Cold stress-induced bladder overactivity in type 2 diabetic mellitus rats is mitigated by the combination of a M_3-muscarinic antagonist and a β_3-adrenergic agonist

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Running head: Add-on Therapy for Cold Stress-induced Detrusor Overactivity

Abstract

Objective: Goto-Kakizaki (GK) rats with type 2 diabetes mellitus respond to low temperature (LT) environments with bladder overactivity, including increased voiding frequency and decreased voiding interval and micturition volume. We determined if bladder overactivity could be inhibited by treatment with the combination of a M_3-muscarinic receptor antagonist and a β_3-adrenergic receptor agonist.

Methods: Ten-week-old female GK rats were fed a high-fat diet for 4 weeks. Cystometric investigations were conducted at room temperature (RT, 27±2ºC). The rats were then intraperitoneally administered the vehicle, the M_3-muscarinic receptor antagonist solifenacin, the β_3-adrenergic agonist mirabegron, or a combination of solifenacin and mirabegron. Ten minutes after the administrations, the rats were transferred to the LT environment (LT, 4±2ºC) where the cystometric measurements were continued. The expressions of both M_3-muscarinic and β_3-adrenergic receptors were investigated.

Results: After transfer from RT to LT, both voiding interval and bladder capacity of the vehicle-, solifenacin-, or mirabegron-treated rats were significantly decreased. However, the combination of solifenacin and mirabegron significantly mitigated the bladder overactivity. While both M_3-muscarinic and β_3-adrenergic receptors were detected, the expression of M_3-muscarinic receptor mRNA was significantly higher than that of β_3-adrenergic receptor mRNA.

Conclusions: The cold stress-induced bladder overactivity was not improved by either the M_3-muscarinic receptor antagonist or the β_3-adrenergic receptor agonist alone. However, the combined treatment mitigated the cold stress responses. Combined therapy with M_3-muscarinic antagonists and β_3-adrenergic agonists could reduce side effects and improve the quality of life for diabetic patients with bladder overactivity.

Key Words: Lower Urinary Tract Symptoms, Type 2 Diabetes Mellitus, Cold Stress, Muscarinic Receptor Antagonist, β_3-adrenergic Receptor Agonist, GK rat

1. INTRODUCTION

Patients with type 2 diabetes mellitus usually have complications of lower urinary tract symptoms (LUTS).[1,2,3] Approximately 20% of diabetic patients complain about overactive bladder (OAB) syndrome, which is defined as urgency, with or without incontinence, usually with urinary frequency and nocturia.[4,5,6] Thus, diabetic patients need more effective OAB medications compared to others without diabetes. In addition, women patients with the type 2 diabetes mellitus show higher prevalence of urinary urge incontinence.[7,8] These reports suggest that type 2 diabetes mellitus might induce and/or exacerbate bladder storage dysfunctions, including bladder overactivity, of LUTS.

The pharmacological treatment of bladder overactivity in patients with OAB syndrome includes either muscarinic receptor antagonists or β_3-adrenergic receptor agonists.[9,10,11] The muscarinic receptor antagonists can inhibit detrusor contractions; however, long-term administration is often limited by side effects, such as dry mouth and constipation.[12,13] While the β_3-adrenergic receptor agonists mediate relaxation of the detrusors, the suppression mechanisms of detrusor overactivity are not well understood.[12,13] Even so, the combined therapy of OAB patients with muscarinic receptor antagonists and β_3-adrenergic receptor agonists has been proposed as a long-term pharmacological treatment.

Our previous studies have shown that rats stimulated with cold stress by the sudden exposure to low environmental temperature respond by bladder overactivity.[14,15,16] The cold stress-induced bladder overactivity is characterized by increased voiding frequency with decreased micturition volume and voiding interval. The effectiveness of pharmacological agents on bladder overactivity is dependent on the presence or absence of underlying diseases.[17] While bladder tissues of patients with type 2 diabetes mellitus exhibit cooling-stimulated contractions through Rho-kinase pathways,[18] bladder overactivity induced by whole body cooling in the diabetic patients has not been fully investigated. In this study, we conducted cystometric investigations using Goto-Kakizaki (GK) rats as a type 2 diabetes mellitus model with bladder overactivity in our sudden low temperature exposure protocol. We determined if

70 therapy using a M_3-muscarinic receptor antagonist or a β_3-adrenergic receptor agonist, or the
71 combination of the two, could mitigate the cold stress-induced bladder overactivity.

72

2. METHODS

2.1. Animals

Ten-week-old female GK rats (n=54, 180-220 g, Japan SLC, Inc., Shizuoka, Japan) were selected for this study because they are widely accepted as a rodent diabetes model with reduced secretion of insulin and the presence of diabetic peripheral neuropathy. Blood glucose of the GK rats was over 200 mg/dL, which was significantly higher than in normal rats, such as Wistar Kyoto rats in which the blood sugar level is about 180 mg/dL.[17] The GK rats were fed a high-fat diet that was developed for diabetes and obesity research (Rodent Diet Quick Fat; CLEA Japan, Tokyo, Japan), and water was freely available. They were maintained under a 12-hour alternating light-dark cycle for 4 weeks. The animals were treated in accordance with National Institutes of Health Animal Care Guidelines and guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine.

