

Alpha₁-Adrenergic Receptors Mediate Bladder Overactivity Induced by Cold Stress in Rats with Bladder Outlet Obstruction

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Running head: Cold stress induces bladder overactivity in bladder outlet obstruction

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Abstract

Purpose: To determine if alpha1-adrenergic receptors (AR) mediate bladder overactivity induced by cold stress in rats with bladder outlet obstruction (BOO).

Materials and Methods: The urethras of 10-week-old female Sprague-Dawley rats were ligated to create BOO. After 4 weeks, cystometric investigations were performed at room temperature (RT, $27\pm 2^{\circ}\text{C}$) for 20 min. The rats were then given 0.3 mg/kg naftopidil (n=6) or vehicle (n=5) intravenously. Five minutes later, they were transferred to low temperature (LT, $4\pm 2^{\circ}\text{C}$), and the cystometric patterns were again recorded for 40 min. In BOO rats and in sham-operated rats (n=8) the expression levels of alpha_{1A}- and alpha_{1D}-AR mRNAs and the presence of alpha_{1A}- and alpha_{1D}-AR immunoreactivity on calcitonin gene-related peptide (CGRP)-positive nerve cells were investigated.

Results: During LT exposure, the vehicle-treated BOO rats exhibited cold stress-induced bladder overactivity. In the naftopidil-treated rats, the increase of basal pressure and decreases of both voiding interval and bladder capacity induced by LT were significantly reduced compared to the vehicle-treated animals. In the bladders of BOO rats exposed to LT, the expression of alpha_{1D}-AR mRNA was significantly higher than in sham-operated rats, and the immunoreactivity for alpha_{1D}-ARs on the CGRP-positive nerve cells tended to be more pronounced.

Conclusions: alpha1-ARs mediate part of the bladder overactivity induced by cold stress in rats with BOO. Cold stress increases the expression of alpha_{1D}-AR mRNA and the immunoreactivity for alpha_{1D}-ARs on the CGRP-positive nerve cells in BOO rats. Naftopidil partially inhibits the cold stress overactivity, suggesting that it is mediated, at least partially, through alpha_{1D/1A}-ARs.

INTRODUCTION

It is generally considered that in man cold stress, produced by seasonal or continuous environmental low temperature (LT), can elicit lower urinary tract symptoms (LUTS), including urinary urgency, frequency, and nocturia. Experimental animal data supporting this showed that normal rats exposed to cold stress, produced by sudden exposure to environmental low temperature (LT), showed bladder overactivity involving resiniferatoxin-sensitive C-fibers and alpha1- adrenoceptors (AR) ^{1,2}.

Benign prostatic hyperplasia (BPH), may lead to bladder outlet obstruction (BOO) and storage, voiding, and post-voiding symptoms. In rats with BOO, it has been demonstrated that the obstruction-induced changes in the bladder wall are accompanied by neural plasticity resulting in a more prominent spinal reflex that could contribute to the development of bladder overactivity ^{3,4}, a change associated with an upregulated bladder C-fiber activity. Patients with neurogenic bladders may also have an upregulation of C-fiber mediated bladder activity ^{5,6}, and often have a positive ice water test ⁷, suggesting an enhanced sensitivity to cold. Since patients with BOO often have a positive ice water test ⁸, there may be reasons to believe that also BOO can be associated with an enhanced LT-induced bladder overactivity.

In the present study, we have tested in a rat model of BOO 1) if sudden exposure to LT can induce bladder overactivity, 2) if such activity involves alpha1-ARs, and 3) whether sudden exposure to LT changes alpha1-AR expression (RT-PCR), or affects alpha1-AR immunoreactivity on afferent nerves.

MATERIAL AND METHODS

Animals

Ten-week-old female Sprague-Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) were used for the experiments. The animals were treated in accordance with National Institutes of Health Animal Care Guidelines and the guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine (No. 230047).

