Effects of PGE$_2$ EP$_3$/EP$_4$ receptors on bladder dysfunction in mice with experimental autoimmune encephalomyelitis

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MATERIALS AND METHODS

Animals. Female SWXJ mice (8–10 wk old) were purchased from the Jackson Laboratory. All mice were maintained in the animal breeding facility at key discipline laboratories of Zhengzhou University under specific pathogen-free conditions and exposed to 12:12-h light-dark cycles (light from 6 AM to 6 PM). The experimental protocol was approved by the Animal Ethics Committee of Zhengzhou University School of Medicine.
Agonists and antagonists selective for each EP receptor subtype (ONO-DI-004, EP1 agonist; butaprost, EP2 agonist; sulprostone, EP3 agonist; CAY-10598, EP4 agonist; ONO-8711, EP1 antagonist; AH-6809, EP2 antagonist; DG-041, EP3 antagonist; and AH-23848, EP4 antagonist) were purchased from Cayman Chemical (Ann Arbor, MI) and ONO Pharmaceutical (Osaka, Japan). The high specificity of each agonist and antagonist for each EP receptor subtype was confirmed as follows: ONO-DI-004 and ONO-8711 exhibit high affinity ($K_i$: 150 and 1.7 nM, respectively) for the EP1 receptor and low affinity ($3.3 \times 10^{-11}$ nM and $5 \times 10^{-11}$ nM, respectively) for the other EP receptors (EP2,EP3). Butaprost and AH-6809 exhibit high affinity ($K_i$: 150 and 350 nM, respectively) for the EP2 receptor, sulprostone and DG-041 exhibit high affinity ($K_i$: 0.35 and 8.1 nM, respectively) for the EP3 receptor, and CAY-10598 and AH-23848 exhibit high affinity ($K_i$: 12 and 260 nM, respectively) for the EP4 receptor.

16,16-Dimethyl PGE2 (dm-PGE2) and incomplete Freund’s adjuvant (F5881) were purchased from Sigma-Aldrich (St. Louis, MO). PLP$_{139–151}$ peptide was purchased from SBS Genentech (Beijing, China). Pertussis toxin and Mycobacterium tuberculosis H37RA (killed and desiccated) was purchased from Difco (Detroit, MI). Bordetella pertussis was purchased from List Biologicals (Campbell, CA). Rabbit anti-EP$_1$,EP$_4$ receptor polyclonal antibodies and the PGE$_2$ EIA kit were purchased from Cayman Chemicals.

**Induction of EAE.** EAE was induced as previously described (5, 42). Briefly, mice were immunized with a 200-μl emulsion consisting of PLP$_{139–151}$ peptide (200 μg/mouse, OpeRon). The emulsion was a 1:1 (vol/vol) mixture of physiological saline and CFA containing 2 mg/ml M. tuberculosis H37RA (Difco). We injected mice intraperitoneally with 0.2 μg purified B. pertussis toxin (List Biologicals) on days 0, 3, and 7 after immunization. Mice were weighed and scored daily for neurological signs according to the following scale: 0, no sign; 1, tail weakness or slightly clumsy gait; 2, tail paralysis and/or moderately clumsy gait and/or mild hindlimb weakness; 3, moderate to severe hindlimb paraparesis or mild forelimb weakness (or both); 4, complete hindlimb paralysis or moderate to severe forelimb weakness (or both); 5, quadriplegia with uroclepsia or moribund state; and 6, death.

**Frequency-volume chart analysis.** The analysis of micturition was based on the protocol used by Altuntas et al. (5), and the experiment was performed at the Research Center for Obesity and Diseases (Beijing, China). Briefly, before the micturition testing started, mice were fed lactose-free milk instead of solid food for 24 h to reduce the frequency and weight of the feces generated during testing, which prevents skewing of the urine collection and abervations of data analysis (22). The 24-h micturition of mice was detected using a metabolic cage (MED-CYT-M, Med-Associates, St. Albans, VT), which included a wire mesh bottom for unobstructed passage of urine droplets. A plastic try was placed directly below the bottom to collect urine, which was placed on an analytic balance (VI-3mg, Acculab, Huntingdon Valley, PA). The data port of the balance was connected to a computer, and changes in the weight of the collection plastic tray were determined spectrophotometrically using an EIA kit. The molecular weights of EP1, EP2, EP3, and EP4 receptors were 42, 53, 52, and 65 kDa, respectively.

**Western blot analysis.** As the disease progressed, mice were examined daily for neurological deficits at different scores. Due to the fact that EAE mice with a score of 4 or 5 failed to urinate because of their severe symptoms of movement, we chose EAE mice with scores of 1–2 and 3 to measure MF per day and VW per void in the metabolic cage, and the control group was tested at the same times after immunization (Fig. 1B). Compared with the control group, MF and mean void weight (VW) were significantly increased in mice with scores of 1–2 and 3 compared with control mice ($P < 0.05$; Fig. 1C). However, there were no differences in MF between these two groups.
(mice with a score of 1–2 versus a score of 3). VW in mice with scores of 1–2 and 3 was significantly lower than that in the control group \((P < 0.05; \text{Fig. 1C})\). However, the difference in VW between mice with scores of 1–2 and mice with a score of 3 did not achieve statistical significance \((P > 0.05)\).

