Cyclophosphamide induces NR2B phosphorylation-dependent facilitation on spinal reflex potentiation

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It is well-established that cyclophosphamide (CYP) can sensitize the pelvic afferent nerve arising from the urinary bladder and therefore induce suprapubic pain. To test the possibility that CYP might mediate the development of visceral hypereflexia/hyperalgesia by facilitating spinal activity-dependent neural plasticity, we compared the pelvic-urethra reflex activity and spinal N-methyl-D-aspartate receptor NR2B subunit (NR2B) phosphorylation in rats treated with vehicle solution and CYP. Compared with vehicle solution, when accompanied by upregulation of phosphorylated NR2B expression in the lumbosacral (L6–S2) dorsal horn, CYP increased the evoked spikes in spinal reflex potentiation induced by repetitive stimulation (1 stimulation/1 s). Moreover, intraperitoneal pretreatments with Nω-nitro-L-arginine methyl ester and roscovitine, nitric oxide synthase and cyclin-dependent protein kinase 5 (Cdk5) antagonists, respectively, overwrote CYP-enhanced reflex potentiation and NR2B phosphorylation. When compared with the untreated group, the treatment with small-interfering RNA of NR2B, which decreased the expression of NR2B expression, abolished CYP-dependent reflex facilitation and spinal NR2B phosphorylation. These results suggested that CYP might facilitate spinal reflex potentiation mediated by N-methyl-D-aspartate receptors and participate in the development of visceral hypereflexia/hyperalgesia through nitric oxide- and Cdk5-dependent NR2B phosphorylation at the lumbosacral dorsal horn.

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CHRONIC PATHOLOGICAL CONDITIONS such as inflammation or irritation of visceral organs can induce changes in sensory pathway properties, leading to visceral hypereflexia and/or hyperalgesia (37). For example, because of the urotoxicity of its metabolite acrolein, patients that receive cyclophosphamide (CYP) as an anti-neoplasmic agent are known to develop interstitial cystitis, characterized by hematuria (38) and suprapubic pain (12). In vivo animal studies have demonstrated that intraperitoneal administration of CYP induces cystitis symptoms, including irritative voiding, gross hematuria, and lower abdominal pain that resemble interstitial cystitis (33). Therefore, CYP-induced cystitis has been used as an animal model for bladder-related pain and inflammation (5, 33, 42).

In the lumbosacral dorsal horn, glutamatergic N-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission has been implicated in the processing of afferent nociceptive input from the lower urinary tract (4). Spinal administration of NMDAR agonists dose-dependently facilitates both visceralmotor reflexes and pressor responses to noxious pelvic stimulation (13). Conversely, pharmacological blockade of NMDARs using selective antagonists inhibits nociceptive responses caused by visceral irritation (43). These results suggest that spinal NMDARs contribute to visceral nociception/hyperalgesia. Moreover, phosphorylation of N-methyl-D-aspartate receptor NR2B subunit (NR2B) tyrosine residues in NMDARs has been noted as an important determinant of NMDAR-mediated current (20), which defines the role of NMDARs in pain-related neural plasticity (3, 17, 18, 22).

Our laboratory has recently documented that the acute activation of nociceptive TRPV/TRPA-expressing afferent fibers, fulfilled by instilling irritants in the uterus (23, 32) and descending colon (22, 25, 26, 29), has the potential to facilitate spinal reflex potentiation (SRP) (6, 41), a spinal NMDAR-mediated form of pain-related neural plasticity (15). However, the impact of chronic noxious stimulation/inflammation on SRP is unclear. We set out to study the role of chronic inflammation in pain-related neural plasticity by investigating the impact of chronic CYP injection on SRP. In addition, the role of NR2B in CYP-induced modification of SRP was explored using selective pharmacological agents and genetic knockdown. Moreover, in light of the fact that nitric oxide (NO)-dependent PKG activation (7) and cyclin-dependent protein kinase (Cdk)-dependent ERK phosphorylation (28) are known to regulate NR2B phosphorylation and therefore modulate NMDAR-mediated neural plasticity, we employed pharmacological antagonists of NO and Cdk5 to explore the participation of intracellular NO and Cdk5 in the NR2B-mediated modulation of SRP caused by CYP.