Bladder Outlet Obstruction (BOO) Model

SD rats were anesthetized with pentobarbital sodium solution (40 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and then a midline incision was made to expose the urethra. To produce bladder outlet obstruction, a polyethylene tube (PE-50; inside diameter, 0.58 mm; outside diameter, 0.97 mm, Becton Dickinson and Company, Sparks, MD, USA) was placed adjacent to the urethra. Both the urethra and the tube were ligated with a 5-0 silk, and then the tube was carefully removed, leaving the ligature in place to create a mild BOO. Eight SD rats were subjected to sham operation without the ligature. All animals were kept for 4 weeks under a 12-hour alternating light-dark cycle with freely available food and water. To ascertain that the ligated rats were obstructed, only animals with a bladder capacity between 2 and 5 ml at room temperature (RT) were selected for inclusion into the final BOO study group (age-matched, non-ligated SD rats have approximately 1-1.5 ml bladder capacity at RT). Ultimately, we accepted 11 of the ligated rats as meeting the above requirements for BOO for further studies.

Drug

Naftopidil, kindly provided by Asahi Kasei Pharma Co. (Tokyo, Japan), is an α_1 -AR blocker that has a high affinity for $\alpha_{1D/1A}$ -ARs. The naftopidil powder was completely dissolved with 0.1 M phosphate buffer solution in half of the final volume. Then several drops of 0.1 M sodium dihydrogen phosphate solution were

slowly added with vortexing and ultrasonication to achieve the final volume and pH, 4.0. The dissolved naftopidil solution was diluted to desired concentration with 0.9% saline.

Cystometric Investigations

After 4 weeks, the surviving ligated rats were anesthetized as above to insert a catheter for cystometric investigations. Each urinary bladder and the ligated urethra was exposed and incised at the center of the dome. After releasing the urethral ligature, a polyethylene catheter (PE-50, Becton Dickinson and Company) was inserted through the incision and fixed at that site with a 5-0 suture. The free end was tunneled subcutaneously and exteriorized at the back of the neck. The cannulated rats were kept for 3 days as above. Just prior to the cystometric investigations, a polyethylene catheter (PE-10, Becton Dickinson and Company) was inserted into the jugular vein of the rats anesthetized by inhalation of 3% sevoflurane (Abbot Japan Co., Ltd., Tokyo, Japan). Each rat was allowed to recover from the anesthesia in a metabolic cage for 2 hours. After recovery, the bladder catheter was connected through a T-tube to a pressure transducer (P23 DC; Nihon Kohden, Tokyo, Japan) and a syringe pump (TE-351, Terumo, Tokyo, Japan). A fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments, Tokyo, Japan) to measure micturition volume was placed under the metabolic cage. Throughout the experiments, RT saline (25-27°C) was pumped into the bladder at a rate of 10 ml/hr. The bladder pressure and micturition volume were recorded continuously on a pen oscillograph (10 mm/min recording speed; Recti-Horiz-8K; NEC San-ei Instruments). The following cystometric parameters were measured: basal pressure (cmH₂O), maximum pressure (cmH₂O; = maximum pressure during the voiding cycle), voiding interval (min), micturition volume (ml), and bladder capacity (ml). The bladder capacity was calculated by adding the micturition volume and the residual volume that was determined as the difference between the saline infusion volume and micturition volume. The rats were not given food or water during the cystometric investigations.

Cystometric measurements of the catheterized, unanesthetized, unrestricted rats were taken under the following environmental temperature conditions. The rats were placed singly in metabolic cages at RT ($27\pm 2^{\circ}\text{C}$) for 20 min and then intravenously administered 0.2 ml of either 0.3 mg/kg naftopidil or vehicle, delivered slowly by hand-powered injection. After 5 min, they were gently and quickly transferred to the metabolic cages in a cold room for LT exposure ($4\pm 2^{\circ}\text{C}$) for 40 min. After the cystometric investigations, the rats were anesthetized with pentobarbital sodium solution as above, and then the urinary bladders were removed. Afterwards, the rats were euthanized by inhalation of diethyl ether.