2.2 Drugs

The M_3-muscarinic receptor antagonist solifenacin (catalogue# HY-A0002, MedChem Express, Princeton, NJ, USA) was dissolved at 20.0 mg/ml with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Just prior to use, it was diluted 1:10 or 1:100 with 0.9% saline. It was administered intraperitoneally at 0.1 ml/200 g-body weight (final dose: 0.1 or 1.0 mg/kg-body weight). The β_3-adrenergic receptor agonist mirabegron was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). It was dissolved at 20.0 mg/ml with 0.9% saline. Just prior to use, it was diluted 1:10 or 1:100 with 0.9% saline. It was administered intraperitoneally at 0.1 ml/200g-body weight (final dose: 0.1 or 1.0 mg/kg-body weight).

2.3 Cystometric Investigations

After 4 weeks on the high-fat diet, the GK rats were anesthetized with pentobarbital sodium solution (40 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and inhalation of 2% sevoflurane (Pfizer Japan Co., Ltd., Tokyo, Japan). Urinary bladder domes were exposed and incised to allow insertion of a polyethylene catheter (PE50, Becton Dickinson & Company, Sparks, MD, USA). For delivery of vehicle or drugs during the cystometry experiments, another catheter (PE90, Becton Dickinson & Company) was inserted into the intraperitoneal space. The free ends of both catheters were brought out subcutaneously to the back and fixed. Each rat was caged individually for two days after surgery.

For cystometry investigations, the bladder catheter was connected through a T-tube to a pressure transducer (P23 DC; Nihon Kohden, Tokyo, Japan) and a syringe pump (catalogue# TE-351, Terumo, Tokyo, Japan). To measure micturition volume, a fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments, Tokyo, Japan) was placed under the metabolic cage. Throughout the experiments, saline kept at room temperature was pumped into the bladder at a rate of 10 ml/hr. The rats were not given food or water during the cystometric investigations. The bladder pressure, voiding interval (min), and micturition volume (ml) were recorded continuously with LabChart system (AD Instruments, BRC Bioresearch, Inc., Nagoya Japan). Residual volume (ml) was determined as the difference between the saline infusion volume and micturition volume. Bladder capacity (ml) was calculated by adding the micturition volume and the residual volume.

Low temperature exposed-cystometric investigations of the unanesthetized, unrestricted experimental GK rats were conducted. To obtain baseline measurements, bladder pressure and micturition volume were recorded for approximately 20 minutes at room temperature (RT, 27±2ºC). Each rat was then administered one of the following intraperitoneal treatments: vehicle (n=6), 0.1 mg/kg solifenacin (n=6), 1.0 mg/kg solifenacin (n=6), 0.1 mg/kg mirabegron (n=6), 1.0 mg/kg mirabegron (n=6), or the combination of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron (n=6). Ten

minutes after each administration, the cystometric measurements were continued for approximately 10 minutes to estimate effects of the treatments under RT. The rats were then gently and quickly transferred in their metabolic cage to a refrigerator (MPR-513, SANYO Tokyo Manufacturing Co., Ltd., Tokyo, Japan) for exposure to low temperature (LT, 4±2ºC). The LT exposure cystometry was continued for 40 minutes.

After the cystometric investigations, the rats were anesthetized with the pentobarbital sodium solution (as above), and the urinary bladders were removed, and then the rats were euthanized by an overdose of pentobarbital sodium.

2.4 Real-time RT-PCR

Twelve GK rats that were not used in cystometric investigations were anesthetized with the pentobarbital sodium solution (as above), and the urinary bladders were removed. Approximately one third of each bladder, from the top to the trigone, was prepared for real-time reverse transcription-polymerase chain reaction (RT-PCR). The remaining portion was used in immunohistochemical investigations (described below).

Within the bladder tissue, expression levels of M_3-muscarinic receptor and β_3-adrenergic receptor mRNAs were semi-quantitatively estimated by real-time RT-PCR. Total RNA was extracted from each bladder with a RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 0.1 μg of total RNA with SuperScript VILO Master Mix (Thermo Fisher Scientific K.K., Foster City, CA, USA). The synthesized cDNA was mixed in TaqMan Universal PCR Master Mix with the following gene assay probes: M_3-muscarinic receptor (Chrm3, catalogue# Rn00560986_s1), β_3-adrenergic receptor (Adrb3, catalogue# Rn00565393_m1), or beta-actin (Actb, catalogue# Rn00667869_m1), all from Thermo Fisher Scientific K.K. Real-time RT-PCR of the mixed cDNA-probe solution was performed at 50ºC for 2 min followed by 95ºC for 10 min. These were followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. Gene expression was

147 calculated by the delta-delta method as the ratio to threshold cycle value of the internal standard gene
148 beta-actin.
149
150 **2.5 Immunohistochemistry**

151 The presence of M_3-muscarinic receptors and β_3-adrenergic receptors within the urinary bladder
152 tissues was visualized by immunohistochemistry. The harvested portions of the bladders from GK rats
153 not used for cystometry were fixed with 4% paraformaldehyde and embedded in paraffin. Serial
154 sections (5 μm) were deparaffinized and treated with 10 mM sodium citrate (pH 6.0, 100˚C, 5 min)
155 for antigen retrieval. The specimens were then coated with 1.5% normal donkey serum (Chemicon
156 International, Inc., Temecula, CA, USA) and 1.5% non-fat milk in phosphate buffered saline (PBS)
157 for 1 hour at 4˚C.

