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Expression of E-series prostaglandin (EP) receptors and urodynamic effects of an EP₄ receptor antagonist on cyclophosphamide-induced overactive bladder in rats

Yao-Chi Chuang, Naoki Yoshimura[†], Chao-Cheng Huang^{*}, Moya Wu, Pradeep Tyagi[‡], and Michael B. Chancellor[‡]

^{*} Departments of Urology and Pathology, Chang Gung Memorial Hospital, Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan

[†] Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, PA

[‡] Department of Urology, William Beaumont Hospital, Royal Oak, MI, USA

Abstract

OBJECTIVE—To investigate the expression of four subtypes of E-series prostaglandin (EP₁–EP₄) receptors and the urodynamic effects of an EP₄ receptor antagonist (AH23848) in cyclophosphamide (CYP)-induced overactive bladder (OAB) in rats, as intravesical prostaglandin E₂ (PGE₂) induces OAB via activation of EP receptors and sensitization of afferent nerves.

MATERIALS AND METHODS—Experimental and control rats were injected with CYP (200 mg/kg, intraperitoneally) or saline, respectively. Continuous cystometrograms (CMGs) were performed 48 h after CYP or saline injection under urethane anaesthesia. AH23848 was given intravenously at doses of 0.01 and 0.1 mg/kg. The bladder was then harvested for histology. Some bladders were harvested for analysis of EP receptors expression by Western blotting without a CMG study. CMG variables (baseline pressure; intercontraction interval [ICI], pressure threshold [PT], contraction amplitude) and histological changes were measured.

RESULTS—CYP-induced up-regulation of EP₄ receptor (100% increase) accompanied by detrusor overactivity (ICI 70.5% decrease; PT, 67.7% increase). However, CYP down-regulated EP₁ receptor expression (51.9% decrease), but had no significant effects on the EP₂ and EP₃ receptors. AH23848 significantly extended the ICI in CYP-treated rats but it had no effects on other urodynamic variables or in control rats.

CONCLUSIONS—Modulation of EP receptors plays a role in CYP-induced OAB. Antagonists to the EP₄ receptor may be a new target for treatment of patients with OAB.

Keywords

EP₄ receptor; cyclophosphamide; overactive bladder; rat

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Correspondence: Yao-Chi Chuang, 123 Ta Pei Road, Niao Song Hsiang, Kaohsiung Hsien, Taiwan. chuang82@ms26.hinet.net.

CONFLICT OF INTEREST

Yao-Chi Chuang is a consultant and investigator for Allergen and Pfizer, Naoki Yoshimura is a consultant for Astellas, Pradeep Tyagi is a consultant for Lipella, and Michael B. Chancellor is a consultant for Pfizer, Astellas and Allergen.

INTRODUCTION

Prostaglandin E₂ (PGE₂) is a local mediator in the bladder that affects micturition reflex in the normal physiological state and under pathophysiological conditions [1]. PGE₂ has been shown to modulate bladder function at the detrusor muscle as well as bladder afferent and efferent nerves [2,3]. Intravesical administration of PGE₂ stimulates reflex micturition through activation of capsaicin-sensitive afferent nerves and causes overactive bladder (OAB) in rats and in humans [2,4]. Furthermore, urinary PGE₂ has been reported to be increased in patients with OAB compared with controls [5].

The effects of PGE₂ are mediated by G-protein-coupled E-series prostaglandin (EP₁–EP₄) receptors. Previous studies have shown that EP₁ receptor antagonists inhibit OAB induced by spinal cord injury or BOO [3,6]. Su *et al.* [7] reported that the EP₃ receptor is involved in bladder voiding at the supraspinal and spinal centres. Our recent study also showed up-regulation of the EP₄ receptor in cyclophosphamide (CYP)-induced OAB in rats [8]. Lin *et al.* [9] reported that administration of an EP₄ antagonist or EP₄ knockout attenuated inflammatory pain and hypersensitivity in a Freund's adjuvant-induced somatic pain model in rats. Ma *et al.* [10] reported that the EP₄ receptor was up-regulated and associated with an increased interleukin 6 level in a sciatic nerve injury model in rats and that administration of an EP₄ antagonist suppressed the interleukin 6 level.

In the present study, we evaluated the expression profiles of the EP receptor subtypes in normal bladder and OAB. We hypothesized that the modulation of EP receptors expression might contribute to the generation of OAB. We further hypothesized that blocking the EP₄ receptor might suppress CYP-induced OAB.