MATERIALS AND METHODS

Animal preparations. This study was reviewed and approved by the Institutional Review Board of National Chung-Hsing University in Taichung, Taiwan. Two hundred and forty-three adult female Sprague Dawley rats (200–300 g) were used throughout this study. In CYP-induced cystitis, there are 75 animals used to establish baseline reflex activity; 33 received vehicle solution (Veh, ip bolus) and 55 received CYP (150 mg/kg ip bolus) pretreatment 2 days before experiments. In small-interfering (si) RNA experiments, there are each 40 rats that...
received intrathecal negative control (siVeh) or siRNA of NR2B (siNR2B) injection 2 days before experiments.

**Reflex activity recordings.** On the day of experiment, animals were anesthetized (1.2 g/kg ip urethane), and a catheter was inserted through a slit made at the atlanto-occipital membrane to the lumbosacral spinal cord (L~2–S~2). Wire electromyogram electrodes were placed intra-abdominally in the external urethra sphincter to pick up external urethra sphincter electromyogram activity. The left pelvic nerve was carefully dissected and dissected. The central stump of the transected nerve was then mounted on a pair of electrodes for stimulation. Two paradigms were used to test stimulation (TS, 1 stimulation/30 s, lasting for 30 min) for evoking baseline reflex activity and repetitive stimulation (RS, 1 stimulation/1 s, lasting for 30 min) for inducing reflex potentiation. Square wave pulses with a constant intensity causing a single action potential under TS were used throughout each experiment. Intrathecal agents were injected either 5 min following or 1 min before stimulation started.

**Drug applications.** Drugs used in this study were as follows: CYP, an anti-neoplastic agent (150 mg/kg ip, 2 days before experiments), 4-hydroxy-1-[2-(4-hydroxyphenoxo)ethyl]-1-(4-methylbenzyl)lipiperidine (Co-101244, a selective NR2B antagonist, 100 nM, 10 μl it), N^2^-nitro-arginine-methyl ester (l-NAME), a NO synthetase inhibitor (100 μM, 10 μl it and 0.2 ml 2 times/day ip), roscovitine, a Cdk5 inhibitor, 100 nM, 10 μl it and 0.2 ml 2 times/day ip), protoporphyrin IX (PPIX; a soluble guanylate cyclase (sGC) activator, 100 μM, 10 μl it), and N-methyl-D-aspartate (NMDA, a selective glutamate NMDA receptor agonist, 10 μM, 10 μl it). Drugs were dissolved in artificial cerebrospinal fluid or DMSO and applied in a final DMSO concentration of <1%. A solution of NO and Cdk5 in RS-induced SRP by antagonizing CYP-dependent facilitation by decreasing the evoked spike (CYP + Co, P < 0.01 vs. Veh + RS, n = 7) and partially reversed CYP-dependent facilitation by decreasing the evoked spike (CYP + Co, P < 0.01 vs. Veh + RS, n = 7). The involvement of NR2B in CYP-dependent SRP facilitation was then investigated using Western blotting analysis. Immunoblotting showed that, compared with TS (Veh + TS and CYP + TS; Fig. 1D), pNR2B expression in the lumbosacral dorsal horn was significantly increased by RS in both Veh and CYP treatment (Veh + RS and CYP + RS, P < 0.01 vs. Veh + TS, n = 4), although the amounts of the tNR2B-to-β-actin ratio under various treatments exhibited no statistical difference (P > 0.05, n = 4). In addition, with the Veh + RS group, pNR2B expression but not the tNR2B-to-β-actin ratio was significantly increased in CYP + RS animals (P < 0.05 vs. Veh + RS, n = 4).

