An excitatory role for peripheral EP<sub>3</sub> receptors in bladder afferent function


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Su X, Lashinger ES, Leon LA, Hoffman BE, Hieble JP, Gardner SD, Fries HE, Edwards RM, Li J, Lapin NJ. An excitatory role for peripheral EP<sub>3</sub> receptors in bladder afferent function. Am J Physiol Renal Physiol 295: F585–F594, 2008. First published June 18, 2008; doi:10.1152/ajprenal.90273.2008.—The excitatory roles of EP<sub>3</sub> receptors at the peripheral afferent nerve innervating the rat urinary bladder have been evaluated by using the selective EP<sub>3</sub> antagonist (2E)-3-{1-[(2,4-dichlorophenyl)methyl]-5-fluoro-3-methyl-1H-indol-7-yl}-N-{(4,5-dichloro-2-thienyl)sulfonyl}-2-propenamide (DG-041). The bladder rhythmic contraction model and a bladder pain model measuring the visceromotor reflex (VMR) to urinary bladder distension (UBD) have been used to evaluate DG-041 in female rats. In addition, male rats [spontaneously hypertensive rat (SHR), Wistar-Kyoto (WKY), and Sprague-Dawley (SD)] were anesthetized with pentobarbital sodium, and primary afferent fibers in the L6 dorsal root were isolated for recording the inhibitory response to UBD following intravenous injection of DG-041. Intravenous injection of DG-041 (10 mg/kg), a peripherally restricted EP<sub>3</sub> receptor antagonist, significantly reduced the frequency of bladder rhythmic contraction and inhibited the VMR response to bladder distension. The magnitude of reduction of the VMR response was not different in the different strains of rats (SD, SHR, and WKY). Furthermore, quantitative characterization of the mechanosensitive properties of bladder afferent nerves in SHR, WKY, and SD rats did not show the SHR to be supersensitive to bladder distension. DG-041 selectively attenuated responses of mechanosensitive afferent nerves to UBD, with strong suppression on the slow-conducting, high-threshold afferent fibers, with equivalent activity in the three strains. We conclude that sensitization of afferent nerve activity was not one of the mechanisms of bladder hypersensitivity in SHR. EP<sub>3</sub> receptors are involved in the regulation of bladder micturition and bladder nociception at the peripheral level.

EP<sub>3</sub>; visceromotor reflex; bladder rhythmic contraction; bladder distension; afferent nerve

SENSATION ASSOCIATED WITH the urinary bladder is conveyed primarily by pelvic and hypogastric nerves, by which the signal is relayed to the central nervous system (CNS). Most afferent fibers innervating the musculature of the bladder body pass through the pelvic nerve, whereas the majority of afferent endings in the bladder submucosa are derived from the hypogastric nerve (41), suggesting that the afferent fibers in the pelvic and hypogastric nerves have different roles, signaling mechanical stimulation (e.g., bladder distension) and chemical stimulation (e.g., inflammation), respectively (20, 22, 30, 34). Bladder distension is a natural mechanical stimulus to evoke sensations such as fullness, urgency, and pain while the literature suggests a complex regulatory role of prostaglandins (PGs) in multiple aspects of urinary bladder physiology/pathophysiology.

PGE<sub>2</sub>, one of the principal PGs, is synthesized in urothelium and detrusor smooth muscle (19, 24, 25, 27) as well as in neurons and glial cells (18, 23) and is released in response to various physiological (e.g., bladder distension) and pathological (e.g., mediators of inflammation) stimulation. PGE<sub>2</sub> interacts with four EP receptor subtypes (EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub>) (23).

PGE<sub>2</sub> has been detected at a high level in the urine of overactive bladder patients and may contribute to the clinical pathophysiology of bladder hypersensitivity (12, 13). Intravesical administration of PGE<sub>2</sub> induces bladder overactivity in animals by activation of sensory bladder afferent neurons (9, 18, 29, 31). Furthermore, the reduction of PG formation by cyclooxygenase inhibitors has demonstrated in vivo efficacy in hyperactive bladder models (1, 8, 11, 39). Based on studies in EP<sub>3</sub> receptor-deficient mice, this subtype has been reported to mediate the inflammatory nociceptive response to peritoneal irritation (42).