158 M_3-muscarinic cholinergic receptors were stained following incubation (12 hours, 4ºC) with the
159 primary antibody (catalogue# ab87199, 1:200, rabbit polyclonal, Abcom, Cambridge, UK).
160 Simultaneously, the incubation solution contained one of the following other primary antibodies:
161 uroplakin III (catalogue# sc-15186, 1:100, goat polyclonal, Santa Cruz Biotechnology Inc., Santa Cruz,
162 CA, USA), a marker for the bladder urothelium; smooth muscle actin (SMA, catalogue# 61001, 1:100,
163 mouse monoclonal, Progen Biotechnik GmbH, Heidelberg, Germany), a marker for smooth muscle
164 cells; or calcitonin gene-related peptide (CGRP, catalogue# 16013, 1:800, guinea pig polyclonal,
165 Progen Biotechnik GmbH), a marker for afferent nerve fibers. The sections were then rinsed with PBS
166 and incubated with donkey anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:250,
167 Thermo Fisher Scientific K.K.) and donkey anti-goat, -mouse, or -guinea pig IgG secondary antibody
168 (as appropriate) conjugated with Alexa Fluor 594 (1:250, Thermo Fisher Scientific K.K.) for 1 hour at
169 4ºC. After a final rinse, cell nuclei were counterstained with 5 μg/ml 4', 6-diamidino-2-phenylindole
170 dihydrochloride (DAPI, Thermo Fisher Scientific K.K). The slides were coated with Fluorescent

Mounting Medium (Dako Cytomation, Carpinteria, CA, USA), and photographed with a fluorescence microscope (Keyence, Osaka, Japan).

β_3-adrenergic receptors were stained following incubation (12 hours, 4ºC) with the primary antibody (catalogue# SP4073P, 1:100, rabbit polyclonal, Acris Antibody, Inc., San Diego, CA, USA). Simultaneously, the incubation solution contained one of the following other primary antibodies as described above for uroplakin III, SMA, or CGRP. After incubation with the primary antibodies, the sections were rinsed and incubated with the secondary antibodies, stained with DAPI, coated with Fluorescence Mounting Medium, and photographed as described above.

2.6 Statistical Analysis

The results were expressed as means ± standard error of the means. Statistical differences were determined by using the Excel® Statistics program (Esumi Co., Ltd. Tokyo, Japan). Two-way repeated measures analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) post-hoc analysis within each group. To compare values for the cystometric parameters measured in the vehicle-, solifenacin-, mirabegron-, and the combined solifenacin- and mirabegron-treated rats, two-way non-repeated ANOVA and SNK methods were used. To compare expression of M_3-muscarinic and β_3-adrenergic receptor mRNA, two-way repeated t-test was used. P-values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Effects of the M_3-muscarinic receptor antagonist solifenacin and the β_3-adrenergic receptor agonist mirabegron on cold stress-induced bladder overactivity

At 4-weeks after feeding on a high-fat diet, the GK rats treated with vehicle, solifenacin, or mirabegron underwent cystometric investigations at RT and LT. At RT, neither the voiding interval nor the bladder capacity were altered by administration the vehicle ($P>0.05$ for all, Table 1). Similarly, neither solifenacin nor mirabegron at either 0.1 mg/kg or 1.0 mg/kg, affected the RT voiding interval or the bladder capacity ($P>0.05$ for all, Table 1). Thus at RT, for all treatment groups in which either solifenacin or mirabegron were peritoneally infused alone, there were no changes in voiding interval or bladder capacity between the pre- and post-treatment periods.

During the 40 min LT exposure, both the voiding interval and bladder capacity of the vehicle-treated GK rats decreased significantly compared to the RT post-treatment values (Table 1). These parameters of the rats treated with either 0.1 or 1.0 mg/kg solifenacin also decreased compared to the RT post-treatment values during LT exposure (Table 1). Similarly, for the rats treated with either 0.1 or 1.0 mg/kg mirabegron, their voiding interval and bladder capacity decreased compared to the RT post-treatment values during LT exposure (Table 1). Therefore, treatments with neither solifenacin or mirabegron alone at 0.1 or 1.0 mg/kg inhibited the decreases of voiding interval and bladder capacity during LT exposure.

3.2 Effects of combined treatment with the M_3-muscarinic receptor antagonist solifenacin and the β_3-adrenergic receptor agonist mirabegron on cold stress-induced bladder overactivity

At RT, neither the bladder pressure nor the micturition volume were altered by administration of the vehicle (Fig. 1a). Just after transferring to LT exposure, the vehicle-treated GK rats exhibited bladder overactivity induced by cold stress as shown by the increased voiding frequency and decreased

micturition volume and voiding interval (Fig. 1a). For control rats treated with the vehicle, the cold stress-induced bladder overactivity continued during the 40 min of LT exposure. Thus, the voiding frequency increased (Fig. 1a).

Similarly, the bladder pressure and the micturition volume in rats that received the combined treatment of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron at RT did not change from the pre-treatment period (Fig. 1b). Like the vehicle-treated rats, they also exhibited the cold stress-induced bladder overactivity during the 40 min LT exposure (Fig. 1b). In rats receiving the combined treatment of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron at RT, there were no changes in any of the measured post-treatment bladder functions during the RT period (Fig. 1c). However, after transfer to the LT environment, there was a partial inhibition of the cold stress-induced bladder overactivity (Fig. 1c).