**Cdk5 and NO involvement in RS-induced reflex potentiation and NR2B phosphorylation.** We next examined the involvement of NO and Cdk5 in RS-induced SRP by antagonizing each pharmacologically. We found that, compared with animals that received TS and RS, respectively, intrathecal application of l-NAME and ROS, selective nitric oxide synthase (NOS) and Cdk5 inhibitors, respectively, at 5 min after the start of stimulation failed to affect TS-evoked baseline reflex activity (TS + l-NAME and ROS, P > 0.05 vs. TS, n = 7; Fig. 2A) and RS-induced SRP (RS + l-NAME and RS + ROS, P > 0.05 vs. RS, n = 7; Fig. 2, A and D). In contrast, pretreatment with l-NAME and ROS at 1 min before stimulation attenuated RS-induced SRP by decreasing the mean spike count (l-NAME + RS and ROS + RS, P < 0.01 vs. RS, n = 7; Fig. 2, B and D) while exhibiting no effects on TS-evoked baseline reflex activity (l-NAME + TS and ROS + TS, P > 0.05 vs. TS, n = 7; Fig. 2, B and C). Moreover, pretreatment with l-NAME and ROS (l-NAME + RS and ROS + RS, respectively; Fig. 2E) significantly reduced RS-induced phosphorylation of NR2B in the lumbosacral dorsal horn without affecting the tNR2B-to-β-actin ratio compared with animals that received RS only (P < 0.05 vs. RS, n = 4), whereas posttreatment application had no effect (data not shown). We next tested the involvement of NO-dependent sGC activation in RS-induced SRP through intrathecal administration of PPIX, a sGC activator, after RS-induced SRP was attenuated by...
dependent attenuation of RS-induced SRP (ROS
We observed that intrathecal NMDA also reversed the ROS-
activation was also studied using a selective NMDAR agonist.
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other hand, compared with vehicle solution (Veh + TS) and CYP (CYP + TS). The reflex potentiation induced by RS (1 stimulation/1 s) was characterized by an increasing firing in both groups (Veh + RS and CYP + RS) that was antagonized by intrathecal Co-101244 administrations at 1 min before stimulation (Veh + Co + RS and CYP + Co + RS). Ticks at the bottom of the tracing indicate the shocks. B: summary data showed the mean spike count of TS-evoked baseline reflex and RS-induced reflex potentiation in rats treated with vehicle solution (Veh + TS and Veh + RS) and CYP (CYP + TS and CYP + RS). C: also shown were the average spike counts from the last 3 shocks of stimulation in vehicle solution- and CYP-treated animals without (Veh + RS and CYP + RS) and with (Veh + Co + RS and CYP + Co + RS) intrathecal Co-101244 administration (n = 7). D: compared with TS (Veh + TS and CYP + TS), RS significantly increased phosphorylated NR2B (pNR2B) expression in both animals treated with vehicle solution (Veh + RS) and CYP (CYP + RS), despite no statistically significant between-group difference in the level of the total NR2B (tNR2B)-to-β-actin ratio (P > 0.05, n = 4). In addition, compared with the Veh + RS group, pNR2B but not the tNR2B-to-β-actin ratio was significantly increased in CYP + RS animals. B and C: ***P < 0.01 vs. Veh + RS. ###P < 0.01 vs. Veh + RS + Co. D: **P < 0.01 vs. Veh + TS. #P < 0.05 vs. Veh + RS (n = 4).

