Mechanisms underlying the effects of propiverine on bladder activity in rats with pelvic venous congestion and urinary frequency

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ABSTRACT

We investigated the mechanisms by which propiverine hydrochloride influenced bladder activity in rats with pelvic venous congestion (PC) and urinary frequency. To create PC rats, female rats were anesthetized with isoflurane and the bilateral common iliac veins and bilateral uterine veins were ligated. At 4 weeks after ligation, we assessed voiding behaviour, locomotor activity, and urinary 8-hydroxydeoxyguanosine (8-OHdG) and nitric oxide metabolites (NOx). We also performed cystometry and measured mRNAs for nitric oxide synthase (NOS) and several receptors in the bladder wall. PC rats showed a decrease in locomotor activity and an increased frequency of urination. There was a decrease in endothelial NOS (eNOS), M3, and TRPV1 mRNA expression in the bladder wall, as well as an increase in inducible NOS (iNOS) mRNA. Administration of propiverine to PC rats increased locomotor activity to the level in sham rats, improved bladder function, decreased urinary 8-OHdG excretion, and increased urinary NOx excretion. In addition, propiverine increased neuronal NOS (nNOS) mRNA expression, and decreased expression of iNOS, M3 and TRPV1 mRNA in the bladder wall. Therefore, propiverine not only improved bladder dysfunction through its previously reported actions (anti-muscarinic effect, Ca antagonist effect, and inhibition of noradrenaline re-uptake), but also by reducing inflammation.

Unilateral or bilateral ovarian vein incompetence can cause pelvic congestion syndrome, which includes chronic pain, irritable bladder, pelvic organ dysfunction, vulval varices, and lower limb varicose veins (3, 5, 7). Our previous study demonstrated that chronic prostatitis and stress incontinence were related to vena cava reflux caused by tricuspid regurgitation (10). When we performed external iliac venography in a woman with pelvic congestion syndrome, we found that pelvic venous congestion was associated with tricuspid regurgitation and absence of pelvic venous valves (11). Our previous studies have also demonstrated that ligation of the bilateral common iliac veins in male rats or ligation of the bilateral common iliac veins and uterine veins in female rats induces changes of urinary frequency and locomotor activity (12–15). In these rat models of venous ligation, bladder blood flow is decreased by about 80% compared with that in intact rats (15). Therefore, pelvic venous congestion may be one of the causes of pelvic ischemia, and might be related to lower urinary tract symptoms/diseases.

Propiverine hydrochloride (propiverine) is an anticholinergic agent and Ca antagonist that is indicated for treatment of urinary urgency and/or urge incontinence due to involuntary bladder contractions (overactive bladder) (17). Since propiverine also increases the maximum urethral closing pressure by inhibiting noradrenaline re-uptake, it is effective for stress incontinence as well (8, 16). Therefore, we...
investigated the mechanisms by which propiverine influences bladder activity in rats with urinary frequency due to pelvic venous congestion.

MATERIALS AND METHODS

Animals. A total of 48 female Sprague-Dawley rats weighing 200–230 g were used. To create the pelvic venous congestion model (PC rats), 32 rats were anesthetized with 2% isoflurane. An incision was made in the lower abdomen and the bilateral common iliac veins were ligated with metal clips, after which the bilateral uterine veins were ligated en bloc with the uterine arteries and uterine horn at a site near the ovaries. Following ligation of these veins, dilation of the distal common iliac veins was confirmed. After closing the abdomen, all animals were given a subcutaneous injection of ampicillin (30 mg). The 32 PC rats were randomized to a PC group and a PC/propiverine group (n = 16 per group). For the sham group, 16 additional rats were anesthetized with isoflurane and the bilateral common iliac veins were dissected free of the common iliac arteries. The rats were administered 1 mL of distilled water (sham and PC groups) or propiverine dissolved in distilled water (5 mg/mL, PC/propiverine group) by gavage using a fine catheter once daily for 15 days from 2 weeks after surgery. Rats were allowed free access to standard diet and tap water during the treatment period.

At 4 weeks after surgery, the 3 groups underwent the different experimental protocols (described below) in turn and the data obtained were compared among the groups. This study protocol was approved by the President of the University of the Ryukyus based on the judgment of our institutional Animal Care and Use Committee.

Assessment of voiding behaviour. Voiding behaviour was assessed in 8 rats from each group. The rats were placed in a metabolic cage for 24 h at 23°C with free access to standard rat chow and water. The integrating urine weight was recorded by a computer at 1-minute intervals during assessment. Individual voiding volumes and voiding times were investigated during the 12-h dark period from 8:00 p.m. to 8:00 a.m., the 12-h light period from 8:00 a.m. to 8:00 p.m., and the total 24-h period.

