP5, 10 μM, 10 μl), and Co-101244 (RS+Co, 10 μM, 10 μl) all blocked the RS-induced reflex potentiation. The summarized data in Fig. 2C show that intrathecal glutamate (TS+GLU) and NMDA (TS+NMDA, **P < 0.01 to TS, n = 35) significantly increased the mean spike numbers evoked by the TS. Moreover, pretreated CNQX (RS+CQX, 10 μM, 10 μl), AP5 (RS+AP5, ###P < 0.01 to RS, n = 35) significantly decreased the mean spike numbers evoked by the RS.

Capsaicin-Induced Cross-Organ Reflex Sensitization

Neural sensitization, the responsiveness of the small-diameter C-fiber to noxious stimulation, which may be dynamically enhanced following injury or inflammation, is presumed to underlie secondary hyperalgesia and tactile allodynia (72, 78, 79, 81). Thus, we activated capsaicin-sensitive afferent fibers, arising from the uterus, by capsaicin instillation to test the role of these fibers on the induction of cross-organ reflex sensitization. As shown in Fig. 3A, TS on the afferent nerve evoked a baseline reflex activity with a single action potential. Intrauterine saline instillation exhibited no effect (TS+SL), while capsaicin instillation produced sensitization in the evoked activity (TS+CP). In another animal, pretreatment with lidocaine in the uterus before capsaicin instillation did not affect the baseline reflex activity evoked by the TS (TS+LDC), whereas it abolished the uterine capsaicin instillation-induced sensitization (TS+LDC+CP).

B: when compared with a baseline reflex activity with a single action potential evoked by the TS, intrauterine capsaicin instillation with concentrations higher than 100 μM produced cross-sensitization in the evoked reflex activity. The sensitization caused by capsaicin instillation in the same animal was enhanced parallel to the test concentrations when the concentrations were lower than 100 μM [TS+CP (1 μM), TS+CP (10 μM), and TS+CP (100 μM)], but the sensitization weakened when we instilled capsaicin with concentrations higher than 300 μM [TS+CP (300 μM) and TS+CP (1,000 μM), respectively]. C: summarized data (means ± SE) show capsaicin (TS+CP), but not saline (TS+SL), instillation into the uterus significantly increased the spike number evoked by the TS (**P < 0.01 to TS, n = 7). Pretreatment with lidocaine (TS+LDC+CP) abolished the uterine capsaicin instillation-induced cross-sensitization on the evoked reflex activity. ###P < 0.01 to TS+CP, n = 7. D: summarized data (means ± SE) shows the mean spike number increased in a dose-dependent manner when the concentration of instilled capsaicin ranged from 1 to 100 μM. However, capsaicin with concentrations higher than 300 μM did not cause an increase in the spike number (*P < 0.05, **P < 0.01 to TS, n = 7).

Fig. 3. Intrauterine capsaicin instillation produced cross-organ reflex sensitization. A: TS on the afferent nerve evoked a baseline reflex activity with a single action potential in the EUSE. Intrauterine saline instillation exhibited no effect (TS+SL), while capsaicin instillation (0.05 ml) produced sensitization in the evoked activity (TS+CP). In another animal, pretreatment with lidocaine in the uterus before capsaicin instillation did not affect the baseline reflex activity evoked by the TS (TS+LDC), whereas it abolished the uterine capsaicin instillation-induced sensitization (TS+LDC+CP).