MATERIALS AND METHODS

All experimental procedures were performed on female Sprague-Dawley rats (220–280 g), and reviewed and approved by the Institutional Animal Care and Use Committee before the study began. OAB was induced by i.p. injection of CYP, which is metabolized to acrolein, an irritant eliminated in the urine [11,12]. CYP (200 mg/kg; i.p.) or a corresponding volume of saline was injected on day 1 (ten rats in each group). In each group, four rats were used for Western blot analysis, and the other six were used for the cystometrogram (CMG) studies, EP₄ antagonist administration, and histology and immunohistochemistry.

On day 3, the rats were anaesthetized (urethane, 1.2 g/kg; s.c.) for i.v. catheter implantation. Polyethylene (PE)-50 tubing filled with heparinized saline (100 IU/mL) was inserted into the femoral vein for drug administration.

After i.v. catheterization, another PE-50 tubing was inserted into the bladder through the urethra and connected via a three-way stopcock to a pressure transducer and to a syringe pump for recording intravesical pressure and for infusing solutions into the bladder. A control CMG was performed by filling the bladder with saline (0.08 mL/min) to elicit repetitive voiding. The amplitude (the peak pressure minus the basal pressure during each contraction period), pressure threshold (PT, the pressure immediately before the reflex contraction), pressure baseline (PB, the lowest bladder pressure during filling) and intercontraction interval (ICI, the time between contractions) of reflex bladder contractions were recorded. Measurements in each rat represented the average of 3–5 bladder-voiding contractions.

For EP₄ antagonist [AH23848] administration a 18-mg/mL stock solution of AH23848 was made in dimethyl sulfoxide and subsequent drug dilution were made in saline on the day of experiment. Cumulative doses of the EP₄ antagonist AH23848 (vehicle, 0.01, and 0.1 mg/

kg, Sigma Aldrich, St. Louis, MO, USA) was injected i.v. after obtaining a baseline CMG and the effects of the drug were followed up for 30 min.

After CMG, the rats were deeply anaesthetized and killed via transcardiac perfusion, first with Krebs buffer followed by 4% paraformaldehyde fixative. The rats were then dissected to harvest the bladder.

For the histology and immunohistochemistry, the bladder was fixed in 10% buffered formaldehyde for 24–48 h, and then embedded in paraffin. The bladder tissue for histology was cut into 3- μ m sections and stained with haematoxylin and eosin. Alternatively, the tissue sections of bladder for immunohistochemistry were cut into 3- μ m sections, dewaxed in xylene and rehydrated to distilled water through decreasing concentrations of alcohols. Antigen retrieval was achieved by pressure-cooking tissue sections immersed in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase activity was blocked with 3% H₂O₂ solution. Tissue sections were then incubated with rabbit anti-EP₄ receptor polyclonal antibody (Cayman chemical, MI, USA; 1:1000 dilution) at +4 °C over night. Sections were then washed in PBS at pH 7.0 and incubated in BioGenex Super EnhancerTM Reagent (Launch Diagnostics, Kent, UK) for 20 min. After further washing in PBS the sections were incubated for 30 min in BioGenex polymer horseradish peroxidase complex (Launch Diagnostics) followed by a PBS wash. Slides were finally developed with 3,3'-diaminobenzidine chromogen (BioGenex DAB substrate, Launch Diagnostics) and counterstained with Mayer's haematoxylin. Slides were then dehydrated through increasing concentrations of alcohol to xylene and coverslip mounted with Entellan (Merck, Darmstadt, Germany). The result of the immunostaining was defined as negative when no staining or only faint staining was present. The result of the immunostaining was defined as positive when moderate to strong staining intensity was noted.

For Western blot analysis of EP_{1–4} receptor expression, four rats from each group were deeply anaesthetized and killed with no CMG study and without transcardiac perfusion on day 3. The bladder was removed and weighed, and Western blot analysis of EP_{1–4} receptor expression was conducted according to the standard protocol (Amersham Biosciences) and our previous study [8]. The samples were homogenized in protein extraction solution (T-PER; Pierce Biotechnology) before sonication and purification. The amount of total protein was measured with the Bradford protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed using the buffer system of Laemmli. Briefly, an aliquot of the extracts equivalent to 30 μ g protein was loaded onto 8% polyacrylamide gel, electrophoresed at a constant voltage of 100 V for 1 h and transferred to Hybond-P PVDF Membrane (Amersham Biosciences). The membrane was blocked with blocking agent and then immunoblotted overnight at +4 °C with mouse anti-actin monoclonal antibody (Chemicon, USA; 1 : 10 000 dilution) and anti-EP_{1–4} receptor antibodies (rabbit anti-EP₁ receptor polyclonal antibody, Cayman, MI, USA [1 : 200 dilution]; rabbit anti-EP₂ receptor monoclonal antibody, Chemicon [1 : 500 dilution]; rabbit anti-EP₃ receptor polyclonal antibody, Cayman [1 : 500 dilution]; rabbit anti-EP₄ receptor polyclonal antibody, Cayman [1 : 500 dilution]) [13–15]. According to the product information of manufacturer, the molecular weights were 42 kDa, 53 kDa, 52 kDa, and 65 kDa for EP₁, EP₂, EP₃, and EP₄ receptors, respectively.