For the treatment with 0.1 mg/kg solifenacin combined with 0.1 mg/kg mirabegron, voiding interval decreased by 54.8%, from 3.78 min to 1.65 min ($P<0.01$, Fig. 2a). However, for the rats treated with the combination of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron, the decrease in voiding interval was only 15.3%, from 3.09 min to 2.52 min ($P>0.05$, Fig. 2a). Thus, the decrease of voiding interval in the group treated with high doses of the combined solifenacin and mirabegron treatment was mitigated compared to the vehicle- and the combined 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron doses respectively (both $P<0.01$, Fig. 2a).

Similarly, after transferring to LT exposure, the bladder capacities of the vehicle- and the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-treated rats were significantly decreased, from 0.68 ml to 0.39 ml and from 0.65 ml to 0.33 ml, respectively (both $P<0.01$, Fig. 2b). In contrast, the bladder capacity of the rats treated with the combination of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron decreased less, from 0.50 ml to 0.45 ml (Fig. 2b). Thus, the decrease of bladder capacity in the rats with the combined solifenacin and mirabegron at the high dose (1.0 mg/kg each),

239 -6.7%, was less than for the vehicle- or the combined 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-
240 treated rats (-41.6% and -46.0%, respectively; both $P<0.01$, Fig. 2b).
241

242 **3.3 Expression of Urinary Bladder M_3-muscarinic and β_3-adrenergic Receptors**

243 We semi-quantitatively estimated the expression levels of the M_3-muscarinic and β_3-adrenergic
244 receptor mRNAs within the urinary bladder tissues in GK rats that were not used in the cystometric
245 studies. The expression level of M_3-muscarinic receptor mRNA was significantly higher than that of
246 β_3-adrenergic mRNA ($P<0.01$, Fig. 3a).

247 The presence of M_3-muscarinic and β_3-adrenergic receptors were visualized within the
248 urothelium, smooth muscle cells, and afferent nerve fibers in the GK rats. The M_3-muscarinic receptors
249 were detected within the uroplakin III-positive urothelium (Fig. 3b), SMA-positive smooth muscle
250 cells (Fig. 3c), and CGRP-positive afferent nerve fibers (Fig. 3d). Similarly, β_3-adrenergic receptors
251 were also present within the urothelium (Fig. 3e), smooth muscle cells (Fig. 3f), and afferent nerve
252 fibers (Fig. 3g).
253

4. DISCUSSION

Our previous study showed that the cold stress-induced bladder overactivity of Sprague-Dawley (SD) rats without any underlying disease was inhibited by treatments with 1.0 mg/kg CL316243, another β_3-adrenergic receptor agonist.[19] Thus, we concluded that the cold stress-induced bladder overactivity in normal SD rats is mediated with pathways involving β_3-adrenergic receptors. However, in the present study, the bladder overactivity of GK rats was not inhibited by treatments with either 0.1 mg/kg or 1.0 mg/kg mirabegron, even though expression of β_3-adrenergic receptor mRNA and protein were detected within the urinary bladders. Currently, we do not have a good explanation of why mirabegron alone effectively reduced cold stress-induced bladder overactivity in SD rats, but not in GK rats.

The cold stress-induced bladder overactivity of normal Wistar Kyoto rats, maintained under the same conditions (including the high fat diet for four weeks) as the GK rats in the present study, was not inhibited by administration of imidafenacin, another M3-muscarinic receptor antagonist.[17] In contrast, the same dose of imidafenacin, 0.3 mg/kg, partially inhibited the cold stress-induced bladder overactivity of GK rats.[17] In the urinary bladders of diabetic GK rats, the ratio of M_3-muscarinic receptor mRNA to M_2-muscarinic receptor mRNA is higher than that of WKY rats.[17] This suggests that 1.0 mg/kg solifenacin, like imidafenacin, would mitigate the cold stress-induced bladder overactivity of the GK rats. However, the cold stress-induced bladder overactivity of the GK rats was not significantly improved by 1.0 mg/kg solifenacin alone. At this time, we cannot explain why solifenacin alone did not effectively reduce the cold stress-induced bladder overactivity in GK rats.

Based on the ineffectiveness of either solifenacin or mirabegron alone to reduce LT-induced bladder overactivity in GK rats, we determined if the combination solifenacin and mirabegron would be effective. In a previous study, we showed that while the cold stress-induced bladder overactivity of spontaneously hypertensive rats (SHRs) was not inhibited by treatments with either 0.1 mg/kg solifenacin or 0.1 mg/kg mirabegron, the combined treatments with the same doses did mitigate the

cold stress responses.[16] The expression profiling of M_3-muscarinic and β_3-adrenergic receptor mRNA in the urinary bladders of GK rats was similar to those of SHRs.[16] Thus, in the current study, we hypothesized that the same dose of the combined treatments would be effective in reducing the cold stress-induced bladder overactivity. However, the cold stress-induced bladder overactivity of the GK rats was not fully mitigated by the same dose combined treatments. These results suggest that there might be differences in the mechanisms of bladder overactivity between the SHR and GK rats, or that the bladder overactivity of GK rats might exhibit drug resistance due to neurogenic and/or myogenic bladder dysfunctions.[20,21,22]

This study had three major limitations. First, we did not investigate dose-response curves for either the M_3-muscarinic receptor antagonist or the β_3-adrenergic receptor agonist. Therefore, we were not able to determine the optimum dose for either drug. Second, we did not investigate the synergistic effects of the combined therapy on the myogenic and/or neurogenic mechanisms that regulate detrusor activity. Finally, we did not investigate why the urinary bladders of GK rats had higher expression levels of M_3-muscarinic receptor mRNA compared to the β_3-adrenergic receptor mRNA.