B: when compared with a baseline reflex activity with a single action potential evoked by the TS, intrauterine capsaicin instillation with concentrations higher than 100 μM produced cross-sensitization in the evoked reflex activity. The sensitization caused by capsaicin instillation in the same animal was enhanced parallel to the test concentrations when the concentrations were lower than 100 μM [TS+CP (1 μM), TS+CP (10 μM), and TS+CP (100 μM)], but the sensitization weakened when we instilled capsaicin with concentrations higher than 300 μM [TS+CP (300 μM) and TS+CP (1,000 μM), respectively]. C: summarized data (means ± SE) show capsaicin (TS+CP), but not saline (TS+SL), instillation into the uterus significantly increased the spike number evoked by the TS (**P < 0.01 to TS, n = 7). Pretreatment with lidocaine (TS+LDC+CP) abolished the uterine capsaicin instillation-induced cross-sensitization on the evoked reflex activity. ###P < 0.01 to TS+CP, n = 7. D: summarized data (means ± SE) shows the mean spike number increased in a dose-dependent manner when the concentration of instilled capsaicin ranged from 1 to 100 μM. However, capsaicin with concentrations higher than 300 μM did not cause an increase in the spike number (*P < 0.05, **P < 0.01 to TS, n = 7).
the same preparation, which was also characterized by an elongation in evoked activity but subsided slowly (TS + CP). The cross-organ sensitization on the evoked reflex activity caused by uterine capsaicin instillation lasting for ~20 min. To further elucidate whether the capsaicin-induced cross-organ reflex sensitization was mediated by the capsaicin-sensitive afferent fibers arising from the uterus, we intra-uterine pretreated another animal with lidocaine, a nerve conduction blocker, before capsaicin instillation. As shown in Fig. 3A, lidocaine did not affect the baseline reflex activity evoked by the TS (TS + LDC), whereas it abolished the cross-organ reflex sensitization caused by uterine capsaicin instillation (TS + LDC + CP). The summarized data in Fig. 3C show that uterine capsaicin instillation (TS + CP, **P < 0.01 to TS, n = 7) significantly increased the mean spike numbers evoked by the TS. Moreover, lidocaine pretreatment (TS + LDC + CP, ###P < 0.01 to TS + CP, n = 7) significantly decreased the spike numbers caused by the TS in association with uterine capsaicin instillation.

### Dose Dependence

It is well established that a high dose of capsaicin may cause damage in the nociceptive afferent fibers (34), and this phenomenon, known as desensitization, is valid in clinical pain management (9, 83). Next, we tested whether a high concentration of capsaicin may desensitize the cross-organ reflex sensitization caused by uterine capsaicin instillation. As shown in Fig. 3B, the reflex activity potentiated by uterine capsaicin instillation of the same animal was parallel to the test concentrations within the range lower than 100 μM (1 μM TS + CP, 10 μM TS + CP, and 100 μM TS + CP), but the cross-organ reflex sensitization weakened when the capsaicin concentrations were higher than 300 μM (300 μM TS + CP and 1,000 μM TS + CP). To verify the desensitization caused by high-concentration capsaicin instillation was not caused by the timing of drug administration, we then tested cross-organ sensitization in another three animals using repetitive application of low-dose capsaicin (10 μM). The spike numbers evoked by four successive injections of capsaicin with a concentration of 10 μM were 7.92 ± 4.38, 8.22 ± 2.51, 7.46 ± 4.56, and 8.34 ± 4.21 spikes/stimulation and no statistical significance was shown in these treatments (n = 3, P > 0.05). Moreover, the spike numbers evoked by four successive injections of vehicle solution also showed no statistical difference (1.15 ± 0.85, 1.36 ± 1.20, 1.08 ± 0.97, and 1.43 ± 0.32 spikes/stimulation, n = 3, P > 0.05). The summarized data in Fig. 3D show the mean spike numbers, counted 10 min following the TS onset, increased in a dose-dependent manner to the concentrations of instilled capsaicin at a range from 1 to 100 μM. When the capsaicin concentrations were higher than 300 μM (*P < 0.05, **P < 0.01 to TS + CP, n = 7), the increase in spike numbers weakened.

### TRPV1 Antagonist

In animals, TRPV1 is present in visceral neurons (6, 8) and has been shown to participate in physiological (69) and pathological (83) neural responses caused by the activation of capsaicin-sensitive afferent fibers arising from the uterus. We, therefore, tested the participation of TRPV1 in capsaicin-induced cross-organ reflex sensitization by pharmacologically blocking TRPV1 using a selective antagonist, capsazepine (30). As shown in the top trace in Fig. 4A, TS on the afferent nerve evoked a baseline reflex activity with a single action potential (TS), and uterine capsaicin instillation produced reflex potentiation in the same preparation. The bottom trace in Fig. 4A showed intrauterine capsazepine did not affect the baseline reflex activity (TS + CPZ) in another preparation, whereas it abolished the cross-organ reflex sensitization caused by uterine capsaicin instillation (TS + CPZ + CP) in another animal preparation. The summarized data in Fig. 4E show that capsazepine pretreatment (TS + CPZ + CP, ###P < 0.05 to TS + CP, n = 7) significantly decreased the mean spike numbers evoked by the TS in association with uterine capsaicin instillation (TS + CP; Fig. 5).