After washing, the membrane was incubated with secondary antibody using 5% defatted milk powder in Tris-buffered saline for 2 h at room temperature using a horseradish peroxidase-linked anti-rabbit or anti-mouse IgG. Western blots were visualized by enhanced chemiluminescence detection system (Amersham Biosciences). The amount of β -actin was also detected as the internal control. Quantitative analysis was done using LabWorks Image Acquisition and Analysis software.

For statistical analysis the control and CYP-treated bladder weights, CMG variables and EP receptors expression were compared using the unpaired *t*-test. Repeated measures ANOVA followed by Tukey's multiple comparison test was used to compare changes in CMG variables before and after cumulative injections of AH23848. Quantitative data are expressed as the mean (SEM), with $P < 0.05$ considered to indicate statistical significance.

RESULTS

CYP EFFECTS ON BLADDER WEIGHT AND EP RECEPTORS EXPRESSION

As shown in Fig. 1A and Table 1, CYP induced bladder oedema and haemorrhage and increased bladder weight (118% increase). Western blotting analysis (Table 1, and Fig. 1B) showed that CYP induced up-regulation of the EP₄ receptor (100% increase) accompanied by down-regulation of the EP₁ receptor (51.9% decrease). However, CYP had no significant effects on the EP₂ and EP₃ receptors.

CMG RESPONSE TO CYP AND EP₄ ANTAGONIST AH23848

CYP treatment induced a higher PT (67.7%) and a shorter ICI (70.5%) compared with the control (saline injection) group (Table 2 and Fig. 2). These CMG variables reflect the OAB induced by CYP injection.

Cumulative administration of AH23848 (0.01 and 0.1 mg/kg; i.v.) did not induce significant changes in CMG variables in the control rats (Fig. 2A). However, both doses of AH23848 (0.01 and 0.1 mg/kg; i.v.) significantly extended the ICI by 57.7% and 88.5% compared with the pre-drug control value ($P < 0.05$ and $P < 0.01$, respectively) in the CYP-treated group (Fig. 2B). AH23848 did not change other CMG variables. These results indicate the therapeutic effect of AH23848 on OAB.

HISTOLOGY AND EP₄ IMMUNOHISTOCHEMICAL STAINING

Photomicrograph of bladder sections showed mucosal oedematous change (marked thickening of the submucosal layer, Fig. 3D,E), haemorrhage (extravasations of red blood cells) and accumulation of inflammatory cells (polymorphonuclear leukocytes) in the CYP-treated rats (Fig. 3D,E) compared with the control rats (Fig. 3A,B). Furthermore, CYP treatment resulted in more EP₄ (Fig. 3F) expression in the bladder mucosa region, submucosal inflammatory cells and macrophages (mononuclear cells with moderate amount of cytoplasm) than in the control rats (Fig. 3C).

DISCUSSION

The present study showed that CYP injection in rats induced modulation of EP receptors expression in the bladder, comprising of an up-regulation of the EP₄ receptor and down-regulation of the EP₁ receptor. Furthermore, treatment with AH23848 significantly increased ICI compared with the pre-drug control value in the CYP-treated group [16], but had no significant effects on the other CMG variables. These results indicate the therapeutic effect of EP₄ receptor antagonist on OAB.

CYP has long been used as a chemical agent to induce cystitis and OAB in rats. The toxic metabolite of CYP, acrolein, is eliminated in the urine, thereafter, stimulating capsaicin-sensitive bladder afferents, and inducing neurogenic inflammation and OAB [11,12]. A previous study reported up-regulation of EP₄ expression in the bladder urothelium and L6 and S1 spinal cord in accordance with up-regulation of cyclooxygenase 2 (COX-2) expression and OAB in a chronic CYP injection model in rats [8]. The increased expression

of bladder COX-2 could lead to increased production of PGE₂, which might activate peripheral EP₄ receptors and produce OAB.

