Life Sciences 234 (2019) 116772

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Evaluation of a rat model of functional urinary bladder outlet obstruction produced by chronic inhibition of nitric oxide synthase

Katsuhiko Noguchi[a,*], Kimio Sugaya[a], Saori Nishijima[a], Mayuko Sakanashi[b], Katsumi Kadekawa[a], Katsuhiro Ashitomi[a], Shiho Okitsu[c], Hideyuki Yamamoto[c]

[a] Southern Knights' Laboratory Co., Ltd., Japan
[b] College of Pharmacy, Kinjo Gakuin University, Japan
[c] Department of Biochemistry, Graduate School of Medicine, University of the Ryukyus, Japan

ARTICLE INFO

Keywords:
N (omega)-Nitro-L-arginine methyl ester
Nitric oxide
Urinary frequency
Urinary bladder neck obstruction
Overactive detrusor

ABSTRACT

Aims: Ligation of the urethra to create partial bladder outlet obstruction has widely been used as an animal model of bladder obstruction, although obstructive bladder dysfunction may be due to both mechanical and functional obstruction. Previous studies in rodents have demonstrated that long-term nitric oxide (NO) deficiency can lead to detrusor overactivity, and lack of NO may thus cause impairment of bladder outlet relaxation. The aim of this study was to define the characteristics of bladder and urethral dysfunction induced by chronic NO deficiency through both in vivo and in vitro investigations.

Main methods: Rats were divided into two groups, and one group received an NO synthase inhibitor (N_{ω}-nitro-L-arginine methyl ester hydrochloride: L-NAME) in the drinking water for 4 weeks. Bladder and urethral function were evaluated by continuous cystometry and isovolumetric cystometry. In vitro functional studies of detrusor strips and measurement of the mRNA and protein expression of an ischemic marker and a gap junction protein were also performed in separate rats.

Key findings: L-NAME administration raised blood pressure and decreased plasma nitrite/nitrate level compared to the control group. L-NAME treatment increased the frequency of bladder contractions and the residual volume, and elevated urethral pressure and bladder contraction pressure. In addition, carbachol-induced contraction was reduced in isolated detrusor strips from the L-NAME group, and bladder expression of HIF-1 and connexin 43 showed upregulation.

Significance: These findings suggest that chronic administration of L-NAME to rats induces bladder hyperactivity with residual urine, and may provide a useful model of functional bladder obstruction.

1. Introduction

Various studies have documented that numerous patients of both sexes have age-related bladder dysfunction and associated lower urinary tract symptoms [1,2]. Bladder outlet obstruction (BOO) is a particularly common urological problem in men with benign prostatic hyperplasia and it causes obstructive bladder dysfunction characterized by bladder wall hypertrophy, decreased urinary flow and compliance, detrusor instability, and post-voiding residual urine [3]. Placing a suture around the urethra to induce partial BOO has been widely employed to create animal models of bladder obstruction [4], although obstructive bladder dysfunction is not only caused by mechanical obstruction and may be related to functional obstruction. Studies performed in rodents with long-term nitric oxide (NO) deficiency, originally a model of arterial hypertension [5], have identified detrusor overactivity [6–8]. This seems to occur because bladder outlet relaxation is impaired by lack of NO, which promotes neurogenic dilation of the bladder neck and urethra as part of the micturition reflex [9,10]. Because the pathophysiologic features of this alternative model of BOO are not well characterized, the present study was performed for in vivo and in vitro investigation of the characteristics of urinary bladder and urethral dysfunction induced by chronic administration of an NO synthase (NOS) inhibitor, N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME).

* Corresponding author at: Southern Knights' Laboratory Co. Ltd., 1-1-823 Miyagi, Chatan, Okinawa 904-0113, Japan.
E-mail address: knoguchi-ok@umin.ac.jp (K. Noguchi).

https://doi.org/10.1016/j.lfs.2019.116772
Received 19 March 2019; Received in revised form 5 August 2019; Accepted 13 August 2019
Available online 15 August 2019

2. Methods

2.1. Animals

Female Sprague-Dawley rats (9–10 weeks old) supplied by Japan SLC, Inc. (Shizuoka, Japan) were used. The reasons why we did not use male rats but instead female rats in the present study are as follows: Firstly, enlargement of the prostate in rats does not cause to narrow the urethra, because the urethra in rats is not encircled by the prostate unlike humans. Secondly, the rat penile urethra is so thin that catheterization is difficult. Thirdly, it is difficult to perform the measurement of intravesical pressure in male rats without insertion of a catheter via the bladder dome, which will require several days to recover from an injury due to laparotomy and to obtain stable bladder functions [11]. The study protocol was approved by the President of the University of the Ryukyus based on the judgment of the Institutional Animal Care and Use Committee.

2.2. Experimental protocol

Animals were divided into a control group and an L-NAME group. In the L-NAME group, rats received L-NAME (Cayman) in the drinking water for 4 weeks, as described previously [5], while age-matched control rats received water alone. L-NAME was dissolved in the drinking water at a concentration of 2 g/L, resulting in a daily intake of approximately 70 mg/rat.