Real-time RT-PCR

We determined the effect of BOO on the expression of α_{1A} - and α_{1D} -AR mRNA by real-time reverse-transcription polymerase chain reaction (RT-PCR). Approximately one third of the bladder tissue (from the bladder dome to the trigone) was homogenized, and total RNA was extracted with the RNeasy Mini Kit[®] (Qiagen Inc., Valencia, CA, USA). Complementary DNA (cDNA) was synthesized from 0.1 μg of total RNA with the High Capacity RNA-to-cDNA Master Mix[®] (Life Technology Co. [Applied Biosystems], Foster City, CA, USA). The synthesized cDNA was mixed with TaqMan[®] Universal PCR Master Mix (Life Technology Co.) and TaqMan[®] gene expression assays (MGB probes, FAM[™] dye-labeled, Life Technology Co.). The gene probes were α_{1A} -AR (Adra1a, Rn00567876_m1, Life Technology Co.), α_{1D} -AR (Adra1d, Rn00577931_m1, Life Technology Co.), and beta-actin (Actb, Rn00667869_m1, Applied Biosystems Co.). Real-time RT-PCR of the cDNA was performed by using the Opticon 2[®] PCR System and Opticon Monitor ver 3[®] (Bio-Rad Laboratories, Hercules, CA, USA). The reactions of the cDNA-probe mixed solution were performed at 50°C for 2 min followed by 95°C for 10 min. These were followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Gene expression was calculated by the delta-delta method as the relative ratio to threshold cycle value of the internal standard

gene, beta-actin.

Immunohistochemistry Investigations

The harvested and trimmed urinary bladders were fixed in 4% paraformaldehyde phosphate buffer solution (Nalkalai Tesque, Inc., Kyoto, Japan) for 12 hours at 4°C. The tissues were embedded in paraffin, and cut into 5- μ m thick serial sections. The sections were deparaffinized, and then antigen retrieval was achieved by immersion of the sections in 0.01 M sodium citrate (pH 6.0, Mitsubishi Chemical Medience Co., Tokyo, Japan) and microwaving at 100°C for 5 minutes. The specimens were coated with 1.5% normal donkey serum (Chemicon International Inc., Temecula, CA, USA) and 1.5% non-fat milk in 0.01 M phosphate buffered saline (PBS, pH 7.4, Mitsubishi Chemical Medience Co.) for 1 hour at 4°C. The sections were then incubated with the primary antibody for calcitonin gene-related peptide (CGRP, 1:800, guinea pig polyclonal, Progen Biotechnik GmbH, Heidelberg, Germany) as a marker of primary sensory neurons, and either α_{1A} -AR antibody (1:100, rabbit polyclonal, Santa Cruz Biotechnology Inc., CA, USA) or α_{1D} -AR antibody (1:100, rabbit polyclonal, Santa Cruz Biotechnology Inc.) for 12 hours at 4°C. The sections were rinsed with PBS at 4°C, and then incubated with donkey anti-guinea pig IgG secondary antibody conjugated with Alexa Fluor 594 (1:250, Life Technology Co. [Molecular Probes], Eugene, OR, USA) and donkey anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:250, Life Technology Co.) for 1 hour at 4°C. Following rinsing, cell nuclei were counterstained with 5 μ g/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Life Technology Co.). The slides were coated with Fluorescent Mounting Medium (Dako Cytomation, Carpinteria, CA, USA) and observed with a Leica DAS Microscopethe (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical Analysis

The results were expressed as means \pm standard error of the means. Two-tail

paired student's t-tests were used to compare values within each group. Two-tail unpaired student's t-tests were used between groups. Differences with $P < 0.05$ were considered significant.

RESULTS

Effects of Naftopidil on Cold Stress-induced Bladder Overactivity in BOO Rats

Four weeks after urethral ligation and 3 days after bladder cannulation, the rats meeting the inclusion criteria of 2 to 5 ml bladder capacity at RT were prepared for cystometric investigations at LT. The eleven BOO rats included were randomly separated into two groups. The naftopidil-treated group comprised 6 and the vehicle-treated group 5 rats. All BOO rats showed a mild bladder overactivity at baseline, with no differences between the groups (data not shown).