In the present experiments, acute CYP treatment on day 1 induced bladder inflammation and OAB, and increased EP₄ expression on day 3. The EP₁ receptor was down-regulated while there were no significant changes in the expression of the EP₂ and EP₃ receptors. Jiang *et al.* [17] reported over-expression of EP₂ and EP₄ subtypes in the bladder urothelium of human with OAB induced by BOO, while expression of EP₁ and EP₃ were not significantly altered. The difference in obstruction vs chemical irritation models or human vs rat may account for the differences. However, both Jiang *et al.* [17] and the present study showed EP₄ receptor up-regulation.

Furthermore, the present study showed that the EP₄ receptor was up-regulated in the bladder urothelium as well as submucosal inflammatory cells and macrophages, and that the EP₄ antagonist, AH23848, suppressed CYP induced bladder overactivity. The activation of EP receptors can modulate various second messenger signalling pathways. EP₄ activates adenylyl cyclase to increase the level of intracellular cAMP, which might induce excitable effects on the peripheral nerves and change ion channel permeability, resulting in afferent hyperexcitability [18]. Lin *et al.* [9] reported that AH23848 attenuated PGE₂ sensitization of capsaicin currents in dorsal root ganglion culture. Previous studies using a partial sciatic nerve ligation model of rats showed that the EP₄ receptor level was increased in the injured nerves and invading macrophages in association with up-regulation of interleukin 6, and a selective EP₄ antagonist, L-161982, was able to suppress interleukin 6 release. The authors concluded that the EP₄ receptor is the subtype mediating the effects of PGE₂ on macrophage functions [10].

Taken together, up-regulation of EP₄ receptors in the nervous system, bladder urothelium, and immune cells, may be involved in the hyperactive neuroimmune interaction, resulting in neurogenic inflammation and hypersensitivity, which can be suppressed by EP₄ antagonists.

All four EP receptor subtypes were expressed in the control bladder but their physiological effects in normal micturition is unclear. McCafferty *et al.* [19] reported that EP₃ receptor-knockout mice had increased bladder capacity compared with wild-type mice under control conditions. Infusion of the EP₃ receptor agonist, GR63799X, into the bladder of wild-type mice reduced the bladder capacity, which was ineffective in the EP₃ receptor-knockout mice. The data provide evidence supporting a functional role for EP₃ receptors in 'normal' urinary bladder function. In the present study the EP₄ antagonist, AH23848, had no significant effect on the micturition reflex in the control group. The present results were in accordance with a previous study that showed that AH23848 attenuated complete Freund's adjuvant-induced pain hypersensitivity, but produced no change in thermal latency or mechanical withdraw thresholds on the normal side [9]. The characteristic of the EP₄ antagonist, AH23848, which suppressed bladder overactivity without affecting normal physiological function, implies that the EP₄ antagonist, AH23848, may be useful in the control of OAB without compromise of normal micturition.

PGE₂ has been shown to increase the activity of the capsaicin receptor transient receptor potential vanilloid 1 via EP₁ receptor activation in a protein kinase C-dependent manner as well as through protein kinase A via EP₄ [20]. Lee *et al.* [3] also reported that an EP₁ receptor antagonist suppressed intravesical PGE₂ induced detrusor overactivity. It would be interesting to investigate the effect of an EP₄ receptor antagonist on intravesical PGE₂-induced detrusor overactivity in the future. Previous studies have shown that EP₁ knockout or EP₁ antagonists suppressed detrusor overactivity during the storage phase (increased bladder capacity, voiding volume, and voiding interval), but did not mediate important

effects during the voiding phase (without changing the voiding pressure or residual urine volume) in a BOO model [3,21]. The role of PG in the bladder may be to sensitise afferent nerves rather than have a direct contractile effect on detrusor myocytes, and some of these effects may be mediated via the EP₁ receptor [1,3]. In the present study EP₄ receptors, but not EP₁ receptors, were up-regulated, and an EP₄ antagonist increased ICI, but did not affect contraction amplitude. These results suggest that both EP₁ and EP₄ receptors contribute to the sensitization of bladder afferent nerves and overactivity.