2.3. Measurement of blood pressure

In the control group (n = 8) and the L-NAME group (n = 8), the blood pressure and heart rate were measured in conscious animals by the tail cuff method (BP-98A, Softron, Tokyo, Japan).

2.4. Measurement of plasma nitrite and nitrate

Blood samples obtained from 4 rats of the control group and 8 rats of the L-NAME group were deproteinized by addition of an equal volume of methanol. Then the plasma levels of nitrite and nitrate, which are stable metabolites of NO, were measured by the method of Griess using an HPLC system with an automated NO detector (ENO-20, Eicom, Kyoto, Japan), as described previously [12].

2.5. Cystometric experiments

Rats were anesthetized with urethane (0.6 g/kg subcutaneously) and placed in a restraining cage (NAIGAI-CFK-1P; NMS, Tokyo, Japan), after which a polyethylene catheter (PE50; Clay Adams, Parsippany, NJ, USA) was inserted transurethrally into the bladder. This catheter was connected to an infusion pump through polyethylene tubing, and the bladder was filled with physiological saline at a rate of 0.05 mL/min. Continuous cystometry was carried out for at least 90 min, and the bladder activity was recorded.

Rats were anesthetized with urethane (1.2 g/kg subcutaneously). The bladder was exposed via a lower abdominal incision, a polyethylene catheter (PE-50, Clay-Adams, Parsippany, NJ) was inserted through the bladder dome and a purse suture was placed tightly around the catheter (Supplemental Fig. 1 and 2a). This catheter was connected to an infusion pump via polyethylene tubing, allowing the bladder to be filled 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. The residual volume was measured after the last bladder contraction.

After completion of continuous cystometry, the lower abdominal wound was opened again and the bladder neck was ligated. Then another polyethylene catheter (PE-50) was inserted into the urethra through the external urethral meatus and the bladder was filled with physiological saline (0.1 mL/min) beyond the threshold volume to induce isovolumetric rhythmic contractions. Physiological saline was also infused continuously into the urethral catheter (0.05 mL/min) to measure the urethral pressure (Supplemental Fig. 1 and 2b). Bladder and urethral activity were monitored simultaneously for at least 60 min, with the urethral pressure in the storage phase or bladder contraction phase being evaluated during the final 30 min of cystometry.

2.6. Organ chamber study

The urinary bladder was excised from the control group (n = 8) and the L-NAME group (n = 7) under isoflurane anesthesia and rapidly placed in aerated Krebs solution to remove fat and connective tissues. The composition of the Krebs solution was as follows (in mM): 118 NaCl, 4.7 KCl, 1.9 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$ and 11.7 glucose. Then the bladder was cut lengthwise from base to dome into 2 × 8 mm strips, and each strip was transferred to a 25 mL organ bath containing Krebs solution at 37 °C with 95% O_2 and 5%CO_2. One end of each strip was fixed to a metal rod, and the other end was attached to a force transducer (MLTF050/ST, ADInstrument). Strips were subjected to 1 g of resting tension and allowed to equilibrate for at least 1 h. Data were recorded by using a PowerLab 4/26 (ML846, ADInstrument).

Contractile responses to 80 mM KCl, carbachol (CCh; Sigma), electric field stimulation (EFS), a purinergic receptor activator and desensitizer (10 μM α,β-methylene ATP; Calbiochem), and 30 μM $PGF_{2\alpha}$ (Ono Pharmaceutical, Osaka, Japan) were recorded as changes of tension from baseline. Concentration-response curves were constructed by cumulative addition of CCh (10 nM to 0.1 mM). For neural stimulation, the smooth muscle strips were placed between double-ring platinum electrodes in the organ bath. Then EFS (pulse width of 0.5 msec, 10 V, 2-sec duration and 2-min interval) was delivered at increasing frequencies (1, 2, 4, 8, 16 and 32 Hz) by using a stimulator with a pulse-drive amplifier (SEG-3104, Nihon Kohden) [13]. When reproducible frequency-response curves were obtained, the contractile response to EFS was recorded in the presence of 1 μM atropine, after purinoceptor desensitization by addition of 10 μM α,β-methylene ATP three times, and after addition of 1 μM tetrodotoxin (TTX; Wako, Osaka, Japan). EFS-induced contractions after addition of TTX were subtracted from the other EFS-induced contractions to obtain TTX-sensitive EFS-induced contraction. The relaxant response to 10 μM isoproterenol (Kowa, Tokyo, Japan), 100 μM sodium nitroprusside (Sigma), or 100 μM papaverine (Wako, Osaka, Japan) was examined after precontraction of bladder muscle strips with 30 μM $PGF_{2\alpha}$ or 80 mM KCl. All responses were normalized to the contraction evoked by 80 mM KCl.