The role of EP<sub>3</sub> receptors in the regulation of afferent nerve activity from the urinary bladder has not been studied. To evaluate the peripheral role of EP<sub>3</sub> receptors in bladder afferent functions, a peripherally limited EP<sub>3</sub> receptor antagonist, (2E)-3-{1-[(2,4-dichlorophenyl)methyl]-5-fluoro-3-methyl-1H-indol-7-yl}-N-{(4,5-dichloro-2-thienyl)sulfonyl}-2-propenamide (DG-041; see Ref. 44), was tested on the micturition reflex in a bladder rhythmic contraction model and on bladder pain sensation by measurement of visceromotor reflex (VMR) and cardiovascular (pressor) responses to noxious urinary bladder distension (UBD).

The spontaneously hypertensive rat (SHR) exhibits bladder hypersensitivity (26, 33). One mechanism for this hypersensitivity could be an enhanced afferent limb of the micturition reflex pathway. The mechanism of sensitized afferent nerve activity in the SHR is based on an increased production of nerve growth factor by bladder smooth muscle (2, 33). This induces enlargement of bladder sensory neurons (3) and sensitization of the bladder afferent nerve activity (5). However, an increase in sensitivity of the primary afferent fibers to mechanical stimulation has not been documented in the SHR. Indeed, using the VMR response to noxious UBD as a measure of bladder afferent signal transmission, the SHR did not present a sensitized bladder phenotype (16).

In addition to the physiological examination of the bladder afferent/detrusor function in SHR, pharmacological responses of SHR to tool compounds targeting afferent pathways or detrusor muscle provide an alternative way to study the mechanism of bladder hypersensitivity in the SHR. For example, β<sub>3</sub>-adrenoreceptor activation results in an enhanced relaxation of bladder smooth muscle in the SHR but fails to change VMR.
and pressor responses to UBD, indicating the importance of the altered detrusor/efferent function in the SHR (16). Thus it is interesting to compare bladder afferent activity between SHR and Wistar-Kyoto (WKY) rats and to evaluate the effect of antagonism of EP3 receptors by DG-041 on the VMR response to UBD and the afferent responsiveness in SHR and their nonmotensive controls (WKY).

MATERIALS AND METHODS

The experimental protocols were approved by the Institutional Animal Care and Use Committee of GlaxoSmithKline Pharmaceuticals (King of Prussia, PA).

Gene expression by TaqMan study. Rats were anesthetized initially with 3% isoflurane and killed by exanguination. The dorsal root ganglia (DRGs) at level L2/L3 and T12/L1 and bladders were removed immediately and kept at −80°C. Tissues were homogenized in Trizol Reagent (Invitrogen, Carlsbad, CA) and after phase separation with chloroform. Total RNA was extracted using the RNaseA Mini Kit (Qiagen) following the manufacturer’s instructions. Any genomic DNA contamination was removed using DNase I (Ambion, Austin, TX). RNA samples were judged to be free of genomic DNA by no amplification in a standard TaqMan assay using 10 ng of RNA and β-actin (ACTB) primer/probe oligonucleotides. The RNA was quantified using Ribogreen RNA quantitation reagent (Invitrogen) and converted to cDNA by reverse transcription utilizing the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The equivalent of 10 ng mRNA/well was arrayed into 384-well plates using a Biomek FX robot (Beckman Coulter), and quantitative RT-PCR was carried out using a 7900HT Sequence Detector System (Applied Biosystems) in a 5-μl reaction volume. TaqMan Universal PCR Master Mix (2X Applied Biosystems) and universal PCR conditions recommended by the manufacturer were followed. The mRNA expression of EP3 receptors and three splice variants (EP3A, D14968; EP3v1, NM012704; and EP3C, D16443) were studied. All primers and probes (6-carboxyfluorescein, tetramethyl-6-carboxyrhodamine) are listed in Table 1. To normalize the data, samples were scaled relative to each other by the geometric mean of the set of valid housekeeping genes for that sample. Each data point was then expressed as the ratio of housekeeping gene abundance in the sample to those samples with similar tissue types. The housekeeping genes used were ACTB, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cyclophilin (peptidylpropyl isomerase A), and the data was expressed as the ratio of housekeeping gene abundance in the sample to the average of that in all samples and marked invalid if it had a relative abundance of 30.

Pharmacokinetic studies. At least 3 days before the start of the study, three rats received surgically implanted femoral vein and arterial catheters for infusion of DG-041 (1 mg/kg with a volume of 4 ml/kg in 16% Caviron and 3.2% dimethyl sulfoxide, over a 30-min infusion) and blood sampling. Blood (0.25 ml) was drawn at predetermined time points, up to 2 h, postdosing in lithium heparin-containing tubes. Plasma was separated by centrifugation and stored at −20°C before mass spectral analysis.

