Urothelium EP₁ receptor facilitates the micturition reflex in mice

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ABSTRACT

We investigated the presence of EP₁ receptor in the urothelium and its role in micturition reflex by examining the effect of intravesical administration of prostaglandin E₂ (PGE₂), an EP₁ agonist (ONO-DI-004), acetic acid, and capsaicin. Age-matched EP₁-KO mice and C57BL/6 wild-type (WT) mice were used. Western blots and standard immunohistochemical procedures were performed. Cystometrygram (CMG) was performed without anesthesia in a restraining cage. ATP release from the cultured urothelium cells was performed using luciferin-luciferase luminometry. The EP₁ receptor was found to be present in the urothelium. In WT mice, PGE₂ infusion shortened the inter-contraction interval (ICI) in a dose-dependent fashion; however, it did not alter the ICI in EP₁-KO mice. The EP₁ agonist significantly shortened the ICI in WT mice, but not in EP₁-KO mice. Acetic acid and capsaicin shortened the ICI in both WT mice and EP₁-KO mice. EP₁ agonist, PGE₂ and capsaicin provoked ATP release from cultured urothelial cells. These results suggest that EP₁ receptor was present in bladder urothelium, and could be activated by PGE₂ to release ATP. EP₁ receptor in urothelium might be important for reflex voiding in pathological conditions.

Urinary prostaglandin E₂ (PGE₂) is synthesized locally in the kidney and the urinary bladder (4, 11). Intravesical administration of PGE₂ in humans causes detrusor overactivity, urgency, and decreased bladder capacity (14). PGE₂ receptors are classified into four general subtypes based on cloning and pharmacological manipulations (5): the EP₁ receptor, which mediates Ca⁺⁺ mobilization and induces smooth muscle contraction; the EP₂ and EP₄ receptors, which mediate increase in cAMP levels and induce smooth muscle relaxation; and the EP₃ receptor which is an inhibitory receptor that mediates decrease in cAMP levels and inhibits smooth muscle relaxation (13).

In a previous study, we showed that an EP₁ receptor antagonist, ONO-8711, inhibited bladder afferent nerve activity from inflamed bladder in rat (8). Intravesical PGE₂ induced detrusor overactivity in rat (9). Recently, several studies had shown that the release of chemical mediators, such as ATP, from urothelial cells were involved in sensory signaling at the urinary bladder and play a role in the regulation of micturition reflex (6). Interstitial cystitis altered ATP release from bladder urothelium in cat and man (3, 15). The urothelium was not only a barrier between the urinary tract and its contents but also a metabolically highly active tissue (2). A number of studies had shown the presence of a variety of neurotransmitter receptors and ion channels in the urothelium (2). The localization of afferent nerve fibers next to the urothelium and some fibers ending in the basal layer of the urothelium suggested that urothelial cells possess a signal-transduction mechanism, and that the urothelium plays a role in senso-

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ry functions (2). Mechanical stress as well as chemical stimuli evoked ATP release from urothelial cells and influenced bladder function (1). We postulated that EP₁ receptor may be present in the urothelium and may be responsible for the release of ATP from the urothelial cells.

It has been reported that PGE₂ increases transient receptor potential vanilloid 1 (TRPV1) activity through EP₁ receptors in mouse dorsal root ganglion (DRG) neurons (12), and it has been suggested that EP₁ and TRPV1 receptors can functionally interact. We therefore set out to investigate the role of EP₁ receptor in the urinary bladder using EP₁ receptor knockout (EP₁-KO) mice.

MATERIALS AND METHODS

Animals. Age-matched male EP₁-KO mice (10–12 weeks, *n* = 30) and male WT mice (C57BL/6, 10–12 weeks, *n* = 60) were used. The EP₁-KO mice were from Kyoto University. The mouse genes encoding EP₁ receptor was disrupted by gene knockout methods using homologous recombination, as reported previously (16). C57BL/6 mice, which are the background species of the knockout mice, were used as a control. The mice were housed with free access to food and water and maintained on forced 12 h light-dark cycle at 22–24°C. The protocols for the experiments were previously approved by the Animal Research Committee, Akita University. We followed the APS (American Physiological Society) guideline for animal research. Statistical analysis was performed using Mann–Whitney *U* and paired *t*-tests, with *p* < 0.05 considered significant. All data are presented as means ± SEM.