### Glutamate Dependence

TRPV1-expressing afferents are presumed to be glutamatergic (29) both in the spinal/medullary dorsal horn (25, 32, 68) and supraspinal levels (46, 51). Thus, by pharmacologically blocking the glutamatergic NMDA receptor using AP5, we tested the possibility that the cross-organ reflex sensitization, caused by the activation of capsaicin-sensitive afferent fibers arising from the uterus, is mediated by modulating the spinal glutamatergic NMDA neurotransmission. We first confirmed reproducibility of the capsaicin-elicited cross-organ sensitization by two consecutive capsaicin instillations (100 μM) with a time interval of 30 min, and both capsaicin instillations produced cross-organ sensitization on the evoked reflex activity (data not shown). We then tested the role of glutamatergic neurotransmission, and as shown in Fig. 4B, uterine capsaicin instillation induced cross-organ sensitization in the reflex activity (TS + CP). Intrathecal pretreatment with AP5 (TS + CP + AP5, 100 μM, 10 μl) in the same preparation abolished the cross-organ sensitization caused by capsaicin instillation. Since studies demonstrated that the subunit composition of glutamatergic NMDA receptors, especially the NR2B subunit, defines the properties essential for synaptic plasticity (10, 43, 57, 84), we next tested the role of the NMDA NR2B subunit on the capsaicin-induced cross-organ sensitization using the NMDA NR2B-selective antagonist, Co-101244, in the same preparation. As shown in Fig. 4B, uterine capsaicin instillation induced cross-organ sensitization in the reflex activity (TS + CP). Intrathecal pretreatment with Co-101244 (TS + CP + Co, 100 μM, 10 μl) abolished the cross-organ sensitization caused by uterine capsaicin instillation. The summarized data in Fig. 4E show that AP5 and Co-101244 (TS + CP + AP5 and TS + CP + Co, respectively, ###P < 0.01, n = 7) both significantly decreased the spike number evoked by the TS in association with uterine capsaicin instillation.

### Secondary Responses to Cross-Sensitization

We then tested whether the cross-organ reflex sensitization caused by the uterine capsaicin-sensitive afferent fiber affected the physiological functions of the pelvic-urethra reflex activity. As shown in Fig. 4C, TS evoked a baseline reflex activity with a single action potential accompanied by a contraction wave in the IUP. Uterine capsaicin instillation potentiated the evoked activity and elongated the contraction wave in the IUP (TS + CP). Pretreatment with capsazepine in another animal preparation abolished the cross-organ reflex sensitization as
well as the elongated IUP wave caused by the capsaicin instillation (TS+CPZ).

Western Blotting Analysis

To further clarify the role of NR2B subunits in the development of cross-organ sensitization caused by uterine capsaicin instillation, the spinal tissues (dorsal half of the L6-S1 level ipsilateral to the stimulated nerve) were harvested at 10 min following stimulation onset from rats TS in association with uterine saline instillation (TS+SL; Fig. 5) and TS in association with uterine capsaicin instillation without (TS+CP) or with pretreatment of intrathecal capsazepine (TS+CPZ) or intrathecal AP5 (TS+CPZ+CP) for Western blot analysis. As shown in Fig. 4A, when compared with saline, uterine capsaicin instillation increased the levels of phosphorylated NR2B subunit (p-NR2B) but did not affect the level of phosphorylated NR2A (p-NR2A). In addition, both intrathecal capsazepine and intrathecal AP5 reversed the increment in p-NR2B levels induced by uterine capsaicin instillation (TS+CPZ and TS+AP5, respectively) and significantly decreased the spike number caused by the TS with intrathecal capsazepine instillation (**P < 0.01 to TS+CP, n = 7).
cal AP5 (TS+CPZ+CP) were summarized in Fig. 2, B and C, respectively (n = 4).

**DISCUSSION**

The cross-innervation of visceral organs within the nervous system, the visero-visceral convergence, offers a complex neural network involving sensory pathways in the pelvic cavity that are essential for the dynamic regulation and integration of sexual, bowel, and bladder functions (3, 58, 77). Alterations in these convergent sensory pathways cause a pathological mechanism by which injury or inflammation in one organ leads to modifications in the function of other organs (11, 23, 24).