**Bladder PGE2 levels in EAE mice.** The above results reflect the bladder dysfunction induced by EAE, which means that the EAE model is suitable for the study of the neurogenic bladder condition. To investigate the role of the PGE2 system in the development of neurogenic bladder induced by EAE, we first determined the level of PGE2 in the bladder of EAE mice with scores of 0–3. As shown in Fig. 2, the bladder PGE2 level increased as the EAE score increased. Compared with the control group, the PGE2 level was significantly higher in mice with scores of 1–2 and 3 compared with control mice \((P < 0.05)\). In addition, the PGE2 level in mice with a score of 3 was significantly higher than that in mice with scores of 1–2 \((P < 0.05)\).

**Bladder weight and EP receptor expression in EAE mice.** We found that the bladder weight-to-total body weight ratio
EP₃/₄ RECEPTORS AND BLADDER DYSFUNCTION

Changes in MF and VW in control mice (Fig. 4, A and B). However, in EAE mice with scores of 1–2 and 3, sulprostone and CAY-10598 demonstrated an ability to increase MF (sulprostone: P < 0.05 for both; CAY-10598: P < 0.05 for both) as well as decrease VW (sulprostone: P < 0.05 for both; CAY-10598: P < 0.05 for both; Fig. 4, A and B) compared with control mice treated with vehicle. The functions of DG-041 and AH-23848 were inversely related to the agonists, which demonstrated an ability to decrease MF (DG-041: P < 0.01 for both; AH-23848: P < 0.01 for both) as well as increase VW (DG-041: P < 0.05 for both; AH-23848: P < 0.05 for both; Fig. 4, A and B) compared with control mice treated with vehicle. However, the administration of agonists and antagonists of EP₁ and EP₂ receptors did not show any significant changes in VW and MF of EAE mice with scores of 1–2 and 3.

We also found significant differences in VW and MF between EP₁–EP₄ agonist/antagonist-treated mice in the control group (EAE score: 0) compared with the EAE group with scores of 1–2 as well as between the control group and the EAE

Table 1 Bladder weight and EP₁–EP₄ receptor expression in mice with different EAE scores

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>0</th>
<th>1–2</th>
<th>3</th>
<th>F Value</th>
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</tr>
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<tbody>
<tr>
<td>Body weight</td>
<td>27.5 ± 1.20</td>
<td>27.3 ± 1.05</td>
<td>25.7 ± 1.23</td>
<td>23.9 ± 2.36</td>
<td>1.89</td>
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<td>Bladder weight to total body weight, %</td>
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<td>0.18 ± 0.01</td>
<td>0.39 ± 0.02†</td>
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<td>EP₁</td>
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Values are means ± SE; n = 4 mice/group. Densitometric analysis used the ratio of the E-prostanoid (EP) receptor to β-actin. *P < 0.05 by ANOVA among three groups; †P < 0.017 by Bonferroni post hoc analysis between experimental autoimmune encephalomyelitis (EAE) scores of 0 and 1–2; ‡P < 0.017 by Bonferroni post hoc analysis between EAE scores of 0 and 3.

Fig. 2. Bladder PGE₂ levels. A comparison of bladder tissue PGE₂ levels in each group with defined differential levels of neurological disability is shown. PGE₂ levels were significantly increased in mice with severe-grade EAE (score: 3, n = 5) compared with control mice (score: 0, n = 6; ***P < 0.001) and mice with moderate-grade EAE (score: 1–2, n = 6, **P < 0.01). PGE₂ levels in mice with moderate-grade EAE were significantly higher than those in control mice (**P < 0.01).

Fig. 3. E-prostanoid (EP) receptor expression in the bladder. Shown is the Western blot analysis of EP receptor expression in the bladder with defined differential levels of neurological disability. EAE induced the upregulation of EP₁ and EP₄ receptors with a concomitant increase in disease severity. However, no significant changes were detected in the expression levels of EP₁ and EP₂ receptors.

### Table 1: Bladder weight and EP₁–EP₄ receptor expression in mice with different EAE scores

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group with a score of 3. However, no significant differences were found between EP1–EP4 agonist/antagonist-treated mice in the two EAE groups (P<0.05; Fig. 4, A and B). In addition, we also examined individual and combined effects of the EP3 antagonist and EP4 antagonist on micturition during EAE development. As shown in Fig. 5, these antagonists had substantially stronger effects in combination than each modality used alone (P<0.05; Fig. 5, A and B).