Western blot. The urinary bladder was excised from deeply anesthetized (intraperitoneal injection of pentobarbital sodium, 35 mg/kg body weight) WT mice (*n* = 20) and washed in 0.1 M phosphate buffered saline (PBS, pH 7.4). The bladder was weighed and its tissue was incubated for 1 h at 37°C in an essential medium containing 2.5 mg/mL dispase (Godo Syusei, Tokyo Japan), to weaken the association between the urothelial layer and the connective tissue. After incubation, the smooth muscle layers were removed and the stripped mucosa was collected. The urothelial layers and smooth muscle layers were subjected to western blot analysis. The urothelial layers were also examined for cell culture. Whole bladder tissue taken from WT mice (*n* = 5) was homogenized in lysis buffer (pH 7.4) with protease inhibitor mixture (Roche Diagnostics GmbH,

Mannheim, Germany). The samples, each containing 20 µg of total protein, were electrophoresed by SDS-PAGE (100 V, 1.5 h) on 10% Tris-glycine gels. The proteins were transferred from the gel onto a nitrocellulose membrane. The membranes were incubated overnight with rabbit anti-EP₁ receptor polyclonal antibody (Cayman Chemical, Ann Arbor, USA; 1 : 300 in dilution). This antibody (#106367) was raised against a synthetic peptide from the human EP₁ receptor C-terminal amino acids 380–402 (GLTPSAWEASSLRSSRHSGLSHF). Some membranes were incubated with the EP₁ receptor antibody absorbed with the EP₁ receptor blocking peptide (Cayman Chemical). After washing with 0.1% Triton X100 at room temperature, the membranes were incubated for 40 min with peroxidase-labeled anti-rabbit IgG secondary antibody (Pierce Chemical; 1 : 40,000 in dilution). The blot was exposed to FUJI medical film (FUJIFILM Corporation, Tokyo, Japan) and developed.

Immunohistochemistry. WT mice (*n* = 5) were anesthetized with 1% halothane (Fluothane®, Takeda, Chemical Industries Ltd, Japan) and perfused via the aorta with physiological saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The bladder was removed, fixed for 6 h and immersed in 30% sucrose solution before being rapidly frozen in liquid nitrogen. Cryostat sections were cut at 5 µm and incubated overnight at room temperature with rabbit anti-EP₁ receptor antibody (1 : 200). The sections were then incubated at 37°C for 1 h with peroxidase labeled anti-rabbit IgG secondary antibody (1 : 40,000 dilution). For negative control sections, primary antibodies were omitted.

Cell culture. The urothelial layer was detached from the bladder of WT mice by enzymatic digestion (dispase) (Godo Syusei). The layer was further treated with 0.25% trypsin and resuspended in serum-free keratinocyte medium (KURABO, Osaka, Japan). The dissociated cell suspension was plated onto collagen-coated wells at a density of approximately 70,000 cells /cm².

ATP measurement. ATP in the culture medium (cultured for 4–5 days) was measured by the luciferin-luciferase method with a Luminometer (Kikkoman Co., Ltd., Tokyo, Japan). A 100 µL sample of supernatant was added to a 100 µL reaction mix of luciferin-luciferase (Kikkoman Co., Ltd.). All drugs were incubated for a minimum of 10 min before sample collection.

Table 1 The voiding frequency and urine volume in WT mice and in EP₁-KO mice

	Means WT ± SEM (n = 7)	Means EP ₁ -KO ± SEM (n = 7)	p Value
Voiding frequency (times/day)	9 ± 1	8 ± 1	Not significant
Urine volume (mL/day)	1.07 ± 0.1	0.99 ± 0.15	Not significant

Fig. 1 Western blot analysis of EP₁ receptor proteins on bladder tissue in WT mice. A: EP₁ receptor at 68 kDa was detected in urothelial layers (lane 1) and whole bladder tissue (lane 3). The amount of proteins loaded was 20 µg/lane. The positions of molecular weight markers are indicated. B: No stained bands are present after pretreatment with the EP₁ blocking peptide (lane 4–6). The amount of proteins loaded was 20 µg/lane.