Many patients with OAB are treated with long-term administration of muscarinic receptor antagonists; however, 75-80% of these patients suffer from the side effects of this treatment.[23,24] One the strategy to accomplish the long-term therapy is the addition of a β_3-adrenergic receptor agonist to a M_3-muscarinic receptor antagonist treatment. This combination of drugs for diabetic patients with OAB might enable long-term administration while reducing or avoiding side effects, and thus it could improve the quality of life for the patients.

In conclusion, the cold stress-induced bladder overactivity in diabetic GK rats was not improved by either the M_3-muscarinic receptor antagonist solifenacin or the β_3-adrenergic receptor agonist mirabegron alone. However, the combined treatment mitigated the cold stress responses. In the urinary bladders of GK rats, while both M_3-muscarinic and β_3-adrenergic receptors were detected, the expression level of M_3-muscarinic receptor mRNA was significantly higher than that of the β_3-

304 adrenergic receptor mRNA. Our results suggest that the combination of a muscarinic receptor
305 antagonist and a β_3-adrenergic receptor agonist for diabetic patients with OAB might be an important
306 and productive area for future clinical research.
307

Conflict of Interest Statement

The authors declare no conflict of interest.

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374 **Figure Legends**

375 **Figure 1.**

376 Cystometric effects of vehicle or combinations of solifenacin and mirabegron on GK rats at RT and
377 LT. (a and b) Under RT condition, bladder pressure and micturition volume were not altered by
378 administration of vehicle (a) or the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron
379 (b). During 40 min of LT exposure, both the vehicle- and the combination-treated GK rats exhibited
380 bladder overactivity induced by cold stress as indicated by increased voiding frequency and decreased
381 micturition volume and voiding interval. (c) At RT, bladder pressure and micturition volume were not
382 altered by administration by combination of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron. During
383 LT exposure, the combination of drugs mitigated the cold stress-induced bladder overactivity as shown
384 by mitigation of the increased bladder pressure and voiding frequency and decreased micturition
385 volume. Upper: bladder pressure; bottom: micturition volume in each panel. Asterisk: 10 minutes after
386 administration.

387

388 **Figure 2.**

389 Mitigation of decreases in voiding interval and bladder capacity by the combination of solifenacin and
390 mirabegron in GK rats during 40 min LT exposure. (a) During LT exposure, voiding interval in both
391 the vehicle- and the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-treated GK rats
392 were significantly decreased compared to the RT values. However, the voiding interval in the
393 combination of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron-treated GK rats did not decrease
394 significantly. The decrease of the voiding interval in with combination of 1.0 mg/kg solifenacin and
395 1.0 mg/kg mirabegron-treated GK rats were significantly smaller than in the vehicle- and the
396 combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-treated ones. (b) Similarly, bladder
397 capacity in both the vehicle- and the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-

398 treated GK rats were significantly decreased; however, the bladder capacity in the combination of 1.0
399 mg/kg solifenacin- and 1.0 mg/kg mirabegron-treated GK rats did not. The decrease in the combination
400 of 1.0 mg/kg solifenacin and 1.0 mg/kg mirabegron-treated GK rats were significantly smaller than
401 that in the vehicle- and the combination of 0.1 mg/kg solifenacin and 0.1 mg/kg mirabegron-treated
402 ones. White bar, room temperature (RT). Gray bar, low temperature (LT).

403

404 **Figure 3.**

405 Expression of M_3-muscarinic and β_3-adrenergic receptors within the urinary bladders of GK rats. (a)
406 The expression level of M_3-muscarinic receptor mRNA was significantly higher than that of M_3-
407 muscarinic receptor mRNA ($P<0.01$). (b-d) M_3-muscarinic receptors (yellow, arrows) were present
408 within the (b) uroplakin III-positive (red) urothelium, (c) SMA-positive (red) smooth muscle cells, and
409 (d) CGRP-positive (red) afferent nerve fibers. (e-g) β_3-adrenergic receptors (yellow, arrows) were also
410 present within the (e) urothelium (red), (f) smooth muscle cells (red), and (g) afferent nerve fibers (red).

411

412

Table 1. Voiding intervals and bladder capacities at room temperature and low temperature in response to solifenacin and mirabegron alone

	Room Temperature				**Low Temperature**	
	Pre-treatment		**Post-treatment**		**Post-treatment**	
	Voiding interval	Bladder capacity	Voiding interval	Bladder capacity	Voiding interval	Bladder capacity
Vehicle	3.94±0.42	0.68±0.07	3.83±0.30	0.67±0.06	2.10±0.23**	0.40±0.05††
0.1 mg/kg Solifenacin	3.72±0.53	0.60±0.11	4.18±0.44	0.71±0.07	1.95±0.23**	0.38±0.04†
1.0 mg/kg Solifenacin	3.57±0.43	0.62±0.09	3.45±0.47	0.58±0.07	1.73±0.15**	0.33±0.03††
0.1 mg/kg Mirabegron	3.02±0.59	0.48±0.09	2.93±0.57	0.48±0.08	1.69±0.22*	0.31±0.04†
1.0 mg/kg Mirabegron	3.30±0.55	0.58±0.10	4.06±0.63	0.68±0.10	1.76±0.21**	0.34±0.04†