**DISCUSSION**

Voiding dysfunction is a common problem for patients with MS and develops in >90% of patients who have had MS for >10 yr (3). Management of MS patients with bladder dysfunction remains a clinical dilemma, as no effective treatment modality currently exists. Altuntas et al. (6) first proposed that the EAE model induced by PLP139–151 could simulate the micturition abnormalities of a neurogenic bladder, which could be used as a more useful animal model for understanding and treating neurogenic bladder. The present study investigated the features of bladder dysfunction in EAE mice and explored the preliminary mechanism. Our results showed that the bladder weight-to-body weight ratio of EAE mice increased as the disease progressed. A recent study conducted by Altuntas et al. (1) reached the same conclusion and suggested that the mechanism of bladder weight change might be related to the elevated level of connective tissue growth factor and increased growth of connective tissue. A cyclophosphamide-induced OAB model in rats also showed an increase in bladder weight compared with control rats (8). Taken together, these results further support our findings and provide us with a deeper understanding of the EAE-induced pathological features of neurogenic bladder.

PGE2 has been implicated as an endogenous mediator of bladder function because it is synthesized locally in both bladder smooth muscle and the urothelium (4, 9, 15). Our results demonstrated a significant increase of PGE2 levels in the bladder during EAE development, suggesting that the severity of EAE can induce the synthesis of PGE2. In addition, treatment of EAE mice with exogenous PGE2 significantly promoted bladder dysfunction, suggesting a positive feedback regulation between PGE2 and bladder dysfunction. PGE2 exerts its physiological roles through four functionally related EP receptors, which are designated as EP1–EP4. Our research found that as the clinical score of EAE increased, EP3 and EP4 receptors were upregulated, whereas EP1 and EP2 receptors were not. To the best of our knowledge, there has been no conclusive results published to date regarding the expression of EP receptors during OAB progression. It has been...
previously suggested that the EP1 receptor is highly expressed in the bladder urothelium in mice and can be activated by PGE2 to release ATP, which is a sensory neurotransmitter that can trigger bladder overactivity and bladder outlet obstruction (20, 26, 31, 41). Moreover, a study (16) in OAB patients found that EP2 and EP4 receptors were overexpressed in the bladder urothelium of patients with OAB induced by bladder outlet obstruction, whereas expression of EP1 and EP3 receptors was not significantly altered. We postulate that the differences in EP receptor expression in the bladder might be due to the different species (human vs. mouse) and different stimulants used to generate OAB models (obstruction vs. chemical). However, the specific mechanisms should be explored in further studies.

Our results also demonstrated that the administration of agonists/antagonists of EP3 and EP4 receptors, but not EP1 and EP2 receptors, significantly affected bladder function of EAE mice and that the antagonists had an additive effect. In addition, VW and MV of EP1−EP4 agonist/antagonist-treated mice in the control group were significantly different from those in the EAE group with scores of 1–2 and 3, whereas there were no significant differences in VW and MV of EP1−EP4 agonist/antagonist-treated mice between the EAE group with scores of 1–2 and the EAE group with a score of 3. Thus, we hypothesize that the differences in VW and MV between the different treatment groups could be due to the progression of EAE disease.

To date, this is the first study implicating EP3/EP4 receptors in the regulation of urinary bladder dysfunction in the EAE model. EP3 receptors are expressed on dorsal root ganglion neurons and sensory afferent nerve terminals of the bladder (29, 40). Therefore, it is conceivable that EP3 receptors are related to bladder dysfunction. According to a study by McCafferty et al. (27), EP3 receptor knockout mice had an enlarged bladder capacity compared with wild-type mice under control conditions, without any changes in urine composition or volume. Jugus et al. (28) also reported that activation of the EP3 receptor using its agonist resulted in a reduction of functional bladder capacity. Importantly, our data showed that manipulation of the EP3 receptor with agonists/antagonists may be useful in the control of bladder dysfunction in the neurogenic bladder.

The EP4 receptor is thought to be involved in afferent hyperexcitability (37, 45). Lin et al. (21) found that an antagonist of the EP4 receptor attenuated PGE2 sensitization of capsaicin currents in dorsal root ganglion cultures. Recently, Chuang et al. (8) found that cyclophosphamide-induced upregulation of the EP4 receptor was accompanied by detrusor overactivity and that an antagonist of EP4 receptors, AH-23848, significantly extended the intercontraction interval in cyclophosphamide-treated rats compared with control rats. Ma et al. (25) showed that a selective EP antagonist, L-161982, was able to suppress IL-6 release from invading macrophages with EP4 receptor upregulation in a partial sciatic nerve ligation model of rats. Therefore, our findings confirmed that the EP4 receptor may play a role in bladder dysfunction.

In this study, we found that administering agonists/antagonists of EP1 and EP2 receptors in mice did not affect bladder dysfunction in the EAE model, which is not consistent with previous conclusions. A previous study (31) has shown that EP1 knockout or treatment with an EP1 antagonist suppressed detrusor overactivity in a bladder outlet obstruction model. In addition, Lee et al. (20) reported that an EP1 receptor antagonist suppressed intravesical PGE2-induced detrusor overactivity. Chuang et al. (8) suggested that although EP1 receptor expression was found to be reduced in the bladder of an OAB model, it is still possible that EP1 receptors contribute to the sensitization of bladder afferent nerves and overactivity. In addition, EP2 receptors have been reported to mediate bladder and urethra relaxation (38). However, additional studies are needed to clarify the true physiological role of EP receptors in bladder function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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