Although the administration of capsaicin, a natural vanilloid substance, may activate sensory nerves and induce a consecutive neuropeptide release, it mimics the sequel that is also triggered during local inflammation. Therefore, capsaicin has been routinely used in studies that identify nociceptive C-fibers to explore their contribution to the regulatory process during inflammation (31, 35). In the present study, the possible participation of the capsaicin-sensitive sensory nerve in cross-organ sensitization was studied in in vivo animal preparations using capsaicin instillation in the uterine horn. Our data in this study demonstrated that capsaicin instillation may activate the primary afferent fibers arising from the uterus, and therefore, sensitize the evoked pelvic-urethra reflex activity, and elongate the contraction wave of the IUP in a cross-organ manner. Although the detailed physiological or pathological relevances of this cross-organ sensitization need further investigations to be elucidated, we suggested that the pelvic-urethra reflex activity may be a biological marker to assess the degree of visceral afferent sensitization in the pelvic organ, which is similar to the visceromotor response (VMR) that measures the electromyogram activity of abdominal striated muscle during afferent sensitization between visceral organs. The cross-organ sensitization on the pelvic-urethra reflex activity caused by uterine capsaicin instillation implies that activation of uterine capsaicin-sensitive afferent fibers may sensitize the visceral afferent arising from the urinary bladder. We propose that this phenomenon, at least in part, underlies the clinical finding that chronic pelvic pain syndrome, characterized by hyperactivity or dysynergia in the lower urinary tract in association with urogenital pain, often occurs in association with obstetrical or gynecological pelvic inflammation (1, 60, 75).

The complex mechanisms and pathways that contribute to the pathophysiology of chronic pain have made it difficult for clinicians to treat patients with current therapies. Most drugs
lack significant efficacy, have dramatic side effects, or have serious long-term safety issues. Capsaicin may activate nociceptive primary afferent fibers causing acute burning pain, and chronic or high-dose applications have been shown to work as an analgesic, but such treatments have been proven to damage the small-diameter nociceptive afferent fibers (34). This phenomenon, known as desensitization, has been widely investigated because it has the potential for developing strategies in pain therapeutics (2, 35). As shown in Fig. 2B in this study, we instilled capsaicin with increasing concentrations into the uterine horn to activate the nociceptive C-fiber, and this procedure sensitized the evoked pelvic-urethra reflex activity in a cross-organ manner. Meanwhile, a dose-dependent relationship was noted between the sensitized reflex activity and the concentration of capsaicin when the test concentrations were lower than 100 μM. However, at a concentration of 100 μM, capsaicin reached a ceiling of maximal response and the induced response of the cross-organ sensitization weakened, even when the concentration of capsaicin was increased from 300 to 1,000 μM. 

Correlating with previous pharmacological studies (35, 73, 74), our data demonstrate that desensitization of the capsaicin-sensitive primary afferent fiber may attenuate the cross-organ sensitization in the pelvic-urethra reflex activity caused by intrauterine irritation. This result implies that vanilloid compound-induced desensitization in the cross-organ interaction between the uterus and the lower urinary tract could be a potential candidate for clinical therapy of secondary neuropathic pain in the lower urinary tract induced by uterine pathology. On the other hand, administration of a high concentration of topical capsaicin may degenerate the C-fiber, and therefore, diminish the neural response mediated by the nociceptive C-fiber. Further investigation is needed to discover whether the weakening of cross-organ sensitization is a result of a high-dose neurotoxic effect when the concentration of capsaicin increases from 300 to 1,000 μM.

TRPV1 is expressed by the capsaicin-sensitive nociceptive afferent fibers arising from the viscera and peripheral tissues. The understanding of the role of TRPV1 in pain-related behavior has advanced rapidly in recent years. TRPV1 is now recognized not only as necessary for visceral nociception (7, 8, 33, 63), but also the agonist-induced desensitization of TRPV1 is one of the key strategies that offers a way to alleviate neuropathic and inflammatory pain (21, 27, 36, 44). Although clinical trials using TRPV1 agonists are still controversial (53), several clinical studies used these agents for the treatment of interstitial cystitis and chronic pelvic pain syndromes (19, 26). Histochemical studies demonstrated TRPV1 immunoreactivity in the uterine capsaicin-sensitive primary afferent fibers (69); meanwhile, electrophysiological recordings showed these afferent fibers to respond to noxious heat (42). In this study, pharmacological blockade of the TRPV1 receptor, using the selective antagonist capsazepine, abolished sensitization on the pelvic-urethra reflex activity caused by uterine capsaicin instillation, indicating that uterine TRPV1 participates in the cross-organ sensitization between the uterus and the lower urinary tract. Studies investigating the intracellular cascades mediating the agonist-induced desensitization of TRPV1 suggested that activation of the TRPV1 receptor may induce the subsequent entry of extracellular Ca<sup>2+</sup> through the channel to the sensory neurons. One of the prominent mechanisms responsible for TRPV1 desensitization is dephosphorylation of the TRPV1 protein by the Ca<sup>2+</sup>/calmodulin-dependent enzyme, phosphatase 2B. Of the several phosphorylation sites identified so far, the most notable are the two sites for Ca<sup>2+</sup>/calmodulin-dependent kinase II where the dynamic equilibrium between the phosphorylated and dephosphorylated states presumably regulates agonist binding. However, the detailed mechanism involved in the TRPV1-mediated desensitization, which has been suggested to alleviate neuropathic and inflammatory pain, is still obscure and needs further investigation.