*P<0.05 and **P<0.01 compared to room temperature in voiding interval of each post-treatment. †P<0.05 and ††P<0.01 compared to room temperature in bladder capacity of each post-treatment

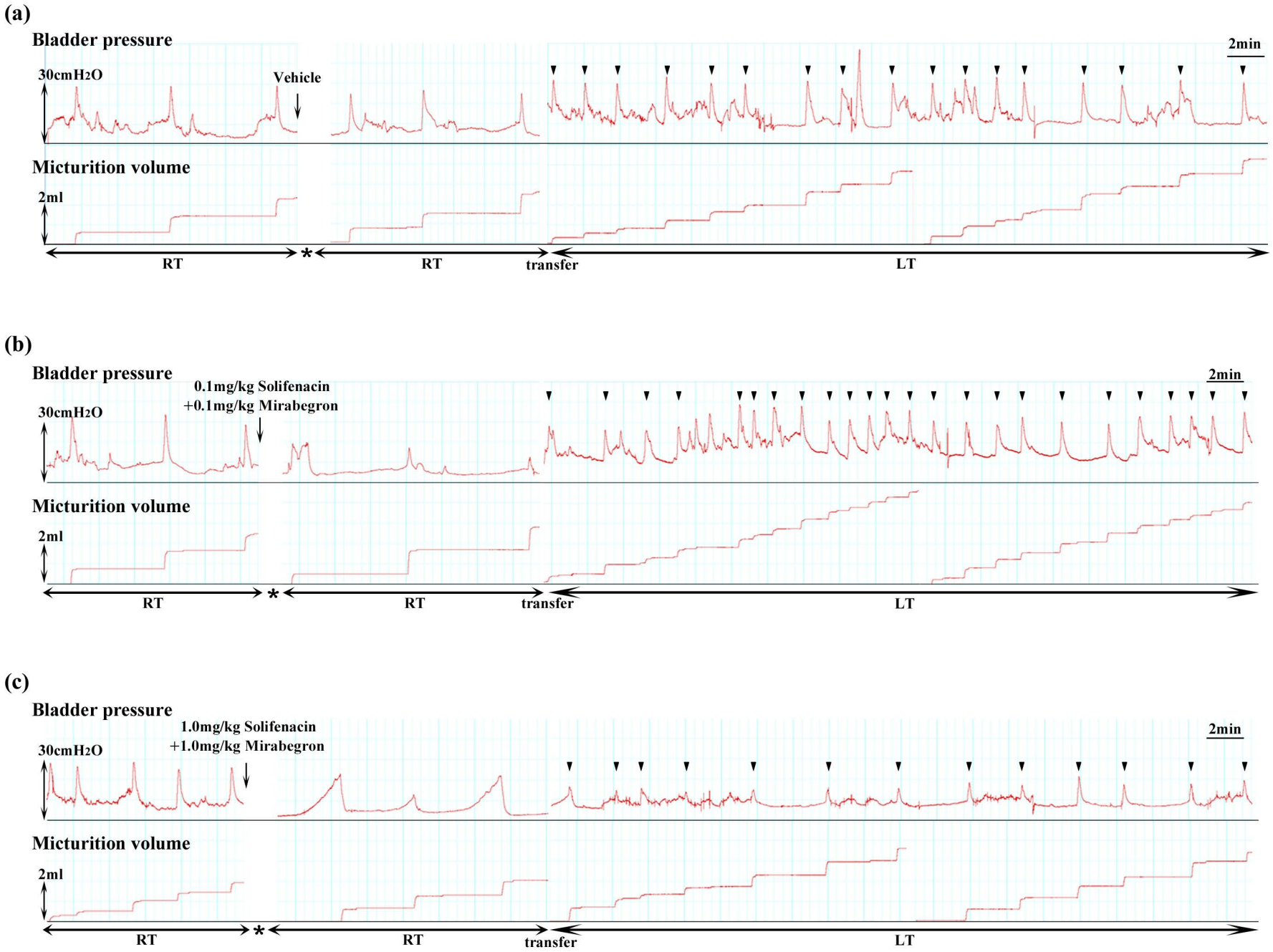
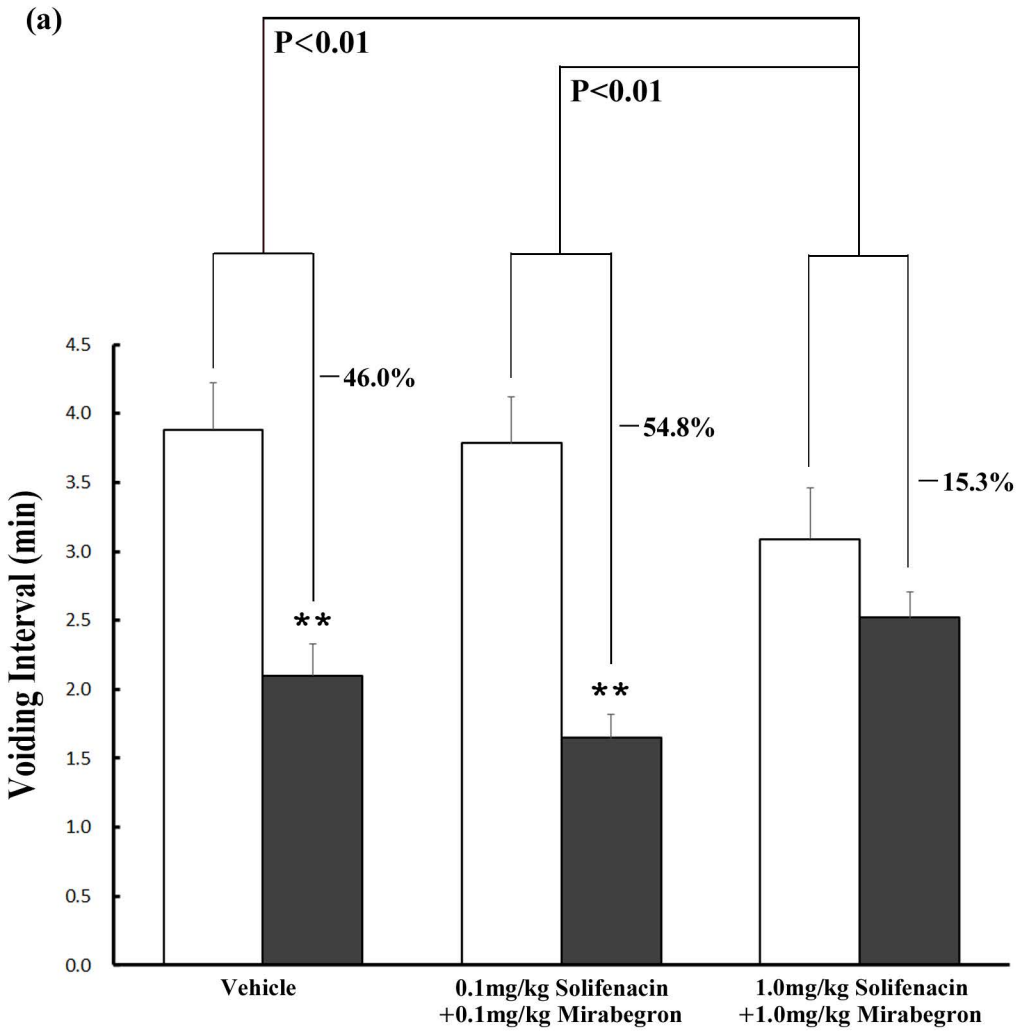