2.7. Quantitative PCR

Bladder wall expression of mRNAs for cholinergic and adrenergic receptors, endothelial NOS, hypoxia inducible factor (HIF)-1α, and the gap junction protein connexin 43 was examined in the control group (n = 4) and the L-NAME group (n = 4). After the bladder was dissected free and weighed, total RNA was extracted from bladder tissue by using an RNeasy Mini Kit (Qiagen Hilden, Germany). Following treatment with DNase I to avoid genomic contamination, 1 μg of RNA was used for synthesis of cDNA with SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientifics). The primer sequences of M_2 receptors, M_3 receptors, α_{1A} receptors, eNOS, HIF-1α, connexin 43 and GAPDH are shown in Table 1.

Relative expression of target genes was analyzed by performing qRT-PCR with TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) and the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) under the following conditions: initial denaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Expression of the target genes was normalized for the geometric mean level of GAPDH expression.

Table 1
Primer sequences used in qRT-PCR experiments.

Gene name	Forward primer	Reverse primer
Chrm2	5′-ggctatcctgttggctttca-3′	5′-tccatactgtattggggatgc-3′
Chrm3	5′-tgctagccttcatcatcacg-3′	5′-cacagccagtagcccagatt-3′
Adra1a	5′-gtgggttgcttcgtcctct-3′	5′-ttaaaaacggtttccgaagg-3′
Nos3	5′-ctgctgcccgagatatcttc-3′	5′-caggtactgcagtccctcct-3′
Hif1a	5′-aacaggatggaatggagcag-3′	5′-tcgtaactggtcagctgtgg-3′
Cx43	5′-ggaagcaccatctccaactc-3′	5′-gagttcatgtccagcagcaa-3′
Gapdh	5′-ggcattgctctcaatgacaa-3′	5′-atgtaggccatgaggtccac-3′

2.8. Western blot analysis

Immediately after euthanasia, the bladder and urethra were freshly isolated and frozen in liquid nitrogen. The tissue was homogenized in 0.45 or 0.25 mL of ice-cold homogenization buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA and 0.5% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitor cocktail. After homogenization, the tissue homogenate was centrifuged at 15,000*g* for 10 min at 4 °C, and the supernatant was used for Western blot analysis. To detect connexin 43, HIF-1α and NOS, 120 or 45 μg protein was solubilized in a Laemmli sample buffer containing 2.5% 2-mercaptoethanol at 98 °C for 5 min, and subjected to 8 or 10% SDS-PAGE at room temperature. The gels were transferred to PVDF membranes, and the membranes were blocked with 5% nonfat milk. They were then incubated with specific primary antibodies against connexin 43 (#3512, Cell Signaling, 1:1000), HIF-1α (#3716S, Cell Signaling, 1:1000), NOS (pan) (#2977S, Cell Signaling, 1:1000), or GAPDH (#2118 L, Cell Signaling, 1:10000) in Can-Get immunoreaction enhancer solution (Toyobo Life Science, Osaka, Japan) or Tris-buffered saline-Tween. After being washed and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000), the membranes were developed with an enhanced chemiluminescence system (Western Lightning ECL Pro, PerkinElmer Inc., Waltham, MA). Quantitative densitometry was performed by a lumino-image analyzer (LAS-4000 mini EPUV, Fuji Film) and Multi Gauge software (version 3.0).

2.9. Statistical analysis

Data were analyzed by Student's *t*-test or ANOVA, followed by a post hoc Bonferroni's multiple comparisons test. Results are expressed as the mean ± SEM. All statistical tests were done by using Prism 7.0 (GraphPad Software, Inc., CA, USA), and $p < 0.05$ was considered to indicate statistical significance.

3. Results

Chronic administration of L-NAME, a selective NO synthase inhibitor, led to a significant increase in blood pressures compared with age-matched control rats, along with a slight decrease in body weight (Table 2). In the L-NAME group, the plasma level of nitrite/nitrate (stable metabolites of NO) was decreased to about one quarter of that in the control group (Table 2), indicating that oral administration of L-NAME for 4 weeks diminished endogenous NO production.

Table 2
Systemic hemodynamic parameters and plasma nitrite/nitrate levels in the control and L-NAME groups.

	Control group	L-NAME group
Body weight (g)	267.8 ± 4.8 (16)	254.0 ± 5.3* (15)
Systolic blood pressure (mmHg)	120.3 ± 2.4 (8)	152.4 ± 2.1** (8)
Diastolic blood pressure (mmHg)	84.8 ± 4.2 (8)	120.4 ± 2.8** (8)
Heart rate (bpm)	343.4 ± 5.9 (8)	325.6 ± 3.6* (8)
Plasma nitrite/nitrate (μmol/L)	16.7 ± 1.2 (4)	4.6 ± 0.6* (8)

Values are means ± SEM. The numbers of rats are in parentheses.
*$P < 0.05$; **$P < 0.01$ vs. the control group (Student's unpaired *t*-test).