Assessment of locomotor activity. After assessment of voiding behaviour, locomotor activity was investigated as a surrogate marker of pelvic pain (14). Rats were housed individually in plastic cages with woodchip bedding, food, and water from 8:00 a.m. Locomotor activity was measured by using a digital counter and an infrared sensor (NS-ASS01; Neuro-science, Inc., Tokyo, Japan). Room lights were on from 8:00 a.m. to 8:00 p.m. Therefore, locomotor activity during the dark period when rats are active was calculated as the sum of all movements from 8:00 p.m. to 8:00 a.m. (the period least affected by switching the lights on and off).

Measurement of urinary 8-hydroxydeoxyguanosine (8-OHdG) and nitric oxide metabolites (NOx). Following assessment of locomotor activity, spontaneously voided urine was collected from each rat for measurement of 8-OHdG (a marker of oxidative stress) (6), nitrite and nitrate (NOx, a smooth muscle relaxant), and creatinine. Measurement of 8-OHdG and creatinine was performed by Cosmobio Co. Ltd. (Tokyo, Japan). After urine samples were deproteinized with an equal volume of methanol, NOx was measured by the Griess method using a high-performance liquid chromatography system with an automated NO detector (ENO-20; Eicom, Kyoto, Japan) (18).

Continuous cystometry. After collection of spontaneously voided urine, each rat was anesthetized with urethane (0.6 g/kg subcutaneously) and placed in a restraining cage (NAIGAI-CFK-1P; NMS, Tokyo, Japan). Then a polyethylene catheter (PE50; Clay Adams, USA) was inserted transurethrally into the bladder. This catheter was connected to an infusion pump and pressure transducer via polyethylene tubing and was used to fill the bladder with physiological saline at a rate of 0.05 mL/min. Continuous cystometry was performed for at least 90 min and bladder activity was recorded.

Measurement of bladder wall expression of receptor mRNAs. In the remaining 24 rats (n = 8 per group), under deep anesthesia with 4% isoflurane, the bladders were harvested and cut into halves longitudinally, with one half being used for mRNA analysis. Expression of mRNAs for the following genes was examined: eNOS, nNOS, iNOS, beta3 adrenoceptor (ADRB3), muscarinic acetylcholine receptor3 (M3), nicotinic acetylcholine receptor (NicotR), vanilloid receptor subtype 1 (TRPV1), and purinergic receptor (P2X3). Total RNA was extracted from bladder tissue with an RNasy Mini Kit (Qiagen, Hilden, Germany). After treatment with DNase I to avoid genomic contamination, 1 μg of RNA was used for synthesis of cDNA with SuperScript™ II Reverse
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Locomotor activity
During the dark period from 8:00 p.m. to 8:00 a.m., locomotor activity was significantly lower in the PC group than in the sham group (13,253 ± 904 movements vs. 16,824 ± 1,413 movements, *P* = 0.045) (Fig. 2). There were no significant differences in locomotor activity between the sham group and the PC/propiverine group (14,921 ± 980 movements), or between the PC group and the PC/propiverine group. These findings suggested that PC rats had pain and/or discomfort, and these symptoms were not completely controlled by propiverine.

Transcriptase (Thermo Fisher Scientific). Relative expression of the target genes was analyzed by qRT-PCR using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus; Takara, Japan) and the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). PCR involved incubation at 95°C for 30 s for initial denaturation, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Target gene expression was determined relative to the geometric mean level of expression of β-actin.

Statistical analysis. Results are reported as the mean ± standard error of the mean (SEM). Student’s *t*-test for unpaired data was used as appropriate, with *P* < 0.05 being considered to indicate statistical significance.

RESULTS

Voiding behaviour
There were no significant differences in the total voided volume over 24 h among the three groups. There were also no significant differences in the frequency of urination during the light period or the dark period. However, the frequency of urination over 24 h (light period + dark period) was significantly higher in the PC group (29 ± 3 times) than in the sham group (22 ± 3 times, *P* = 0.039) or the PC/propiverine group (22 ± 3 times, *P* = 0.027) (Fig. 1). In addition, the single voided volume over 24 h was significantly larger in the PC/propiverine group than in the PC group (1.28 ± 0.32 mL vs. 0.77 ± 0.12 mL, *P* = 0.038). Thus, the PC group displayed urinary frequency, and propiverine improved frequency by increasing the single voided volume.

Locomotor activity
Fig. 2 Locomotor activity during the dark period (8:00 p.m. to 8:00 a.m.) in the sham, PC, and PC/propiverine groups. Mean ± SEM.