During LT exposure, vehicle-treated BOO rats exhibited enhanced bladder overactivity as shown by increased urinary frequency compared to RT (Fig. 1A). Naftopidil-treated rats also had LT-induced bladder overactivity (Fig. 1B); however, this was reduced compared to the vehicle-treated rats.

The basal pressure of the vehicle-treated rats at LT increased significantly, while maximum pressure did not change (Table 1). The voiding interval was reduced (from 18.75 ± 1.82 min) to 7.27 ± 0.10 min ($P < 0.01$ compared to RT, Fig. 1C), and the bladder capacity was reduced (from 3.12 ± 0.52 ml) to 1.17 ± 0.18 ml ($P < 0.01$ compared to RT, Fig. 1D). Similarly, during LT exposure, basal pressure of the naftopidil-treated rats also increased significantly, while maximum pressure did not change (Table 1). The voiding interval decreased (from 15.75 ± 0.67 min) to 9.29 ± 0.99 min ($P < 0.01$ compared to RT, Fig. 1C), and bladder capacity decreased (from 2.60 ± 0.13 ml) to 1.51 ± 0.13 ml ($P < 0.01$ compared to RT, Fig. 1D).

As mentioned, there were no differences between the vehicle- and naftopidil-treated rats in micturition parameters at RT. However, the increase of basal pressure and decreases of voiding interval and bladder capacity due to LT exposure in the naftopidil-treated rats were significantly reduced compared to the vehicle-treated rats (Table 1, Fig. 1C and D).

Expression Level of alpha1-AR mRNAs and Presence of alpha1-ARs in BOO Rats

To investigate the effects of naftopidil on cold stress-induced bladder overactivity, we semi-quantitatively estimated α_1 -AR mRNAs within the urinary bladder tissues. The expression level of α_{1A} -AR mRNA in the BOO rats was similar to the sham-operated rats (Fig. 2A). However, the expression level of α_{1D} -AR mRNA in the BOO rats was significantly higher than that in the sham-operated rats (Fig. 2B).

The α_{1A} -AR immunoreactivity on the CGRP-positive nerve cells in the sham-operated rats (Fig. 2C) was similar to that in the BOO rats (Fig. 2D). Both sham-operated and BOO rats also exhibited α_{1D} -ARs on the CGRP-positive cells (Fig. 2E and F, respectively); however, there appeared to be more in the BOO rats compared to the sham-operated rats. **Alpha₁-ARs were also present on cells other than the CGRP-positive neurons and also appeared to be present in greater numbers in the BOO rats.** This was consistent with the higher expression levels of α_1 -AR mRNAs in the bladders of these animals.

DISCUSSION

This study showed that rats with BOO and bladder capacities between 2 and 5 ml, exhibited cold stress-induced bladder overactivity when exposed to LT. During LT exposure, basal pressure increased significantly, and both voiding interval and bladder capacity decreased significantly compared to the RT baselines.

It has previously been shown that in normal rats, cold stress-induced bladder overactivity can be reduced, but not eliminated, by administration of α_1 -AR antagonists^{1,2}, and this was demonstrated to be the case also in BOO rats. Thus, the increased basal pressure and decreased voiding interval and bladder capacity found in these animals were significantly reduced by naftopidil. **However, in separate preliminary studies, it was shown that in BOO rats with bladder capacities greater than 5 ml, α_1 -AR antagonists had no effect on cold stress-induced bladder overactivity (data not shown).** While the reason for this refractoriness has not yet been investigated, it suggests that the effectiveness of α_1 -AR antagonists might be dependent on the degree of obstruction-induced changes in the bladder wall and consequent effects on neuronal plasticity. It also suggests that factors besides α_1 -ARs may contribute. In previous studies^{9,10} we determined if transient receptor potential melastatin 8 (TRPM8) channels were involved in the bladder overactivity induced by exposure to LT. It was found that the TRPM8 channel agonist, BCTC, effectively inhibited LT-induced bladder overactivity, and concluded that TRPM8 channels together with α_1 -ARs mediated this activity. Other factors for the incomplete response to the α_1 -AR antagonists that needs to be taken into consideration are that 1) the optimum concentration of naftopidil to completely inhibit the cold stress-induced bladder overactivity was not established, and 2) we did not explore the possibility that naftopidil caused an improvement of microcirculation in the bladder. Impeded blood flow is one of the mechanisms of bladder overactivity in BOO rats^{11,12}, and α_1 -AR antagonists improve storage function due to reduction of the impediment to blood flow^{13,14}.