3.1. Continuous cystometry

The interval between bladder contractions was significantly shorter in the L-NAME group than in the control group (Fig. 1). Significant reduction of voiding volume and a significant increase in residual volume were also seen in the L-NAME group. However, there were no significant differences between the two groups with regard to the baseline bladder pressure, threshold pressure of voiding bladder contractions, maximum bladder contraction pressure, duration of bladder contraction, and bladder capacity (Fig. 1). Small non-voiding contractions were seen in some rats of the L-NAME group but not in the control group (Fig. 1a and b).

3.2. Urethral pressure analysis

When we simultaneously measured the isovolumetric intravesical pressure and urethral pressure, we found that the baseline urethral pressure, threshold urethral pressure, and minimum urethral pressure were significantly increased in the L-NAME group compared with the control group. These changes of urethral pressure were associated with a significant increase in maximum bladder contraction pressure and significant shortening of the interval between bladder contractions (Fig. 2). The duration of bladder contraction was also shorter in the L-NAME group, as shown in Fig. 2i. These abnormalities of bladder and urethral function observed in the L-NAME group are typical features of bladder obstruction.

3.3. Organ chamber study

The contractile response of bladder strips to carbachol (CCh) at concentrations from 3×10^{-6} M to 10^{-4} M was significantly weaker in the L-NAME group compared with the control group (Fig. 3a). The logED50 values for carbachol contraction of the control group (−5.93 ± 0.05 M) and the L-NAME group (−5.90 ± 0.05 M) were indistinguishable, indicating that affinity of muscarinic receptors for CCh was comparable between the L-NAME group and the control group. The two groups showed no significant difference in their response to EFS, although contraction was somewhat lower at every stimulus frequency in the L-NAME group (Fig. 3b). Whole bladder weight adjusted for body weight showed no significant difference between the two groups (Fig. 3c). Chronic administration of L-NAME did not significantly influence the contractile response to high potassium (80 mM KCl), $PGF_{2\alpha}$ (30 μM) or the purinergic agonist α,β-methylene ATP (10 μM), as well as the relaxant response to the NO donor sodium nitroprusside (0.1 mM), isoproterenol (10 μM) and papaverine (0.1 mM).

3.4. Quantitative PCR

Whole bladder gene expression of HIF-1α and connexin 43 was significantly increased the L-NAME group compared with the control group, while expression of the muscarinic receptors (M_2/M_3), α_{1A}-adrenergic receptor, and endothelial NOS did not differ between the two groups (Fig. 4).

3.5. Western blot analysis

Since the L-NAME group showed significant increases in bladder expression of HIF-1α and connexin 43 at the transcriptional level, we then examined whether chronic treatment with L-NAME affects the protein expression of these molecules in separate rats. As shown in Fig. 5a and b, bladder HIF-1α protein significantly increased in the L-NAME group compared with the control group, consistent with the gene