1-NAME pretreatment. Electrophysiological tracings show that intrathecal PPIX reversed 1-NAME-elicited SRP attenuation (1-NAME + RS + PPIX, P < 0.01 vs. 1-NAME + RS, n = 7; Fig. 2, B and D). On the other hand, Cdk-dependent NR2B activation was also studied using a selective NMDA agonist. We observed that intrathecal NMDA also reversed the ROS-dependent attenuation of RS-induced SRP (ROS + RS + NMDA, P < 0.01 vs. ROS + RS, n = 7). Roles of NO and Cdk5 in CYP-dependent facilitation of reflex potentiation. The involvement of NO and Cdk5 in NR2B-mediated SRP facilitation caused by CYP was then elucidated by daily intraperitoneal injection of 1-NAME and ROS in CYP-treated animals. We found that, compared with animals receiving saline injection, intraperitoneal pretreatments of 1-NAME and ROS did not affect TS-evoked baseline reflex activity and RS-induced SRP (data not shown). On the other hand, compared with vehicle solution [Fig. 3A (Veh) and Fig. 3D (Veh + RS)], CYP facilitated RS-induced SRP (CYP + RS, P < 0.05 vs. Veh + RS; Fig. 3, B and D) and upregulated pNR2B expression without affecting the tNR2B-to-β-actin ratio in the lumbosacral dorsal horn (P < 0.01 vs. Veh; Fig. 3A). Peritoneal pretreatments of 1-NAME and ROS, while exhibiting no effects on TS-evoked baseline reflex activity (1-NAME + CYP + TS and ROS + CYP + TS, P > 0.05 vs. CYP + TS, n = 7; Fig. 3, B and C), prevented CYP-dependent reflex facilitation (1-NAME + CYP + RS and ROS + CYP + RS, P < 0.01 vs. CYP + RS, n = 7; Fig. 3, B and D) and NR2B phosphorylation (1-NAME + CYP + ROS + CYP, P < 0.01 for CYP, n = 4; Fig. 3A). siNR2B prevention of CYP-dependent facilitation and NR2B phosphorylation. We next knocked down NR2B expression in the lumbosacral spinal cord by daily intrathecal injection of mis-sense (siVeh) or NR2B siRNA (siNR2B) for 5 days. Compared with the siVeh group, tNR2B protein expression in siNR2B animals was significantly reduced compared with β-actin (tNR2B, P < 0.01 vs. siVeh, n = 4; Fig. 4A), indicating that this treatment indeed knocked down spinal NR2B expression. In the siVeh group (Fig. 4B), CYP administration
statistically enhanced NR2B phosphorylation (CYP + RS, P < 0.05 vs. RS) compared with animals that received RS only. In animals of the siNR2B group treated with only RS, NR2B phosphorylation was significantly lower than that in the siVeh group (RS in siNR2B, P < 0.01 vs. RS in siVeh). In the siNR2B group, there was also a statistically significant decrease in NR2B phosphorylation in CYP-treated animals compared with those in the siVeh group (CYP + RS in siNR2B, P < 0.05 vs. CYP + RS in siVeh). On the other hand, siNR2B attenuated the RS-induced SRP (siNR2B, P < 0.01 vs. siVeh, n = 7; Fig. 5, A and B). Moreover, compared with siVeh (siVeh + CYP), siNR2B significantly overwrote the CYP-dependent facilitation of SRP (siNR2B + CYP, P < 0.01 vs. siVeh + CYP, n = 7; Fig. 5, A and C).

**DISCUSSION**

Interstitial cystitis is a chronic inflammatory condition of the urinary bladder that affects an estimated one million people in the United States. Administration of CYP, which is an antineoplastic agent metabolized by the liver to produce the bladder irritant acrolein, is known to induce cystitis in rodents and humans (14). We injected CYP intraperitoneally to develop mild but chronic bladder inflammation to explore its possible effects on SRP modulation. Compared with vehicle solution injection, CYP administration facilitated SRP. This finding supports the notion that CYP administration induces
visceral pain in the lower urinary tract (5). Because SRP has been known to serve as a form of neural plasticity that relates to pelvic visceral pain (24, 27, 30), our results prove an extended role of CYP-induced inflammation in hypereflexia/hyperalgesia development following bladder inflammation. Glutamatergic NMDARs are known to underlie nociception at the spinal cord level (2). Among the NMDAR NR1–3 subunits, the NR2 subunit is of great interest, since it is essential for calcium gating and thus defines the electrophysiological properties of the NMDAR (22). In addition, there are four genes that encode NR2 subunits (NR2A-D), and studies have revealed that NR2B-containing NMDARs play crucial roles in activity-dependent neural plasticity induction (3, 17, 18) because NR2B phosphorylation has been described as an important determinant for NMDAR-mediated current (20). In the present study, chronic CYP administration facilitated SRP and simultaneously enhanced spinal NR2B phosphorylation. Genetic knockdown of NR2B expression using specific siRNA attenuated CYP-induced facilitation and NR2B phosphorylation. These findings agree well with studies showing that, in rats with an intact spinal cord, administration of NMDAR antagonists strongly decreases spinal c-Fos expression caused by bladder irritation (4) and noxious colorectal distension (43), suggesting that NMDARs are probably involved in visceral nociception resulting from CYP-induced cystitis in rats. Moreover, to-
gether with the results showing that NR2B phosphorylation caused by nerve injury-induced neuropathic pain is prevented by NR2B knockout (1) and knockdown (39), our data suggest that NR2B phosphorylation at the dorsal horn is essential for neuropathic pain maintenance and/or hyperalgesia.