α_1 -ARs are subdivided into three subtypes: α_{1A} -, α_{1B} - and

alpha_{1D}-AR¹⁵. The alpha₁-ARs expressed within the lower urinary tract mediate functions related to neurotransmission and endocrine responses¹⁶. A number of experimental studies showed that antagonism of alpha_{1D}-AR in the bladder, urothelium, prostate, peripheral ganglia, and spinal cord improves urinary storage^{17, 18, 19}. Thus, the antagonists of these alpha₁-ARs improve not only voiding symptoms, but also storage symptoms in patients with BPH.

Alpha₁-ARs play important roles in the activation of afferent pathways^{18, 19, 20, 21}, and we have previously shown that cold stress-induced bladder overactivity was mediated by resiniferatoxin-sensitive nerves that express CGRP¹. Thus, to investigate the mechanisms involved in the LT-induced overactivity, we focused on the expression of alpha₁-AR mRNAs within the urinary bladders and the presence alpha₁-AR immunoreactivity on the CGRP-positive nerves. Prior to these investigations, we confirmed that a single administration of vehicle or 1.0 mg/kg naftopidil did not change the expression level of mRNAs or receptors within urinary bladder tissues (data not shown).

The expression level of alpha_{1A}-AR mRNA in the BOO rats was the same as that in the sham-operated rats with intact urinary bladders. However, the expression level of alpha_{1D}-AR mRNA in the BOO rats was significantly higher than that in the sham-operated rats. Alpha₁-AR immunoreactivity was demonstrated on bladder CGRP-positive primary sensory neurons of both BOO and sham-operated rats. The immunoreactivity for alpha_{1A}-ARs in the BOO rats were similar to that the sham-operated rats. In contrast, the presence of alpha_{1D}-AR immunoreactivity on the BOO rat CGRP-positive nerves tended to be greater than that in the sham-operated rats. Further studies are required to establish with certainty the differences in distribution of these receptors in BOO and sham-operated rats.

Even with the limitations of this study, we have shown that the cold stress-induced bladder overactivity of the BOO rats is mediated, at least in part, through alpha₁-ARs, particularly the alpha_{1D}-AR. It may be speculated that the alpha_{1D/1A}-AR antagonist,

naftopidil may have the potential to improve storage symptoms exacerbated by cold stress in patients with BPH.

CONCLUSION

In rats with BOO sudden exposure to LT can induce bladder overactivity. This activity involves α_1 -ARs and can be partly reduced by the $\alpha_{1D/A}$ -AR antagonist, naftopidil. In BOO rats, sudden exposure to LT increases α_{1D} -AR expression and α_{1D} -AR immunoreactivity on afferent nerves.

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Figure Legends

Figure 1. Micturition patterns with change from RT to LT. **A:** After transfer to LT, the vehicle-treated BOO rats exhibited cold stress-induced bladder overactivity patterns including increased micturition frequency (upper tracing) and decreased of micturition volume (lower tracing). **B:** Naftopidil-treated BOO rats transferred to LT also exhibited the cold stress-induced bladder overactivity. The cold responses were reduced compared to vehicle-treated rats. Top: bladder pressure; Bottom: micturition volume. **C and D:** During LT exposure, voiding interval (C) and bladder capacity (D) of both vehicle- and naftopidil-treated BOO rats were significantly decreased. However, the decrease of these values in the naftopidil-treated rats was significantly less compared to the vehicle-treated ones. White bar: RT, black bar: LT. ** $P < 0.01$; compared to RT baseline values. † $P < 0.05$; compared to vehicle-treated rats.