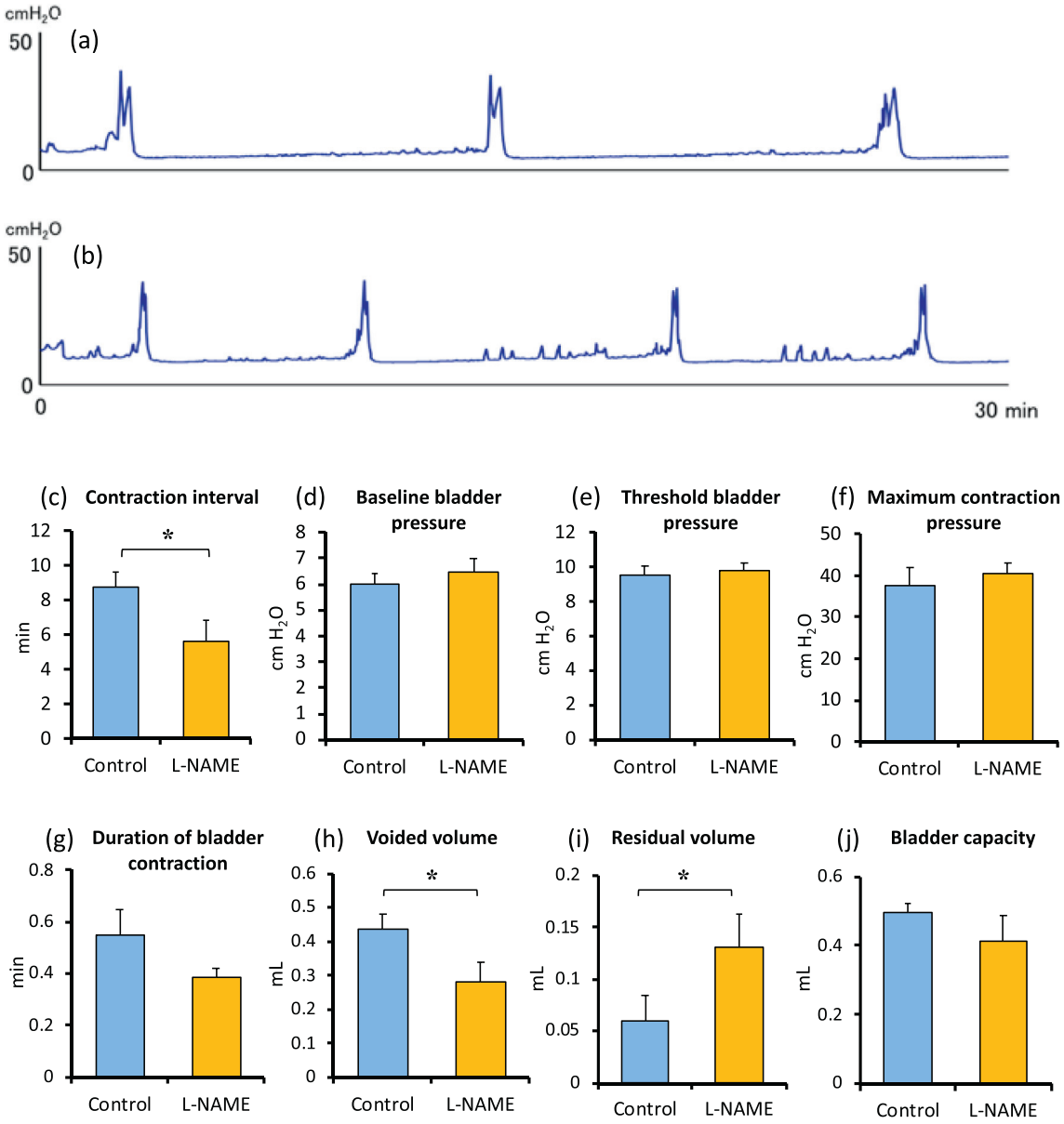

Fig. 1. Representative continuous cystometrograms for the control group (a) and the L-NAME group (b), and continuous cystometry parameters: (c) interval between bladder contractions, (d) bladder baseline pressure, (e) threshold pressure, (f) maximum bladder contraction pressure, (g) duration of bladder contraction, (h) voided volume, (i) residual volume, and (j) bladder capacity. Mean ± SEM, $n = 7$–8. *: $P < 0.05$.

expression. The expression of connexin 43 protein tended to increase in the L-NAME group compared with the control group (Fig. 5a and c), although the difference did not reach a statistically significant level ($P = 0.067$).

In addition, we also examined protein expression of NOS in the bladder and urethra. Although bladder NOS protein was not detected in the present experimental conditions, we found that there was a tendency of downregulation of urethral NOS expression in the L-NAME group compared with the control group ($P = 0.070$, Supplemental Fig. 3).

4. Discussion

In the present study, oral administration of the NOS inhibitor L-NAME for 4 weeks caused elevation of the blood pressure, as reported previously [5,14], since acute or chronic inhibition of NO biosynthesis provokes hypertension due to lack of vasodilatory NO in the systemic microvessels. The L-NAME group also showed a marked decrease in the plasma level of stable NO metabolites, indicating that chronic L-NAME administration led to systemic NO deficiency.

To our knowledge, this is the first study to simultaneously assess the effect of chronic L-NAME treatment on bladder and urethral function in vivo. Our cystometric analysis showed that L-NAME treatment led to frequency and increased the residual volume, along with elevation of urethral pressure and the maximal bladder contraction pressure. These data clearly indicate an increase in bladder outlet resistance during reflex micturition, probably due to impairment of NO-mediated urethral relaxation, since NO is thought to be the primary non-adrenergic and non-cholinergic inhibitory mediator of lower urinary tract smooth muscle [15]. Our findings are consistent with the results of previous investigations into the acute effect of NOS inhibitors on urodynamic parameters [10,16], and also correspond with recent reports that chronic NO inhibition leads to bladder hyperactivity [6–8] with the exception that the earlier study demonstrated decreased frequency of micturition cycles in addition to increased number of non-voiding contractions [6]. Thus, both acute and chronic inhibition of NO