The rat brain-to-blood ratio of DG-041 was also studied. Rats were anesthetized with isoflurane (3%) and dosed with DG-041 (10 mg/kg iv). Arterial blood (0.4 ml) was drawn every 15 min postdose in lithium heparin-containing tubes. Rats were then killed by exanguination. The rat brain was removed and placed in a preweighed container for homogenization, followed by the addition of 2 ml/g acetonitrile to obtain a threefold final dilution. The brain tissues were then homogenized with a Polytron homogenizer and centrifuged. The supernatants were transferred and stored at −20°C before analysis.

Quantification of DG-041 was performed using liquid chromatography/tandem mass spectrometric (LC/MS/MS) detection. Samples were thawed, plasma proteins were precipitated with 200 μl of 95:5 acetonitrile-0.1% formic acid in water, containing an appropriate mass spectral internal standard, and the resulting mixture was vortex mixed for 2 min followed by centrifugation for 30 min at >2,000 g. With the use of a sensitive and selective LC/MS/MS method on an HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled to a Sciex APISQ0500 triple-quadrupole mass spectrometer (Applied Biosystems), samples were analyzed for quantitative concentrations of DG-04. Analytical standards (1–2,500 ng/ml) were prepared in rat plasma or brain homogenate to ensure accurate calibration of the mass spectrometer for each biological matrix.

In vivo bladder rhythm contraction. Female Sprague-Dawley (SD) rats weighing 200–300 g (n = 17) were anesthetized with 3% isoflurane during surgery. Anesthetized rats were maintained with a hot water-circulating heating pad underneath during the studies and were killed upon completion of experimental procedures by an intravenous overdose of pentobarbital sodium (120 mg/kg; Vortech Pharmaceuticals, Dearborn, MI).

One jugular vein was cannulated with polyethylene tubing for intravenous administration of urethane and DG-041. A cannula (PE-50) was placed in the bladder via the urethra, and the urethra was ligated to ensure an isovolumetric bladder. Upon completion of surgical procedures, slow intravenous infusion of urethane (1.2 g/kg, ethyl carbonate; Sigma, St. Louis, MO) was given over 15 min. Saline bladder infusion procedures were begun 30 min after the final dose of urethane.

The urethral cannula was connected with a T connector and linked with a low-volume transducer (model MLT0380D; ADInstruments, Colorado Springs, CO). The signal was amplified through a direct current (DC) amplifier (ADInstruments, ML119). The other end of the T connector was linked to a 20-ml syringe with a perfusion pump. For the pharmacological study on bladder rhythmic contraction, the saline infusion in bladder was at a rate of 50 μl/min to induce miciturition reflex (here defined as bladder contraction with intensity <10 mmHg). The infusion rate was then lowered to 10 μl/min until three to five rhythmic bladder contractions were established; the infusion was then terminated. The vehicle or test compound was administered after a 15-min control period. Following administration, bladder rhythmic contractions were recorded for 20–30 min. Two parameters of the bladder rhythmic contraction were evaluated (frequency/interval and amplitude). The mean controls were calculated by the average of readouts during the last 5-min interval of the control period. The

### Table 1. Primers of Taqman study

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>EP3</td>
<td>CAACTGCGGACGACATCAAGG</td>
<td>AAGCTCGGATACGCGGTTCGCC</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>EP3v1</td>
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<td>CGCTTTGTTGTTCTGATCTCGTGCT</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>EP3A</td>
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<td>CGCTTTGTTGTTCTGATCTCGTGCT</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>EP3C</td>
<td>TGCGCACTGCAACCTGCTGCCT</td>
<td>CCGTCTCTTCTTCTTCTTCGG</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>ACTB</td>
<td>CACATGAGACTGTCGGCCGCCT</td>
<td>CTGGTCTCTTCTTCTTCTTCGG</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>AAGCTCGGATACGCGGTTCGCC</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
</tr>
<tr>
<td>PPIA</td>
<td>TTTATCGTACCTGCAAGGACCTGA</td>
<td>CCAACATGAGACTGTCGGCCCT</td>
<td>FAM-CTGCCAGCTGCGGCCTAGC</td>
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GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
effects of compounds were calculated by the mean response in every 5-min period after injection (16). In vivo VMR and pressor responses to urinary bladder distension. Female rats (SD, n = 23; SHR, n = 13; WKY, n = 11) were anesthetized initially with 3% isoflurane. Cannulas for intravascular administration and for bladder distension were implanted as described above. To measure blood pressure, the right carotid artery was catheterized with PE-50 tubing. The arterial catheter was linked with a low-volume transducer (ADInstrument; MLT0380D), and signal was amplified through a DC amplifier (ADInstrument; ML119). The bladder catheter was also linked to a bladder distension control device. The bladder was distended with saline by regulating air inflow into a Mariott bottle from a valve interface distension control device (University of Iowa, Bioengineering, B482C-1; see Ref. 37). Two needle electrodes were sutured in the oblique abdominal musculature just above the inguinal ligament. Abdominal contractions were quantified by action potentials of electromyographic activity. Action potentials were initially amplified through a low-noise alternating current (AC) differential amplifier (ADInstrument; EC100D), processed using the AD data acquisition program (PowerLab 16/30, ML880). Raw action potentials of myoelectric activities, bladder pressure, and blood pressure were displayed on-line continuously. All data were analyzed off-line using the ADInstrument power lab program (Chart 8).