Although the details have yet to be established, defining the precise neural mechanisms of cross-organ sensitization would have implications for the development of effective pharmacological therapy for the treatments of neurogenic pain and hyperalgesia. It is now presumed that cross-organ neural sensitization may be the result of peripheral and central mechanisms. The convergence of sensory fibers, coming from adjacent pelvic structures or bifurcating afferent fibers, accounts for the peripheral mechanism for the induction of cross-organ sensitization (45). On the other hand, central integrations of neural activity at various levels including the spinal cord, brain stem, thalamus, and amygdala have also been suggested to be involved in the central mechanisms (13, 47, 63). Since the cross-organ sensitization present in this study was defined by peripheral actions, i.e., uterine capsaicin instillation sensitized the evoked urethral electromyogram activity, the possibility that the convergence of sensory afferent fibers or axon collaterals mediates the cross-organ sensitization cannot be excluded. On the other hand, the cross-organ sensitization induced by uterine instillation in this study was abolished by intrathecal NMDA antagonists, indicating (in contrast to studies showing peripheral mechanisms) that the central mechanism at the spinal level, at least in this model, participated in the induction of cross-organ sensitization. This proposal correlates with the neurophysiological basis for CNS-mediated sensitization coming from animal models of chronic somatic pain which showed that following injury or inflammation, there is heightened activity of the small-diameter C-fiber neurons, and this induces activation of the NMDA receptors, expressed in the dorsal horn spinal neurons, to increase their excitability and responsiveness (66, 76, 78–80, 85, 86).

Spinal reflex potentiation, similar to central sensitization, is a spinal-mediated dynamic neural plasticity that manifests as a potentiation in reflex response to stimuli (40, 41). Since in vivo studies demonstrated that the induction and maintenance of SRP depend on spinal glutamatergic NMDA neurotransmission (16, 17, 37, 38, 52, 54–56), we tested the role of the spinal NMDA receptor on cross-organ sensitization caused by uterine capsaicin instillation. Our results showed that pharmacological blockade, using AP5, a NMDA antagonist, reversed such a sensitization caused by uterine capsaicin instillation, indicating that a dynamic increase in the efficacy of glutamatergic NMDA-dependent neurotransmission underlies the cross-sensitization. In addition, we further tested the NMDA NR2B receptor subunit, a subunit that is presumed to be essential for Ca<sup>2+</sup> gating, to define the role of the NMDA receptor for dynamic synaptic plasticity (43, 57) and to verify the role of the NR2B receptor in NMDA-dependent cross-organ sensitization. Our data showed that NR2B dependence in the cross-sensitization was caused by the uterine capsaicin instillation, suggesting that the NR2B subunit has a role in the cross-organ sensitization. This result correlates with studies investigating LTP in the hippocam-
pal CA1 area that showed NR2B participation in NMDA-dependent LTP (10, 84). However, since the NMDA receptor family is made up of NR1, NR2, and NR3 subunits (20, 61), the roles of the other subunits cannot be excluded.

In summary, these findings not only establish the feasibility of this unique model to study inflammatory disorders of the uterus, but these findings may also enable an eventual characterization of the pathophysiological mechanisms involved in the development of and overlapping of uterine inflammation and chronic pelvic pain syndrome characterized by urogenital pain. These data support the notion that the comorbidity of pelvic and urogenital pain is not coincidental but rather causal in nature. Furthermore, spinal NMDA-dependent reflex potentiation triggered by capsaicin-sensitive primary afferent fibers via the activation of the TRPV1 receptor may set the foundation for the pathophysiological development of cross-organ sensitization. It may be hypothesized that long-term or ongoing stimulation of these pelvic sensory pathways and reflexes (i.e., pelvic organ cross-talk) may eventually lead to more permanent sensory changes in the nonirritated organ, perhaps leading to neurogenic inflammation and sensitization via the peripheral and central release of neurotrophic factors and other mediators of this phenomenon (58). Clearly, further research is warranted to expand on the current findings.

REFERENCES

TRPV-mediated cross-organ reflex sensitization


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