Surgical procedures and cystometrygram (CMG). Mice (WT mice, n = 30 and EP₁-KO mice, n = 30) were anesthetized with 1% halothane (Fluothane®, Takeda Chemical Industries Ltd, Japan). The abdomen was opened by a lower midline incision, and the urinary bladder was cannulated with polyethylene tubes (PE50). Abdominal incisions were closed with 4-0 nylon sutures. The bladder catheter was connected to a pressure transducer and an infusion pump using a T-connector. The mouse was placed in a Ballman restraining cage (Natsume, Tokyo, Japan). After recovery from anesthesia, room temperature saline was infused into the bladder at a rate of 0.4 mL/h or 0.5 mL/h. Vesical pressure was recorded on an AP601 polygraph (Nihonkoden, Tokyo, Japan) and digitized with a converter for recording on a PowerLab® system, version 5.0. The parameters used were intercontraction interval (ICI) and maximal voiding pressure (MVP). When the reflex micturition was stabilized, the saline solution was changed to vehicle or drugs. During the course of saline infusion (control condition), three voiding cycles were recorded per animal and each parameter

Fig. 2 Photomicrographs show mice bladder stained with (A, C) and without (B, D) anti-EP₁ receptor antibody. The labeling in the bladder is more intense on the umbrella cells. Calibration bar represents 100 µm in A and B, 50 µm in C and D. U: urothelium. SM: smooth muscle.

was averaged. After the drug was administered, the parameters were recorded for 30 min; these data were used for evaluation. During continuous cystometrygram, animals were kept at room temperature.

Drugs and vehicle. The following 4 compounds were tested in WT or EP₁-KO mice: PGE₂ (1–60 µM), EP₁ receptor agonist (ONO-DI-004, 40 µM), 0.1% acetic acid, and capsaicin (60 µM). PGE₂ (1 mM) and capsaicin (30 mM) were dissolved in absolute ethanol, and stored at –70°C. When used for the CMG experiment, capsaicin with vehicle (VEH, 0.1% ethanol) was infused into the urinary bladder at a concentration of 60 µM. Similarly, PGE₂ was infused at a concentration of 1–60 µM. The PGE₂ and EP₁ receptor agonist (ONO-DI-004) were kindly gifted from Ono Pharmaceutical Co., Ltd (Osaka, Japan).

RESULTS

We assessed both bladder and body weight between WT mice (n = 7) and EP₁-KO mice (n = 7). There was no significant difference in bladder weight (19.0 ± 0.7 mg and 21.1 ± 1.1 mg, respectively) or in body weight (19.9 ± 0.2 g and 21.0 ± 0.6 g, respectively). We also measured both daily voiding frequency and daily urine volume from WT mice and EP₁-KO

Fig. 3 Effect of PGE₂ (30 μM) on cystometrograms in WT mice (A) and EP₁-KO mice (B). Line graphs demonstrate the effect of PGE₂ on ICI (left) and MVP (right). In WT mice, PGE₂ shortened ICI to 47.5% of control (418.4 ± 75.4 s to 198.9 ± 40.3 s). However, PGE₂ did not alter ICI in EP₁-KO mice (624.3 ± 58.5 s to 744.9 ± 73.6 s, 119.3% of control). *Significantly different from control, $p < 0.05$.

mice. There was no significant difference in daily voiding frequency (9 ± 1 times and 8 ± 1 times, respectively, Table 1) or in daily urine volume (1.07 ± 0.1 mL and 0.99 ± 0.15 mL, respectively, Table 1).

Western blot and immunohistochemistry

The protein samples from the WT mice bladder tissue were shown in Fig. 1. The urothelial cell layer and whole bladder tissue showed a prominent immunoreactive band at around 68 kilodaltons (kDa). No band of that size was present in the muscle layer. No stained bands were present in the controls after pretreatment with EP₁ receptor blocking peptide (Fig. 1B).

A strong reaction product for EP₁ receptor was observed in the urothelium. Most urothelial cells showed immunoreactivity for EP₁ receptor in the cytoplasm; the umbrella cells were more intensely stained than basal cells (Fig. 2, A and C). The smooth muscle showed weak reactions. When the tissue was processed with the same immunostaining protocol only omitting the primary antibody, there was no immunoreactive staining in the urothelium (Fig. 2, B and D).