Following completion of the surgical preparation, isoflurane anesthesia was reduced until flexion reflex response could be evoked by pinch of the foot without spontaneous escape behaviors (~1% isoflurane). For UBD, all rats received a series of at least six phasic bladder distensions at 60 mmHg for 30 s at 3-min intervals to evaluate response stability to repeated bladder distension. The compound or vehicle was administered only after four consistent responses were elicited and given 2 min before the onset of distension.

The electromyographic activity was integrated and calculated as the area under the curve. The VMR response to the stimulus was defined as the increase in electromyographic activity during UBD from the baseline activity before each response. Pressor response was quantified as the peak change in mean arterial pressure during UBD compared with the average level during a baseline period immediately before UBD. Following compound administration, response was normalized to the percentile of mean control response: the average of four UBD responses before compound treatment.

Afferent nerve recording. Male rats weighing 400–500 g (16 SD, 12 SHR, and 10 WKY) were used for this study. Rats were anesthetized initially with pentobarbital sodium (Nembutal) at a dose of 40–45 mg/kg ip and maintained thereafter with supplementary intravenous doses of pentobarbital sodium (5–10 mg·kg⁻¹·h⁻¹). The trachea was cannulated to provide artificial ventilation with room air. For injecting drugs, a catheter was passed to the descending aorta via the left common carotid artery. The femoral artery and vein were cannulated to provide arterial pressure and administration of pentobarbital sodium, respectively.

The lower abdomen was exposed by a 3- to 4-cm-long incision laterally at the left flank. The urinary bladder was emptied and catheterized (PE-100) through the fundus. As described above, for UBD, the bladder catheter was connected to a saline bottle from a distension control device. The urethra was ligated close to its entry to the penis, and urine was evacuated constantly via the fundic catheter. The left testis, vas deferens, and seminal vesicle were tied and isolated from the penis, and urine was evacuated constantly via the fundic catheter. The urethra was ligated close to its entry to the bladder. Action potentials of electromyographic activity. Action potentials were initially amplified through a low-noise alternating current (AC) differential amplifier (ADInstrument; EC100D), processed using the AD data acquisition program (PowerLab 16/30, ML880). Raw action potentials of myoelectric activities, bladder pressure, and blood pressure were displayed on-line continuously. All data were analyzed off-line using the ADInstrument power lab program (Chart 8).

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The left L6 dorsal root was identified and decentralized at its entry to the spinal cord. Recordings were made from the distal cut end of the central processes of dorsal root fibers. A length of nerve fiber was draped over a black microbase plate immersed in warm mineral oil. The dorsal root then was split into thin bundles, and a fine filament was teased from the bundle to obtain a single unit. Electrical activity of the single unit was recorded monopolarly by placing the teased fiber over one arm of a bipolar silver-silver chloride electrode; a fine strand of connective tissue was placed across the other pole of the electrode. Action potentials were monitored continuously by analog delay and displayed on a storage oscilloscope after initial amplification through a low-noise AC differential amplifier (Neurolog NL104; Digitimer). The bandpass filter was set between 0.5 Hz and 5 kHz. Action potentials were processed through a window discriminator and counted (1-s bin width) on-line using the spike2/CED 1401 program (CED, Cambridge, UK). Peristimulus time histograms, urinary bladder, and blood pressure were displayed on-line.