Figure 2. Alpha_{1A}- and alpha_{1D}-AR mRNA and protein expression within the urinary bladders. **A and B:** The relative expression of (A) alpha_{1A}- and (B) alpha_{1D}-AR mRNA in the urinary bladders. The expression level of alpha_{1A}-AR mRNA in BOO rats was the same as in sham-operated rats with intact urinary bladders. However, the expression level of alpha_{1D}-AR mRNA in the BOO rats was significantly higher than in the sham-operated rats. * $P < 0.01$, compared to the sham rats. **C and D:** For each triad of images, the alpha_{1A}-ARs (green) are in the upper left micrograph, the CGRP-positive nerve cells (red) are in the lower left micrograph, and the merged images are in the larger micrograph to the right side of each group. There was no difference between the sham-operated (arrows, C) and BOO (arrows, D) rats in the presence of alpha_{1A}-ARs within the CGRP-positive nerve cells. **E and F:** For each triad of images, the alpha_{1D}-ARs (green) are in the upper left micrograph, the CGRP-positive nerve cells (red) are in the lower left micrograph, and the merged images are in the larger micrograph to the right side of each group. The expression of alpha_{1D}-ARs within the CGRP-positive nerve cells in the BOO rats (arrows, F) tended to higher compared to the sham-operated rats (arrow, E). Bar: 20 μm . Blue: nuclei.

Abbreviations and Acronyms

LT = low temperature

RT = room temperature

AR = adrenergic receptor

LUTS = lower urinary tract symptoms

BPH = benign prostatic hyperplasia

QOL = quality of life

BOO = bladder outlet obstruction

RT-PCR = reverse transcription polymerase chain reaction

CGRP = calcitonin gene-related peptide

Table 1. Effects of temperature and naftopidil on basal and micturition pressures in BOO rats

		RT	LT
Basal pressure (cmH ₂ O)			
	vehicle-treated	10.77±3.49	21.63±4.23*
	naftopidil-treated	4.83±0.54	10.81±1.49*†
Micturition pressure (cmH ₂ O)			
	vehicle-treated	37.37±7.68	40.37±7.84
	naftopidil-treated	31.40±5.50	33.34±4.51

RT, room temperature; LT, low temperature; *P<0.01; compared to RT in each group; †P<0.05; compared to vehicle-treated rats; vehicle-treated rats, n=5; naftopidil-treated rats, n=6.