Fig. 1 Voiding behavior (mean single voided urine volume [left], total voided urine volume [center], and frequency of micturition [right]) during the light period and the dark period (24 h) in the sham, PC, and PC/propiverine groups. Mean ± SEM.
Urinary 8-OHdG and NOx

The urinary 8-OHdG level corrected for creatinine (urinary 8-OHdG/cre ratio) was higher in the PC group than in the sham group (32.2 ± 7.2 ng/mg cre vs. 27.8 ± 5.1 ng/mg cre), but the difference was not significant. However, the urinary 8-OHdG/cre ratio of the PC/propiverine group (16.7 ± 2.1 ng/mg cre) was significantly lower than that of the sham group (P = 0.041) or the PC group (P = 0.037) (Fig. 3), suggesting that oxidative stress was decreased by propiverine.

The urinary NOx level corrected for creatinine (urinary NOx/cre ratio) was lower in the PC group than in the sham group (29.7 ± 2.4 μM/mg cre vs. 34.5 ± 4.8 μM/mg cre), although there was no significant difference. However, the urinary NOx/cre ratio of the PC/propiverine group (40.2 ± 4.3 μM/mg cre) was significantly higher than that of the PC group (P = 0.028) (Fig. 3), suggesting that production of NOx in the bladder wall was increased by propiverine.

Continuous cystometry

The interval between bladder contractions was significantly shorter in the PC group (10.2 ± 0.5 min) than in the sham group (15.0 ± 0.6 min, P < 0.001) or the PC/propiverine group (18.8 ± 1.2 min, P < 0.001) (Figs. 4 and 5). The interval was also significantly longer in the PC/propiverine group than in the sham group (P = 0.010). There were no significant differences in baseline bladder pressure or maximum bladder contraction pressure among the 3 groups. These findings showed that propiverine increased bladder capacity without influencing the contraction pressure.

NOS and receptors mRNA of the bladder wall

When relative eNOS mRNA expression corrected for β-actin was compared among the groups, those level in the PC group (0.31 ± 0.02 × 10^{-3}) or the PC/propiverine group (0.27 ± 0.05 × 10^{-3}) was significantly lower (both P < 0.001) than in the sham group (1.18 ± 0.21 × 10^{-3}) (Fig. 6). In contrast, relative nNOS mRNA expression was significantly higher in the PC/propiverine group (1.01 ± 0.29 × 10^{-5}) than in the sham group (0.31 ± 0.05 × 10^{-5}, P = 0.016) or the PC group (0.35 ± 0.10 × 10^{-5}, P = 0.025). Relative iNOS mRNA expression was significantly higher in the PC group (0.43 ± 0.11 × 10^{-6}) than in the sham group (0.16 ± 0.05 × 10^{-6}, P = 0.035) or the PC/propiverine group (0.13 ± 0.05 × 10^{-6}, P = 0.026).

Relative M3 mRNA expression of PC group (9.75 ± 1.40 × 10^{-5}) or the PC/propiverine group (4.37 ± 0.60 × 10^{-5}) was significantly lower (P = 0.021 and P = 0.001, respectively) than in the sham group (17.48 ± 3.16 × 10^{-5}), while it was significantly lower (P = 0.002) in the PC/propiverine group than in the PC group.

Relative TRPV1 mRNA expression of PC group (5.24 ± 0.64 × 10^{-5}) or the PC/propiverine group (3.54 ± 0.38 × 10^{-5}) was significantly lower (P = 0.002 and P < 0.001, respectively) than in the sham group (11.17 ± 1.62 × 10^{-5}), while it was significantly lower (P = 0.019) in the PC/propiverine group than in the PC group.

There were no significant differences in relative ADRB3, NicotR, and P2X3 mRNA expression among the 3 groups. However, PC and propiverine
congestion caused a decrease in bladder blood flow to about 80% of the level in intact rats and also increased bladder vascular permeability (13, 15). In the present study, pelvic venous congestion induced an increase in the 24 h frequency of urination and decreased the interval between bladder contractions on continuous cystometry, indicating that this rat model showed urinary frequency. Pelvic venous congestion also led to a decrease in locomotor activ-

**DISCUSSION**

Our previous study demonstrated that pelvic venous congestion had an influence on eNOS, nNOS, iNOS, M3, and TRPV1 mRNA expression, suggesting that PC and propiverine both affected epithelial, smooth muscle, and nervous systems regulating bladder function.

![Fig. 4](image1.png)  
**Fig. 4** Typical continuous cystometry in the sham rat (upper), PC rat (center), and PC/propiverine rat (lower).