Pelvic nerve input to the L6 dorsal root was identified first by electrical stimulation of the pelvic nerve (10.5-ms square wave pulse at 3–8 mA). Fibers were classified on the basis of their conduction velocities; those with conduction velocities <2.5 m/s were considered unmyelinated C-fibers, and those with conduction velocities >2.5 m/s were considered thinly myelinated A-fibers. The organ innervated was identified by response to brief phasic UBD (60 mmHg, 2–3 s). If a fiber responded to UBD, a stimulus–response function (SRF) to phasic isometric distending pressures of 5, 10, 20, 30, 40, 60, and 80 mmHg, 30 s each at 3-min intervals, was determined. The effects of DG-041 or mexiletine were tested on responses of UBD-sensitive afferent fibers (60 mmHg UBD), by intravenous bolus injection followed by 0.1 ml saline, 2 min before bladder distension. DG-041 was administered intravenously at 10 mg/kg in 30 μl. Mexiletine was injected in a cumulative dosing paradigm. In some experiments, mexiletine was injected 1 h after testing DG-041 if DG-041 failed to change the afferent response.

The resting activity of a fiber was counted for 60 s before distension, and the response to a stimulus was determined as the increase in discharge during distension (30 s) over its resting activity. A SRF to graded UBD was plotted for each individual unit, and a least-squares regression line was obtained from the linear part of the SRF. The regression line then was extrapolated to the ordinate (representing distension pressure) to estimate the response threshold.

Data analysis. All data were expressed as means ± SE. Results were analyzed with Student’s t-test or ANOVA with repeated measures by Prism 4 (GraphPad Software, San Diego, CA). A value of P < 0.05 was considered statistically significant.

Compounds. DG-041 (mol wt 592.326) was synthesized by the Department of Medicinal Chemistry, GlaxoSmithKline. For intravenous administration, DG-041 was dissolved in 30 μl polyethylene glycol (PEG)-400. Mexiletine hydrochloride (mol wt 215.72) was purchased from Sigma-Aldrich and dissolved in saline.

RESULTS

Gene expression by quantitative PCR. The three housekeeping genes (ACTB, GAPDH, and cyclophilin) were expressed in a stable manner to each other across all of the samples, allowing for proper normalization. The localization of mRNA of EP3 receptors in rat bladder and DRG normalized to the mean of housekeeping genes is shown in Fig. 1. The mRNA expression levels of EP3α and EP3β were high in bladder, and mRNA of EP3C was high in DRG and brain. A similar expression pattern was observed in SHR and WKY rats (data not shown). Overall, the EP3 receptors were abundant in all tissues, bladder, and neurons.
Pharmacokinetic studies. DG-041 has pharmacokinetics suitable for in vivo studies in rats. Following intravenous infusion of 1 mg/kg DG-041 over 30 min (n = 3), the maximal concentration was 957 ± 164 ng/ml, the half-time for the elimination phase was 103 ± 26 min, and systemic plasma clearance is 30.4 ± 4.5 ml·min⁻¹·kg⁻¹. DG-041 had a volume of distribution approximately equal to total body water, 0.78 ± 0.29 l/kg. In another group of rats, at 15 min post-10 mg/kg bolus injection (n = 4), the plasma level was 4.39 ± 1.22 μg/ml. At the same time point, the brain level of DG-041 was much lower than blood levels, yielding a brain-to-blood ratio of 0.027 ± 0.004 (n = 4).

Effect of intravenous administration of DG-041 on the micturition reflex. Intravenous administrations of vehicle (PEG-400) did not produce significant changes in bladder rhythmic contractions. The administration of DG-041 produced an inhibition of the bladder rhythmic contraction, reducing frequency or eliminating the contraction (“disappearance” of contractions). The disappearing intervals were 9.03 ± 1.28 min (5 out of 6 rats) and 9.72 ± 1.89 min (4 out of 5 rats) at 3 and 10 mg/kg, respectively.

If the contractions were not abolished, their amplitude was not reduced by DG-041. Figure 2, C and D, summarizes the effect of DG-041 on bladder rhythmic contraction following intravenous administration.

Effect of intravenous administration of DG-041 on nociceptive responses to bladder distension. Figure 3, A and B, demonstrates typical examples of intravenous administration of vehicle and DG-041 on both VMR and pressor responses to noxious UBD at 60 mmHg. Intravenous administration of DG-041 produced a significant inhibition of both VMR and pressor responses to UBD. The maximal inhibition occurred at 2 and 5 min postinjection. Figure 3, C and D, summarizes the effects of DG-041 on the VMR response and the pressor response to UBD following intravenous injection.

The inhibition by DG-041 (10 mg/kg iv) on the VMR response to UBD was compared in three strains of rats (SD, SHR, and WKY). DG-041 produced an equal degree of inhibition on the mean VMR response 5 min postinjection (Fig. 4A).