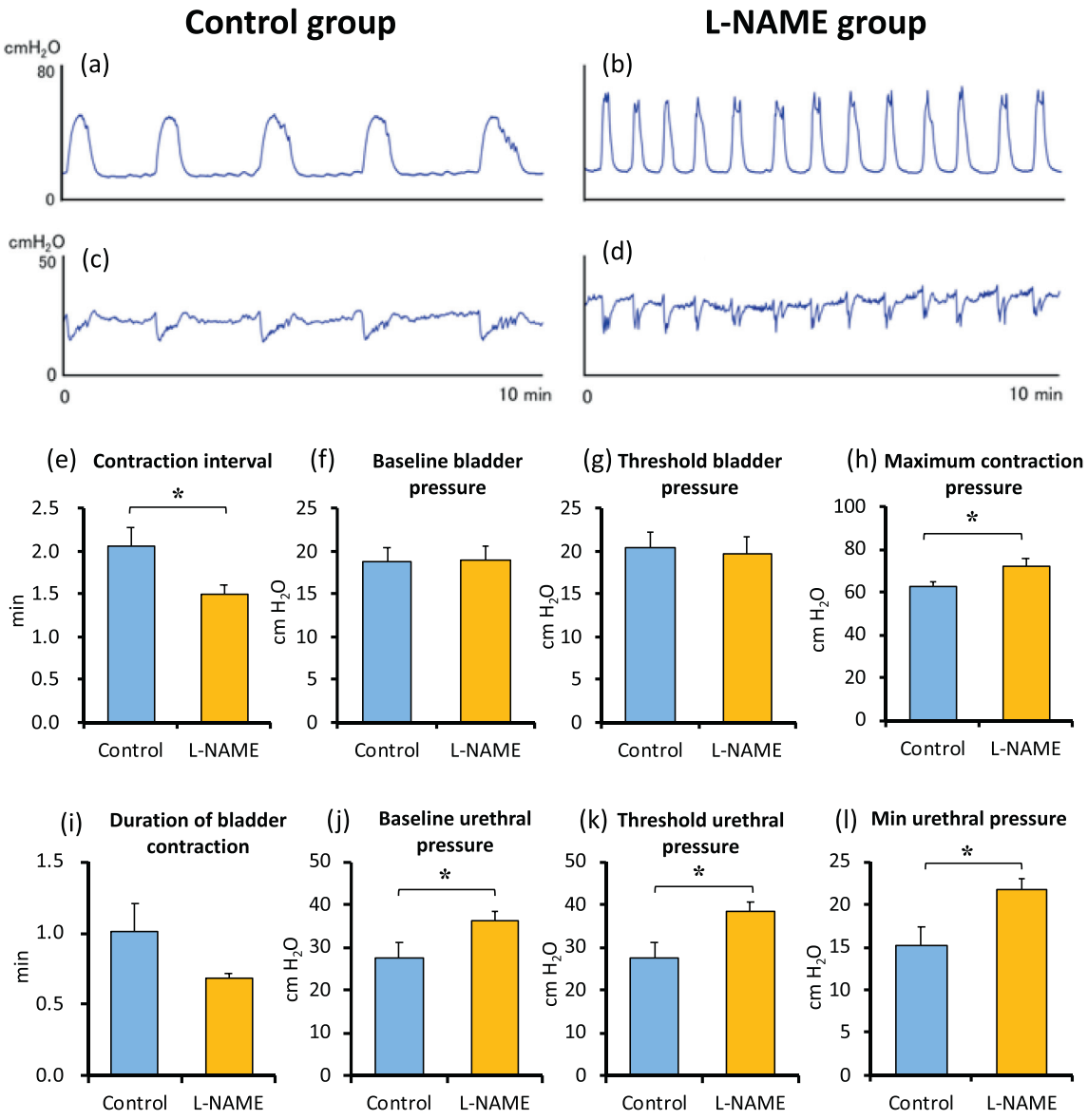

Fig. 2. Representative tracings of intravesical pressure (a and b) and urethral pressure (c and d) obtained during isovolumic cystometry in the control group and the L-NAME group, and cystometry parameters and urethral pressure data during isovolumic cystometry: (e) interval between bladder contractions, (f) baseline bladder pressure, (g) threshold pressure, (h) maximum bladder contraction pressure, (i) duration of bladder contraction, (j) baseline urethral pressure, (k) threshold urethral pressure, (l) minimum urethral pressure. Mean ± SEM, n = 7–8. *: $P < 0.05$.

biosynthesis may cause bladder dysfunction by decreasing NO-mediated effects such as inhibition of bladder afferent nervous activity [17,18].

Although the precise mechanisms underlying the induction of bladder hyperactivity by long-term administration of L-NAME remain unclear at present, it is conceivable that hypoxia or hypoperfusion resulting from loss of NO-mediated vasodilation may provoke bladder instability, since bladder ischemia was recently suggested to make an important contribution to overactive bladder syndrome [19,20]. In this study, L-NAME treatment significantly increased bladder wall gene and protein expression of HIF-1α, which is considered to be a useful marker of hypoxia [21], suggesting that the bladder was affected by chronic continuous or intermittent hypoxia. Upregulation of HIF-1α expression in bladder tissue has previously been demonstrated in rodent models of bladder obstruction induced by partial ligation of the proximal urethra [22–24]. When obstruction occurs, bladder blood flow is reduced by raised intravesical pressure during voiding and/or the increased bladder wall tissue pressure during filling, which might lead to cyclic ischemia/reperfusion injury [2]. Since nerves are sensitive to ischemic injury, chronic bladder ischemia could cause partial damage to both motor and sensory nerves that might have contributed to abnormal detrusor function in the L-NAME group. We also found upregulation of expression for the gap junction protein connexin 43 in bladders harvested from the L-NAME group. Both animal and clinical research has suggested that increased connexin 43 expression may contribute to overactive bladder symptoms by promoting intercellular communication via gap junctions in the bladder [25–27]. Taken together, these findings suggest that bladder ischemia and an increase in gap junctions might be involved in induction of detrusor hyperactivity by chronic administration of L-NAME (Fig. 6). However, further investigations will be needed to clarify the molecular mechanisms and pathophysiological significance of bladder dysfunction related to long-term NO deficiency.