CMG in WT and EP₁-KO mice

Fig. 4 Effect of PGE₂ (1–60 μM) infusion on cystometrograms in WT (A) and EP₁-KO mice (B). In WT mice, PGE₂ infusion shortened ICI and decreased MVP in a dose-dependent fashion. Line graphs demonstrate the effect of PGE₂ on ICI (left) and MVP (right). Infusion with PGE₂ shortened ICI and decreased MVP significantly at a concentration of 10 μM ($p < 0.05$). Horizontal: PGE₂ doses. Vertical: ICI and MVP. *Significantly different from control, $p < 0.05$.

In conscious animals, during continuous infusion (with saline), the ICI in EP₁-KO mice (624.3 ± 58.5 s, $n = 9$) was longer than in WT mice (418.4 ± 75.4 s, $n = 9$, Fig. 3, bottom left). In WT mice, PGE₂ infusion (30 μM) shortened ICI to 47.5% of control (418.4 ± 75.4 s to 198.9 ± 40.3 s, $n = 9$) (Fig. 3A). However, the PGE₂ did not alter ICI in EP₁-KO mice (624.3 ± 58.5 s to 744.9 ± 73.6 s, 119.3% of control, $n = 9$, Fig. 3B). PGE₂ did not change the MVP in both mice (Fig. 3, A and B). In WT mice ($n = 5$), PGE₂ infusion (1–60 μM) shortened ICI and decreased MVP in a dose-dependent fashion; infusion with PGE₂ shortened ICI and decreased MVP significantly at a concentration of 10 μM ($p < 0.05$) (Fig. 4A). In EP₁-KO mice ($n = 5$), PGE₂ infusion (1–60 μM) did not change the ICI and MVP significantly (Fig. 4B).

In WT mice, EP₁ agonist shortened ICI from 342.6 ± 27.1 s to 140.4 ± 13.0 s (41% of control, $n = 7$, Fig. 5A). However, EP₁ agonist did not alter ICI in EP₁-KO mice (from 401.1 ± 39.1 s to 408.1 ± 56.8 s, 101% of control, $n = 7$, Fig. 5B). EP₁ agonist did not change the MVP in either mouse type (Fig. 5A, B).

Fig. 5 Effect of 40 μ M EP₁ agonist (ONO-DI-004) on cystometrograms in WT (A) and EP₁-KO mice (B). Line graphs demonstrate the effect of EP₁ agonist on ICI (left) and MVP (right). In WT mice, EP₁ agonist shortened ICI from 342.6 ± 27.1 s to 140.4 ± 13.0 s, 41% of control. However, EP₁ agonist did not alter ICI in EP₁-KO mice (from 401.1 ± 39.1 s to 408.1 ± 56.8 s). *Significantly different from control, $p < 0.05$.

In WT mice, 0.1% acetic acid shortened ICI from 298.3 ± 40.2 s to 192.6 ± 32.2 s (64% of control, $n = 4$, Fig. 6A). However, 0.1% acetic acid did not alter MVP in WT mice (38.6 ± 5.1 cmH₂O to 33.8 ± 3.6 cmH₂O, 87% of control, $n = 4$, Fig. 6A). In EP₁-KO mice, 0.1% acetic acid shortened ICI from 312.1 ± 44.9 s to 198.2 ± 43.9 s (64% of control, $n = 4$), and decreased MVP from 30.3 ± 2.6 cmH₂O to 22.5 ± 1.7 cmH₂O (73% of control, $n = 4$, Fig. 6B). In WT mice, capsaicin (60 μ M) shortened ICI from 468.2 ± 56.1 s to 228.9 ± 30.9 s (49% of control, $n = 5$, Fig. 7A). In EP₁-KO mice, capsaicin (60 μ M) shortened ICI from 686.3 ± 62.9 s to 342.6 ± 62.93 s (50% of control, $n = 5$, Fig. 7B). capsaicin did not alter MVP in either mouse type (Fig. 7A, B).

ATP release from cultured urothelial cells in WT mice

Administration of PGE₂ (1 μ M) elicited significant ATP release from urothelial cells ($121.7\% \pm 6.7\%$ of control, $p < 0.05$, $n = 5$). EP₁ agonist (1 μ M) elicited greater ATP release from the urothelial cells ($285.6\% \pm 30.7\%$ of control, $p < 0.05$, $n = 5$). Capsaicin sig-

nificantly increased the release of ATP from the urothelial cells ($227.9\% \pm 29.1\%$ of control, $p < 0.05$, $n = 5$). The ATP release was not evoked by 1% ethanol (VEH, $91.3\% \pm 8.5\%$ of control, $p > 0.05$, $n = 5$).