In another group of SHR, responses to three steps of UBD were evaluated in a staircase matter before and after DG-041 (10 mg/kg iv). DG-041 tended to produce stronger inhibition of the response to 60 mmHg UBD than that to 40 mmHg UBD (Fig. 4B).

Effect of DG-041 on response of afferent nerve to bladder distension. A total of 44 mechanosensitive pelvic nerve afferent fibers in the L6 dorsal root were identified by electrical stimulation of the pelvic nerve and UBD (16 from SHR, 12 from WKY, and 16 from SD). The proportions of myelinated
and unmyelinated afferent fibers in SHR, WKY, and SD rats were similar (Table 2). Most of the afferent nerves exhibited an ongoing discharge as we have previously described (30, 34) with similar mean frequencies in three strains. All fibers were characterized for responses to graded intensities of UBD (5–80 mmHg) and exhibited an increased response to increasing pressures of UBD. The response patterns of afferent fibers from SHR were significantly different from those of WKY rats...
Fig. 4. Effect of iv administration of vehicle (PEG-400) and DG-041 on the VMR response to UBD (30 s in 3 min) in anesthetized rats. A: effect of DG-041 on the mean VMR response 5 min postinjection to UBD (60 mmHg) in Sprague-Dawley (SD), spontaneously hypertensive rats (SHR), and Wistar-Kyoto (WKY) rats. The responses are represented as %control, where the baseline response before administration of drugs is defined as 100%. B: responses of the VMR to UBD at 20, 40, and 60 mmHg before and after iv administration of vehicle and DG-041. *P < 0.05 and **P < 0.001. The significance of difference between vehicle and DG-041 value was determined by nonpaired Student’s t-test.

(P < 0.05), but not from SD rats. Afferent fibers from WKY rats exhibited a relatively low mean response threshold and high response magnitude. The mean SRFs are plotted in Fig. 5A. The linear regression analysis of response threshold to the conduction velocity (CV) of afferent fibers did not reveal a correlation between those two parameters (Fig. 5B).

The effects of a bolus injection of vehicle (PEG-400; n = 8, SHR; n = 4, WKY; n = 12, SD) or and DG-041 (10 mg/kg; n = 10, SHR; n = 9, WKY; n = 9, SD) were tested on the responses to noxious UBD (60 mmHg, 30 s). Figure 6 illustrates responses of a C afferent nerve (CV: 1.63 m/s, response threshold: 26 mmHg) before and after receiving DG-041. Vehicle solution did not attenuate the response of this afferent nerve. However, DG-041 produced an immediate reduction of response following injection. The inhibition lasted for >20 min. However, DG-041-induced inhibition was not observed in some other afferent nerves and was not significantly different with the vehicle control in each group, probably due to variability in sensitivity to inhibition. We found no apparent difference in drug effects on afferent fibers from SHR, WKY, or SD rats. Thus the data from the three groups were pooled. An unpaired t-test analysis reveals a significant different response following vehicle and DG-041 application (P = 0.04) in the pooled data group (Fig. 7A). Interestingly, the magnitude of the inhibition correlated well with response threshold and conduction velocities of afferent fibers. The

Table 2. Summary and comparison of mechanosensitive pelvic afferent fibers in SHR, WKY, and SD rats

<table>
<thead>
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<th>SHR</th>
<th>WKY</th>
<th>SD</th>
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<tbody>
<tr>
<td>C-fiber CV, m/s</td>
<td>1.60±0.14 (67)</td>
<td>1.77±0.19 (75)</td>
<td>1.61±0.17 (66)</td>
</tr>
<tr>
<td>Aδ-fiber CV, m/s</td>
<td>5.48±1.71 (33)</td>
<td>3.93±0.51 (25)</td>
<td>4.68±0.97 (33)</td>
</tr>
<tr>
<td>Threshold, mmHg</td>
<td>9.39±1.90</td>
<td>4.01±1.21</td>
<td>8.17±1.16</td>
</tr>
<tr>
<td>Resting firing, impulses/s</td>
<td>0.55±0.22 (25)</td>
<td>0.88±0.31 (17)</td>
<td>1.08±0.37 (13)</td>
</tr>
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</table>

Values are reported as means ± SE. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto; SD, Sprague-Dawley; CV, conduction velocity. For C-fiber and Aδ-fiber, values in parentheses are %. For resting firing, nos. in parentheses indicate % silent.