PGE₂ has been implicated as an endogenous mediator of bladder function in the normal physiological state and under pathophysiological conditions through activation of different EP receptors at the peripheral level and/or central level. EP₁, EP₃, and EP₄ receptors have been shown to affect the micturition reflex in different animal models and different bladder conditions. The interaction and relationship between these EP receptors are not clear at present. It is possible that in an inflammation-induced OAB model there is a reverse correlation between the function of EP₁ and EP₄ receptors, which is different to a BOO-induced OAB model where EP₁ receptors are increased. Additional studies are needed in different animal models to clarify the real role of EP receptors in bladder function.

In conclusion, up-regulation of EP₄ receptors played a role in CYP-induced OAB, which was suppressed by an EP₄ receptor antagonist. Blockade of the EP₄ receptor mechanism may be a potential treatment for patients with OAB.

Acknowledgments

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Abbreviations

PGE₂	prostaglandin E ₂
OAB	overactive bladder
EP	E-series prostaglandin (receptors)
CYP	cyclophosphamide
CMG	cystometrogram
PE	polyethylene
PT	pressure threshold (pressure immediately before reflex contraction)
PB	pressure baseline (lowest bladder pressure during filling)
ICI	intercontraction interval
COX-2	cyclooxygenase 2

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FIG. 1.

A, Gross view, on day 3, CYP significantly induced swelling and haemorrhage of bladder (left) compared with the control rat (right). **B**, Western blotting for detecting EP receptors expression in the bladder of saline- (right) and CYP- (left) treated rats. CYP induced up-regulation of EP₄ receptor accompanied by down-regulation of EP₁ receptor. However, CYP had no significant effects on EP₂ and EP₃ receptors.

FIG. 2.

Representative traces of *in vivo* continuous CMGs in urethane-anaesthetized rats. CMG was performed in a control rat (**A**) with a normal voiding pattern and in a rat with CYP-induced OAB (**B**) before and after i.v. administration of AH23848 (0.01 and 0.1 mg/kg; i.v).

FIG. 3.

Photomicrographs of bladder sections. Control rat with intact urothelium (**A,B**) and rare EP₄ immunostaining (**C**); CYP-induced inflammatory cells accumulation, oedematous change, haemorrhage of urothelium (**D,E**) and up-regulation of EP₄ expression (**F**) at urothelium (arrow), inflammatory cells (white arrow head), and macrophage (black arrow head). **A, D** ×40; **B, C, E, F** ×200.

TABLE 1

Bladder weight and EP1–4 receptors expression in CYP- and saline-treated (control) rats (four rats in each group; densitometric analysis, ratio to β -actin)

Variable	Group		<i>P</i> *
	Control	CYP-treated	
Mean (SEM):			
Bladder weight mg/100 g bw	42.5 (7.5)	92.5 (4.8)	0.001
EP ₁	0.27 (0.04)	0.13 (0.01)	0.013
EP ₂	0.43 (0.07)	0.36 (0.10)	0.602
EP ₃	0.21 (0.03)	0.15 (0.01)	0.116
EP ₄	0.16 (0.02)	0.32 (0.02)	0.002

* unpaired t-test; bw, body weight.

TABLE 2

Effects of i.v. administration of AH23848 on CMG variables in CYP- and saline-treated (control) rats (six rats in each group)

Variable	Control		CYP-treated		
	Baseline	AH23848, 0.01 mg/kg	AH23848, 0.1 mg/kg	Baseline	AH23848, 0.01 mg/kg AH23848, 0.1 mg/kg
Mean (SEM):					
ICI, min	8.8 (1.3)	9.8 (1.6)	9.5 (1.5)	2.6 [‡] (0.3)	4.1 (0.6) [*] 4.9 (0.9) [‡]
Amplitude, cmH ₂ O	64.0 (13.0)	62.5 (10.6)	72.8 (17.5)	64.0 (11.3)	58.3 (12.7) 57.4 (14.5)
BP, cmH ₂ O	3.1 (0.5)	2.3 (0.4)	3.0 (0.4)	3.8 (0.5)	2.9 (0.3) 3.4 (0.6)
PT, cmH ₂ O	6.5 (0.8)	5.3 (1.2)	5.4 (0.8)	10.9 [§] (1.5)	9.7 (1.3) 9.9 (1.1)

* $P < 0.05$ vs baseline;

[‡] $P < 0.01$ vs baseline, repeated measures anova followed by Tukey's multiple comparison test for intragroup test;

[‡] baseline ICI, control vs CYP-treated, $P = 0.001$, unpaired t-test;

[§] baseline PT, control vs CYP-treated, $P = 0.03$, unpaired t-test.