![Fig. 5](image2.png)  
**Fig. 5** Continuous cystometry parameters (interval between bladder contractions [left], baseline bladder pressure [center], and maximum bladder contraction pressure [right]) in the sham, PC, and PC/propiverine groups. Mean ± SEM.
Expression of eNOS mRNA in the bladder wall was decreased by pelvic venous congestion, while expression of nNOS mRNA, but not eNOS mRNA, was increased by administration of propiverine. These findings suggested that propiverine might increase neural NOS expression and increase the urinary NOx level. Studies have shown that nitric oxide displays both relaxant and facilitatory effects, as well as acting directly on bladder smooth muscle (including the detrusor) and having a modulatory effect on the afferent nerves, and it has been suggested that nitric oxide primarily inhibits afferent nerve signalling (4). The decrease in urinary NOx in our rats with pelvic venous congestion might suggest that bladder tissue hypoxia is one of reasons for alteration of urinary frequency. Administration of propiverine reversed the decrease in urinary NOx, suggesting that propiverine improved bladder tissue hypoxia, possibly via relaxation of the bladder and the pelvic vessels. In the present study, bladder wall iNOS mRNA expression was increased in the rats with pelvic venous congestion, but it was reduced by propiverine, suggesting improvement of bladder inflammation. Therefore, an anti-inflammatory effect did not influence urinary 8-OHdG (13).

Expression of eNOS mRNA in the bladder wall was decreased by pelvic venous congestion, while expression of nNOS mRNA, but not eNOS mRNA, was increased by administration of propiverine. These findings suggested that propiverine might increase neural NOS expression and increase the urinary NOx level. Studies have shown that nitric oxide displays both relaxant and facilitatory effects, as well as acting directly on bladder smooth muscle (including the detrusor) and having a modulatory effect on the afferent nerves, and it has been suggested that nitric oxide primarily inhibits afferent nerve signalling (4). The decrease in urinary NOx in our rats with pelvic venous congestion might suggest that bladder tissue hypoxia is one of reasons for alteration of urinary frequency. Administration of propiverine reversed the decrease in urinary NOx, suggesting that propiverine improved bladder tissue hypoxia, possibly via relaxation of the bladder and the pelvic vessels. In the present study, bladder wall iNOS mRNA expression was increased in the rats with pelvic venous congestion, but it was reduced by propiverine, suggesting improvement of bladder inflammation. Therefore, an anti-inflammatory effect

Fig. 6 Bladder wall expression of mRNAs for eNOS, nNOS, iNOS, and several receptors (corrected by β-actin mRNA) in the sham, PC, and PC/propiverine groups. Mean ± SEM.
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of propiverine might have decreased iNOS mRNA expression and the urinary 8-OHdG level, although such an anti-inflammatory action has not been reported. On the other hand, iNOS mRNA expression \((\times 10^{-6})\) in the bladder wall was very low compared to eNOS mRNA expression \((\times 10^{-3})\) and nNOS mRNA expression \((\times 10^{-5})\). Therefore, the total NOx level in the bladder wall might have been increased by administration of propiverine, leading to relaxation of the bladder and the pelvic vessels. Saito et al. reported that bladder blood flow is significantly lower in spontaneously hypertensive rats than in normotensive Wistar rats, and is increased by vasodilators such as nicorandil, a KATP channel opener and NO donor (9). Their findings may also support our data on the relationship between bladder blood flow and NOx.

Activation of the M3 or TRPV1 receptor increases bladder activity (1, 2). In the present study, pelvic venous congestion decreased the expression of M3 and TRPV1 mRNA, and their expression was reduced further by propiverine. Pelvic venous congestion induces an increase in bladder vascular permeability, resulting in extravascular leakage of stimulatory substances that may contribute to the occurrence of lower urinary tract symptoms. However, the increase in stimulatory substances may induce downregulation of various receptors, including the M3 and TRPV1 receptors. Propiverine blocks M3 receptors because of its anti-muscarinic action and blocks TRPV1 because of its Ca antagonist action. Therefore, administration of propiverine might have induced further downregulation of the M3 and TRPV1 receptors, which is another possible mechanism leading to improvement of bladder activity.

In conclusion, rats with pelvic venous congestion showed a decrease in locomotor activity, an increased frequency of urination, and a shorter interval between bladder contractions on continuous cystometry, as well as decreased bladder wall expression of eNOS, M3, and TRPV1 mRNA, and increased expression of iNOS mRNA. When propiverine was administered to these rats, locomotor activity was increased to the level in sham rats along with improvement of bladder function. Also, decrease in urinary 8-OHdG and increase in urinary NOx, increased bladder wall expression of nNOS mRNA, and decreased bladder wall expression of iNOS, M3, and TRPV1 mRNA were also observed. Therefore, propiverine not only improved bladder dysfunction via its previously reported anti-muscarinic, Ca antagonist, and noradrenaline re-uptake inhibitory actions (8, 17), but also by reducing inflammation, as demonstrated by the decrease in urinary 8-OHdG, decrease in iNOS mRNA expression, increase in total NOx due to nNOS activation, and downregulation of M3 and TRPV1 receptor expression.

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