Mexiletine dose-dependently attenuated responses of mechanosensitive afferent fibers to noxious UBD. A total of 19 fibers was studied from SD, SHR, and WKY rats; 5 were A6 and 14 were C-fibers. We found no apparent difference in drug effects on any of these subgroups. The mean ID50 values in SD, SHR, and WKY rats were 9.24 ± 1.46 (6.31–12.17) mg/kg, 7.38 ± 2.37 (1.62–13.14) mg/kg, and 9.53 ± 1.32 (6.81–12.26) mg/kg, respectively. There were no differences in the slopes of the dose-response functions, being −0.13 ± 0.04, −0.14 ± 0.07, and −0.12 ± 0.07, respectively. Figure 8 summarizes the dose-response functions of mexiletine on responses of afferent nerve innervating bladders from SD, SHR, and WKY rats.

DISCUSSION

The present study evaluated the role of peripheral EP3 receptors on bladder afferent function in the rat. Systemic administration of the peripherally limited EP3 receptor antagonist DG-041 inhibited volume-induced rhythmic bladder contraction and the VMR response to bladder distension. The ability of DG-041 to inhibit bladder nociception was confirmed by directly recording afferent nerve activity. Responses of mechanosensitive bladder afferent nerves to UBD were inhibited, with strong suppression on the slow-conducting, high-threshold afferent fibers. In addition, we quantitatively characterized the mechanosensitive properties of bladder afferent nerves in SHR, WKY, and SD rats. Compared with the other rat strains, the mechanosensitive afferent nerves of SHR were not more sensitive to bladder distension, nor were they more sensitive to inhibition by DG-041. This argues against afferent sensitization as a mechanism for bladder hyperactivity in the SHR.
The pelvic afferent nerve was recorded from the L₆ dorsal root, since 84% of the pelvic afferent fibers from the lower urinary tract enter the spinal cord via the L₆ dorsal root and a smaller fraction enters in S₁ (43). The present study shows that the mechanosensitive pelvic afferent fibers in the L₆ dorsal root from SHR, WKY, and SD rats have the proportion of unmyelinated afferent fibers and exhibit ongoing resting activity, similar to observations in SD rats (30, 34, 43). We defined the response threshold by extrapolation but did not separate to the “high threshold” or “low threshold” afferents due to small sample size and the lack of clear criteria to differentiate the groups. As we reported previously in SD rats (32), response thresholds of the afferent fibers did not correlate with conduction velocities. Unexpectedly, afferents from WKY rats had a lower response threshold and a higher response magnitude. In contrast, the SRFs of mechanosensitive afferent nerves were

Fig. 5. Stimulus-response functions of pelvic nerve afferent fibers to graded intensities of UBD (5–80 mmHg, 30 s in 3 min, A). The response threshold and the conduction velocity of afferent fibers did not correlate (B).

Fig. 6. Examples of effects of vehicle (PEG-400) and DG-041 (10 mg/kg iv) on responses of a pelvic nerve afferent fiber to UBD (60 mmHg, 30 s in 3 min). A: responses of the fiber are illustrated as peristimulus time histograms in 0.01-s bandwidth before and after DG-041 (shadow area). Phasic distending pressure is presented below. B: time course for the effect of iv administration of vehicle and DG-041 on responses of the fiber to UBD.
not enhanced in SHR compared with SD rats. This is consistent with our previous study where a sensitized afferent signal was not detected in SHR as measured by the VMR response to bladder distension (16). In addition, inhibition by DG-041 of the VMR response and afferent response to UBD in SHR was not enhanced. The SHR detrusor showed decreased compliance and was more sensitive to inhibition by β3-adrenoceptor activation (16). If an afferent mechanism is involved in the hypersensitivity in SHR, mechanisms targeting afferent nerves, like an EP3 receptor antagonist, would be expected to be more effective against the responses to UBD, although the pathological mechanism of hypersensitivity is not necessarily linked to an altered pharmacology. However, DG-041 did not produce an enhanced inhibition on bladder nociception in the SHR. Thus both physiological and pharmacological arguments do not support afferent sensitization in the SHR.