Our previous work has shown that acute irritation of TRPV1-expressing afferent fibers coming from the descending colon may activate spinal Cdk5 activity, which induces subsequent NR2B phosphorylation, resulting in cross-organ SRP sensitization. In the current study, administration of ROS, a Cdk5 inhibitor, attenuated CYP-induced SRP facilitation and pNR2B expression, suggesting that Cdk5-dependent NR2B phosphorylation is involved in CYP-induced SRP facilitation. On the other hand, NO-dependent guanine cyclase activation has also been implicated in SRP (7). In the present study, pharmacological blockage of NOS using a selective inhibitor, l-NAME, also attenuated CYP-induced SRP facilitation and NR2B phosphorylation, suggesting a role for NO in NR2B-dependent SRP facilitation caused by CYP. This finding is in line with the idea that spinal NO plays roles in the modulation of pain-related central sensitization (10). To our knowledge, no studies have shown interaction between cascades depending on NO and Cdk5 in activity-dependent neural plasticity development. We suspect that CYP might activate different intracellular pathways to induce NR2B phosphorylation, mediating subsequent SRP facilitation. Studies investigating CYP-induced cystitis have shown multiple pathological changes in the lower urinary tract. For example, in vivo histological modifications have been documented (40). Considerable secondary neurological responses, including changes in neurochemical (21, 33–35) and electrophysiological properties (42), have also been noted. Yoshimura and de Groat also demonstrated that, in primary sensory fibers coming from the urinary bladder, the initial stimulating effects of CYP are mediated by the release of inflammatory mediators such as prostaglandin, serotonin, histamine, ATP, and nerve growth factor to afferent terminals. CYP could also produce delayed effects that alter ion channel properties in bladder afferent neurons (42).

In this study, we pharmacologically antagonized NOS and Cdk5 using acute intrathecal injections when we tested their roles in the RS-induced reflex potentiation, whereas daily intraperitoneal administrations were used when we explored their participation in CYP-dependent facilitation. Although these experiments shed light on the involvement of NOS and Cdk5 in different conditions, further studies are required to elucidate the proper doses of these agents administered through different routes that will yield comparable bioavailability. Moreover, studies investigating leukemic cell apoptosis (36) and macrophage NO production (9) have demonstrated that, in addition to Cdk5, ROS inhibits Cdk1, Cdk2, and Cdk7 as well. In macrophages, both Cdk5 and Cdk7 were shown to inhibit lipopolysaccharide-induced NO production by suppressing the nuclear factor-κB (NF-κB) family (9). Previous work has...
demonstrated that the CaMKII-dependent NO pathway is involved in the RS-induced pelvic-urethra reflex potentiation (7), and our unpublished data showed IKKα/β-dependent NF-κB activation participated in a reflex potentiation. The possibility that whether or not other Cdk5, in addition to Cdk5, might have been involved in the induction of SRP is an interesting question to be investigated further. Finally, because ROS has been reported to regulate calcium channel activity (8), whether the ROS could modulate the intracellular calcium signaling to play roles in the SRP, a calcium-dependent form of neural plasticity (7), should be taken into consideration.

The current study notes that, despite a lack of statistical significance in the relationship between the spike counts for TS-evoked baseline reflex activity for Veh and CYP-treated animals, the expression of pNR2B in CYP-treated rats was significantly higher than that for Veh. Although the detailed mechanism for this phenomenon remains unclear, one cause may be the fact that we used single pulses with an intensity that evoked a single action potential (TS-evoked baseline reflex activity) to standardize reflex activity. Although neural excitability in CYP-treated animals enhanced pNR2B expression upregulation in the dorsal horn, the spike count evoked by the TS would not reflect this change, since the threshold intensity could be reduced. This proposal was supported by the fact that the mean threshold intensity for TS to evoke the baseline reflex activity in CYP-treated animals (2.4 ± 0.8 mA) was statistically lower than that for the Veh group (4.8 ± 1.4 mA). However, further experiments are needed to understand the exact causes for such phenomenon and whether it may limit the applicability of SRP to serve as a biological index for investigation into visceral pain in the pelvic region.

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DISCLOSURES

No conflicts of interest are declared by the authors.
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