DISCUSSION

In Western blot analysis, EP₁ receptor was detected as a single band (68 kDa) in the urothelium and the whole bladder tissue, while smooth muscle tissue did not show the same staining pattern. It showed a weak immunoreactive band with a low molecular mass of 54 kDa. We suspect that the 54 kDa band might be a proteolytic cleavage product. From the immunohistochemical study, the EP₁ receptor was strongly detected in the urothelium. Taken together, our results demonstrate that EP₁ receptor was present in the urinary bladder, with a majority being present in the urothelium.

Our study showed that the intravesical administration of PGE₂ or EP₁ agonist had significant effects only on micturition intervals, while the bladder pressure remained unaffected. This data suggested that the EP₁ receptor might be involved in afferent limb for micturition reflex. EP₁ agonist infusion to cultured urothelium cells released ATP. Since ATP from urothelial cells activates P2X₃ receptor at primary afferent terminals from the urinary bladder, it is likely that PGE₂ could activate EP₁ receptor at urothelium and release ATP which act on P2X₃ receptor at primary afferent terminals. Ikeda *et al.* indicated that EP₁ receptor antagonist diminished inflammatory neural activities from urinary bladder in rat (8).

Capsaicin (TRPV1 ligand) facilitated micturition reflex in WT mice. ICI was reduced to 49% of the control. Capsaicin released ATP from the cultured urothelium cells and shortened ICI to 50% of the control in EP₁-KO mice. It is likely TRPV1-channel at EP₁-KO mice activated at afferent limb and facilitated micturition reflex. Recently, Moriyama *et al.* demonstrated EP₁-antagonist diminished inward current activated by capsaicin at DRG cells (12). Further experiments must explore to study the interaction of TRPV1 channel and EP₁ receptor in bladder afferent pathway.

Acetic acid infusion to urinary bladder shortened ICI in WT mice. Acetic acid could activate acid sensing ion channel (ASIC) and/or TRPV1 channel (10). These two channels could activate primary afferent nerve and cause hyperreflexia of micturition (7). However, acetic acid might cause tissue damage

Fig. 6 Effect of 0.1% acetic acid (AA) on cystometrograms in WT (A) and EP₁-KO mice (B). Line graphs demonstrated the effect of acetic acid on ICI (left) and MVP (right). In WT mice, acetic acid shortened ICI from 298.3 ± 40.2 s to 192.6 ± 32.2 s, 64% of control. In EP₁-KO mice, acetic acid shortened ICI from 312.1 ± 44.9 s to 198.2 ± 43.9 s (64% of control), and decreased MVP from 30.3 ± 2.6 cmH₂O to 22.5 ± 1.7 cmH₂O (73% of control). *Significantly different from control, $p < 0.05$. Infusion speed: 0.5 ml/h.

and produce inflammation locally. In our study acetic acid shortened ICI in EP₁-KO mice, suggesting that acetic acid effect was not involved in the PGE₂-EP₁ receptor.

The ICI in EP₁-KO mice was longer than in WT mice. However, the daily voiding frequency in WT mice was not significantly different from EP₁-KO mice. The ICI was recorded after surgery. Thus it could explain that PGE₂ activates EP₁ receptor in WT mice and facilitates micturition reflex. Therefore the ICI in EP₁-KO mice was longer than in WT mice.

In conclusion, the results of these efforts indicated the EP₁ receptor immunoreactivity was strongly detected in urothelium. The urothelial EP₁ receptor could be activated by PGE₂ to evoke ATP release from urothelial cells and to facilitate bladder mechanosensitive afferent activity and reflex voiding in mice. The urinary PGE₂ from kidney and bladder may contribute to the bladder overactivity, and affect urinary bladder function in pathological condi-

Fig. 7 Effect of 60 μM capsaicin (CAP) on cystometrograms in WT (A) and EP₁-KO mice (B). Line graphs demonstrated the effect of capsaicin on ICI (left) and MVP (right). In WT mice, capsaicin shortened ICI from 468.2 ± 56.1 s to 228.9 ± 30.9 s (49% of control). In EP₁-KO mice, capsaicin shortened ICI from 686.3 ± 62.9 s to 342.6 ± 62.93 s (50% of control). *Significantly different from control, $p < 0.05$. VEH: vehicle.

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