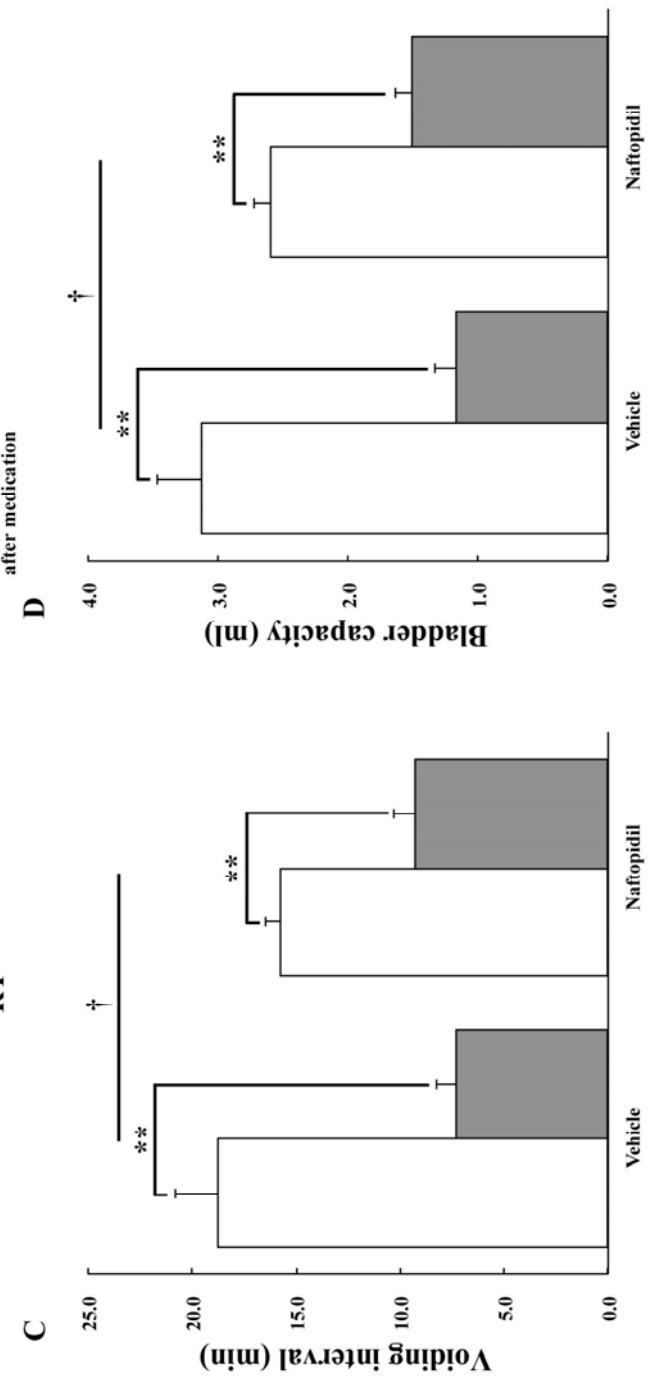


Figure 1.