The results of our organ chamber experiment suggested that chronic NOS inhibition had little influence on detrusor smooth muscle function, because the contractile responses to electrical and pharmacological stimuli were unchanged, except for slightly diminished carbachol-

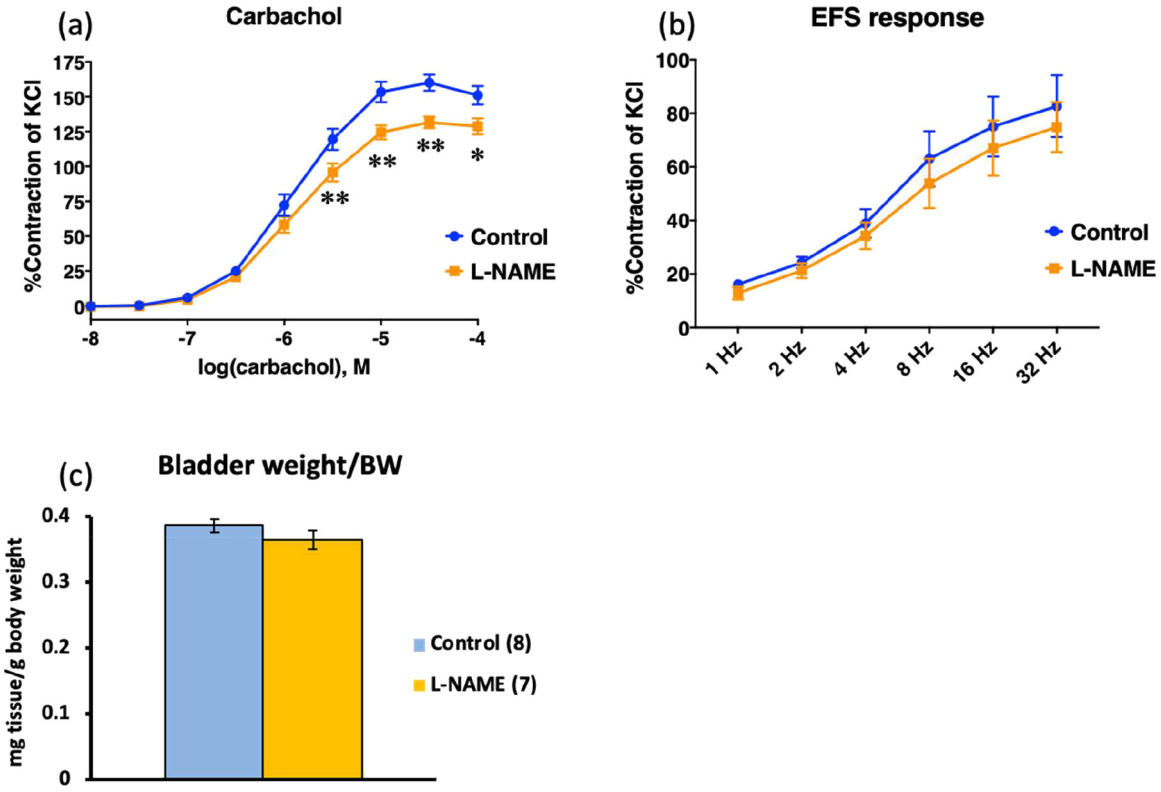

Fig. 3. Concentration-response curves for carbachol ((a) normalized for 80 mM KCl contraction, (b) response to electrical field stimulation (EFS), and (c) bladder weight adjusted for body weight in the control group (n = 8) and the L-NAME group (n = 7). Mean ± SEM. *: $P < 0.05$, **: $P < 0.01$ vs. the control group by two-way ANOVA followed by a post hoc Bonferroni's multiple comparison test.

induced contraction. These results are apparently conflicting with a previous study by Mónica et al. [28] showing increased carbachol-induced contractile responses of detrusor smooth muscle after long-term L-NAME treatment in male Wistar rats. However, diverse data regarding the effect of L-NAME administration on the contractile responses have so far been documented. For example, Nomiya et al. [29] have shown that treatment with 0.3% L-NAME in drinking water plus cholesterol diet for 8 weeks decreases contractile responses of bladder