DG-041 (10 mg/kg iv) inhibited the bladder rhythmic contraction frequency and often temporarily eliminated the rhythmic voiding contractions. Compounds targeting the bladder afferent pathway often produce this effect (6, 7, 17, 40). “Shutdown” of bladder rhythmic contraction reflects an increased threshold for induction of contractions and an increased bladder capacity in the conscious cystometrographic study (17). Because DG-041 is unlikely to achieve effective concentrations in the CNS following systemic administration, its effects are probably due to the inhibition of EP3 receptors located on peripheral neurons or detrusor muscle. DG-041 failed to change the amplitude of the rhythmic bladder contractions; thus, detrusor functions might not be modulated by an EP3 receptor antagonist. On the other hand, we found mRNA for the EP3A and EP3v1 splice variants to be highly expressed in bladder, either detrusor muscle or urothelium. Unfortunately, DG-041 does not differentiate different EP3

Fig. 7. Inhibition by DG-041 on responses of all afferent fibers from SD, SHR, and WKY rats to UBD (60 mmHg, 30 s in 3 min). A: mean inhibition of responses to UBD (as % control) by DG-041 was significantly different from that by vehicle (P = 0.04, nonpaired Student’s t-test). The inhibition correlated well with response thresholds (B) and conduction velocities (C) of afferent fibers but was not dependent on the control response frequencies (D).

Fig. 8. Dose-dependent inhibition by sodium channel blocker mexiletine on responses of pelvic nerve afferent fibers to UBD (60 mmHg, 30 s in 3 min). Mean inhibition of responses to UBD are expressed as % control. There were no apparent differences in effects of DG-041 in SD, SHR, and WKY rats.
splice variants (X. Su, C. W. Wu, D. M. Morrow, and J. P. Jaworski, unpublished observations). Thus inhibition of detrusor muscle contraction cannot be conclusively ruled out. A more selective subtype antagonist could be useful for further clarifying the mechanism of EP3 receptors in bladder function.

The action of an EP3 receptor antagonist on bladder afferent nerves was demonstrated by the inhibitory effect of DG-041 on the VMR response to bladder distension and is consistent with the high expression of EP3 receptor mRNA in primary sensory neurons. The effect of DG-041 on bladder compliance was not measured, but such an effect would not contribute to the antinociceptive activities, since we have demonstrated that an increase in bladder compliance did not change the readout of VMR and pressor responses (16). Thus the antinociceptive effect of DG-041 is predominantly due to inhibition of receptors located on peripheral neurons. It should be noted that the voiding-associated abdominal wall response (4) is triggered by urethra afferent nerve for the physiological voiding (32). It differs from the passive VMR response to noxious bladder distension (60 mmHg), which corresponds to pain sensation. Whether the voiding-associated VMR response contaminates the nociceptive VMR response to 60 mmHg bladder distension should be further investigated.

To further test the interpretations drawn from the VMR experiments, direct effects of DG-041 on the afferent nerve firing were examined. As expected, DG-041 inhibited the response of afferent fibers to bladder distension. This could result from an action at the sensory endings, axon or cell bodies, where the mRNA for EP3 receptors has been detected. The beneficial effects of EP3 antagonism for the inhibition of bladder afferent nerve activity concur with previous studies of hyperalgesia and allodynia associated with the cutaneous pain bodies, where the mRNA for EP3 receptors has been detected. It is known that intravesical administration of PGE2 enhances the micturition reflex (9) and that this effect is mediated in part by lowering the threshold of capsaicin-sensitive bladder afferent nerves (18). PGE2 has been reported to sensitize peripheral terminals of small-diameter, high-threshold, primary afferent fibers to thermal, chemical, and mechanical stimuli (14, 15, 21, 28). In the present study, we have demonstrated for the first time that an antagonist, DG-041, attenuated the response of afferent nerves to bladder distension, selectively on high-threshold and low-conductivity afferent nerves. In contrast, a nonselective sodium channel blocker inhibited responses of all pelvic afferent fibers to vesical organ distensions to the same degree. The nonselective inhibition of afferent activity by sodium channel blockade was reported in our previous studies (35, 36). Accordingly, the inhibition of afferent nerves innervating the urinary bladder by an EP3 antagonist may be more selective for nociceptors and therefore safer for therapeutic intervention. Whether the sites of action of DG-041 are on myenteric and submucosal plexus neurons or on interstitial cells with their associated visceral sensation requires further investigation. Both studies, the rhythmic contraction and the VMR response, require bladder overdistension. Further investigation of EP3 antagonists in conscious cystometry will highlight the role of EP3 receptors in basal urodynamics under conditions of physiology or pathology of bladder overactivity.

In summary, we found that mechanosensitive afferent hyperactivity is not the mechanism of the hypersensitive bladder in SHR. We also have demonstrated for the first time that EP3 receptors are involved in the regulation of the micturition reflex and bladder nociception by an action on afferent nerves and hence added further evidence for the potential usefulness of EP antagonists in the treatment of urgency or pain associated with bladder dysfunction.

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REFERENCES