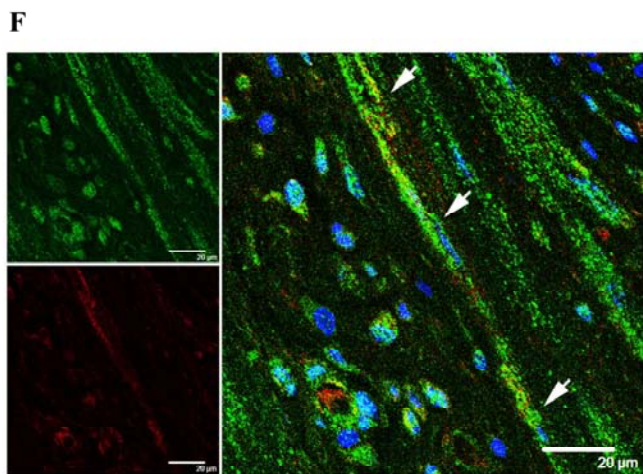
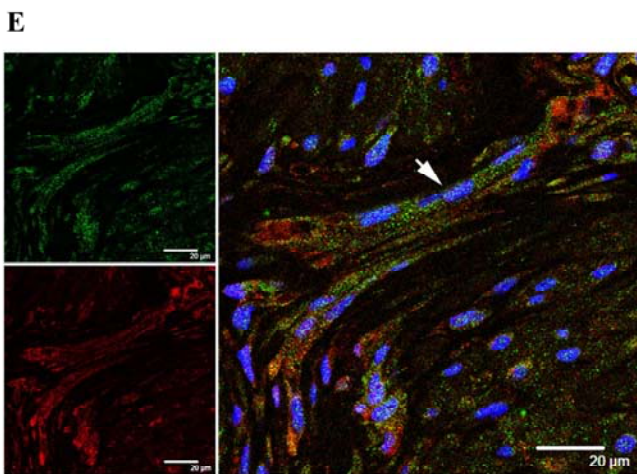
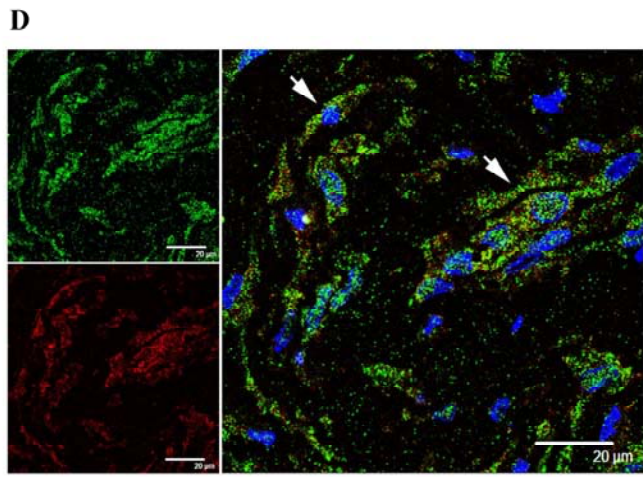
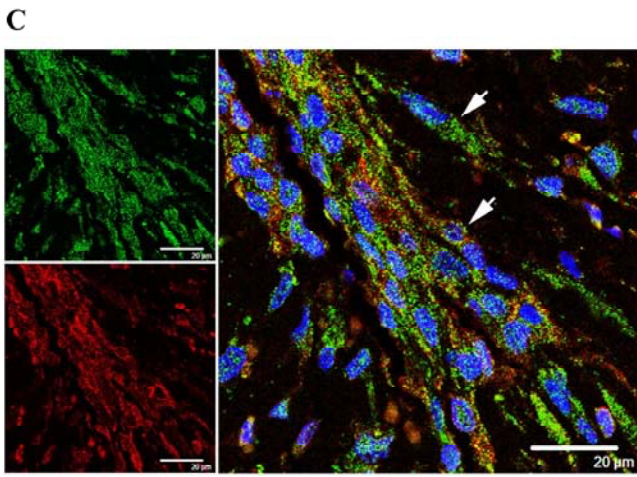
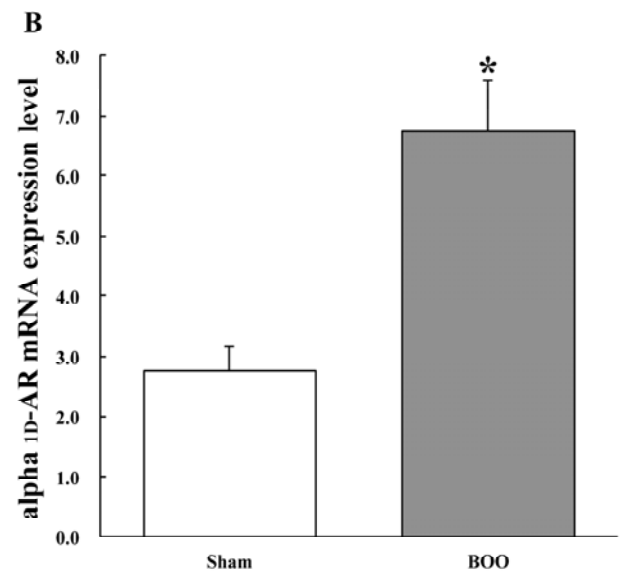
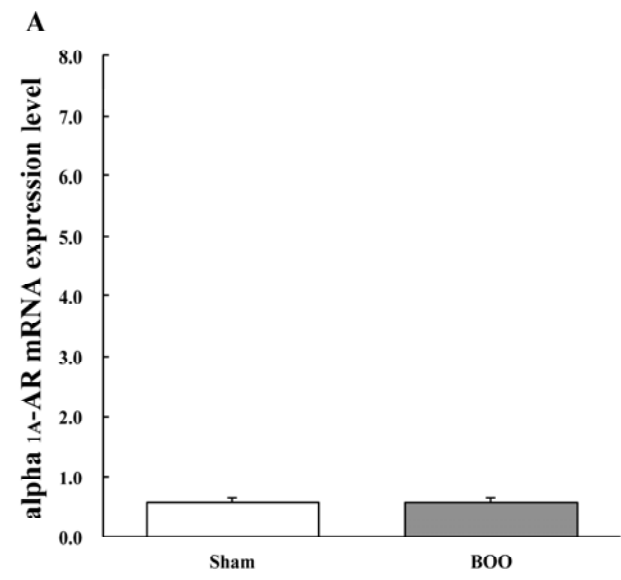


Figure 2.