(a)
Bladder pressure
30cmH2O
Vehicle
2min
Micturition volume
2ml
RT
*
RT
transfer
LT
(b)
Bladder pressure
0.1mg/kg Solifenacin
+0.1mg/kg Mirabegron
30cmH2O
2min
Micturition volume
2ml
RT
*
RT
transfer
LT
(c)
Bladder pressure
1.0mg/kg Solifenacin
+1.0mg/kg Mirabegron
30cmH2O
2min
Micturition volume
2ml
RT
*
RT
transfer
LT

Figure 1


(a)
P<0.01
P<0.01
−46.0%
−54.8%
−15.3%
**
**
Voiding Interval (min)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
0.1mg/kg Solifenacin
+0.1mg/kg Mirabegron
1.0mg/kg Solifenacin
+1.0mg/kg Mirabegron

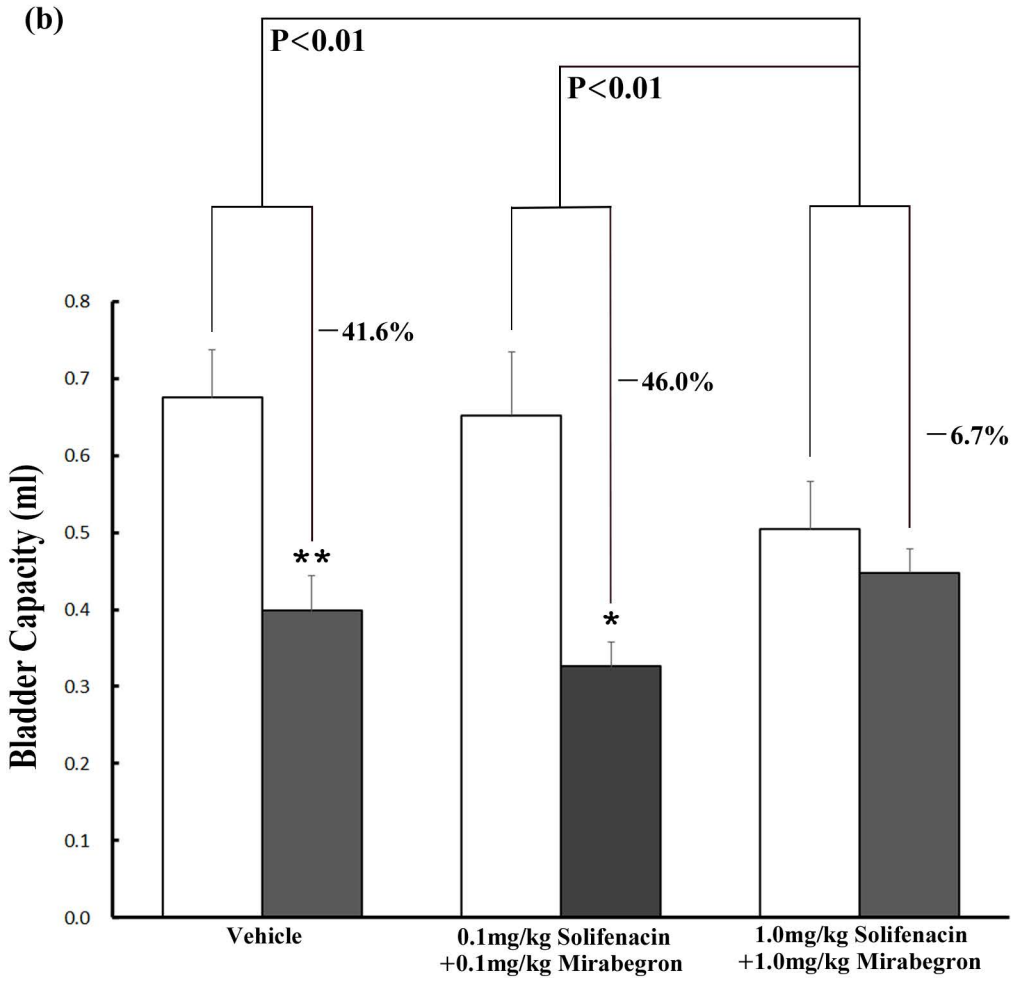

(b)
P<0.01
P<0.01
−41.6%
−46.0%
−6.7%
**
*
Bladder Capacity (ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle
0.1mg/kg Solifenacin
+0.1mg/kg Mirabegron
1.0mg/kg Solifenacin
+1.0mg/kg Mirabegron

Figure 2

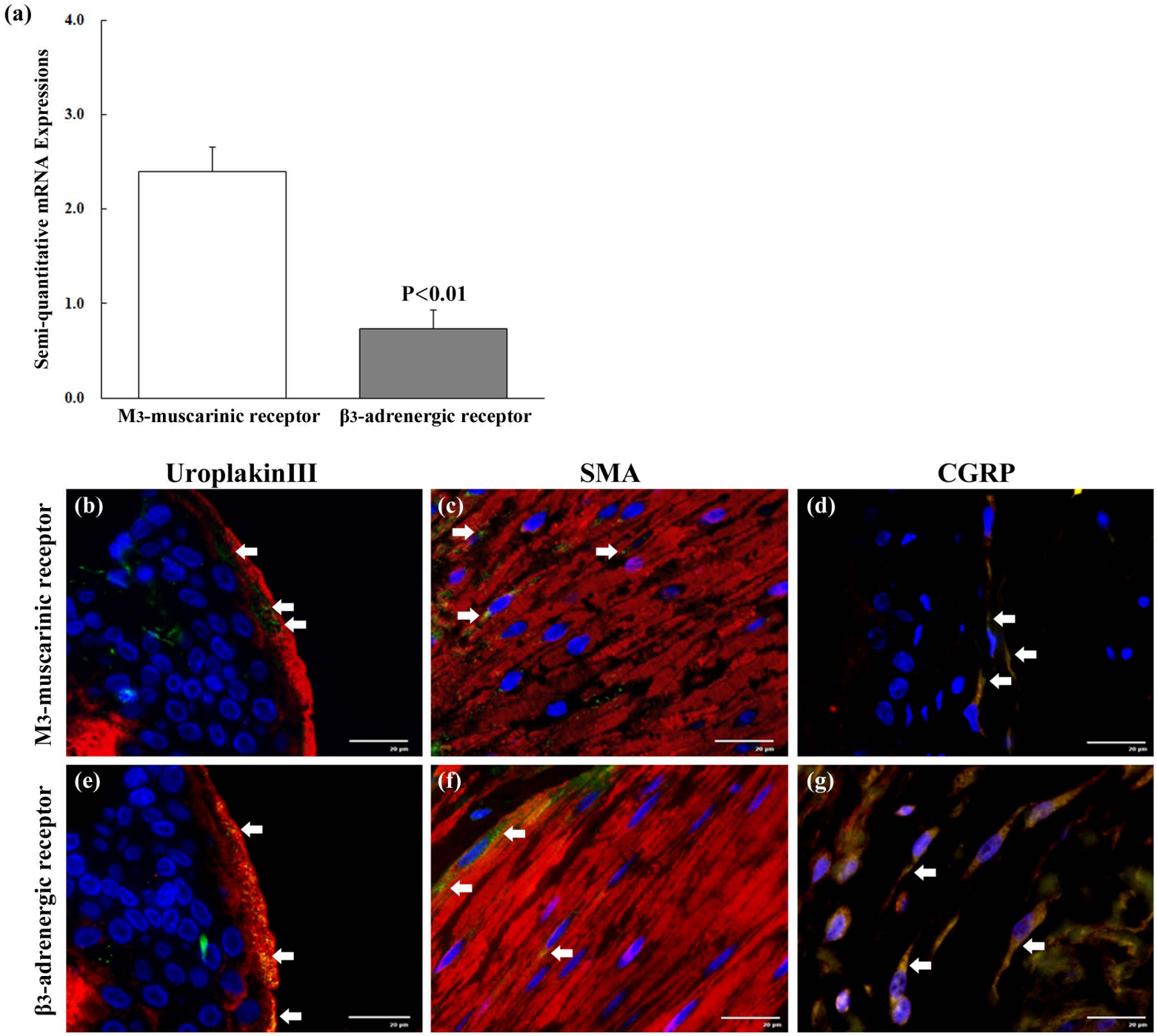

(a)
Semi-quantitative mRNA Expressions
4.0
3.0
2.0
1.0
0.0
P<0.01
M3-muscarinic receptor
β3-adrenergic receptor
UroplakinIII
SMA
CGRP
M3-muscarinic receptor
β3-adrenergic receptor
(b)
(c)
(d)
(e)
(f)
(g)

Figure 3