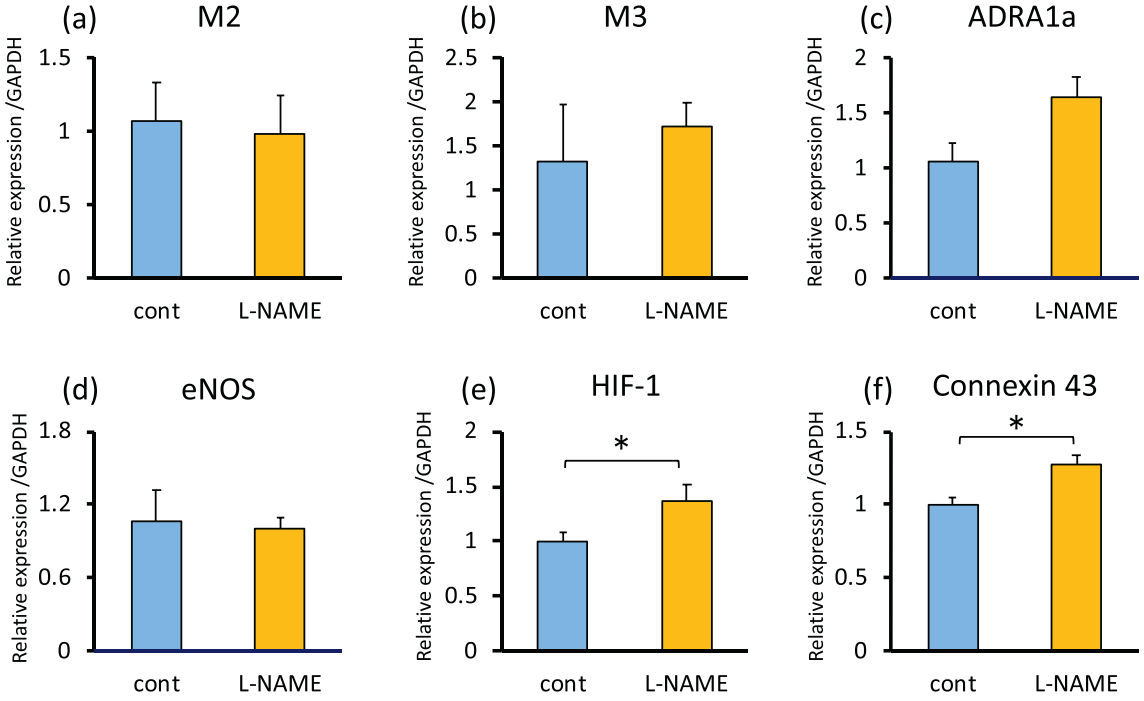

Fig. 4. Gene expression of various receptors, endothelial nitric oxide synthase (eNOS), and hypoxia-inducible factor-1 (HIF-1) in whole bladders from the control group and the L-NAME group. (a) Muscarinic M_2 receptor, (b) muscarinic M_3 receptor, (c) α_{1A}-adrenergic receptor, (d) eNOS, (e) HIF-1 and (f) connexin 43. Mean ± SEM, n = 4. *: $P < 0.05$.

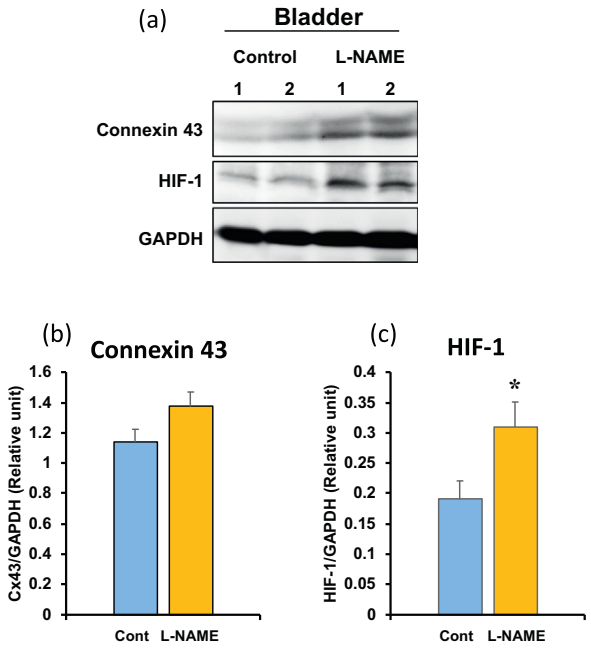

Fig. 5. Protein expression of connexin 43 (Cx43) and hypoxia-inducible factor-1α (HIF-1) in whole bladders isolated from the control and the L-NAME group. (a): Representative Western blots of Cx43, HIF-1 and GAPDH, and (b) and (c): relative densitometric analysis of Cx43 (b) and HIF-1 (c) normalized by GAPDH. Mean ± SEM, $n = 5–6$. *: $P < 0.05$.

strips to carbachol and electrical field stimulation in male Sprague Dawley rats, while Conners et al. [30] have demonstrated that continuous administration of L-NAME with an osmotic pump does not affect contractile function of bladder strips in male rabbits. An early in vitro study [16] has also revealed that electrically induced contractions of the detrusor muscle from female Sprague-Dawley rats are unaffected by addition of L-NAME ($\sim 10^{-4}$ M), which is compatible with the present observation. These inconsistent findings may reflect special features of NOS such as the negative feedback regulation of NOS activity [31] and NOS uncoupling [32]. Furthermore, these discrepancies may arise from the differences in experimental conditions employed, such as species of animal, strain and sex of rats, doses of L-NAME and the treatment duration. Nevertheless, attenuation of the contractile response to carbachol observed in the L-NAME group might contribute in part to the residual urine. In contrast to the present study, marked impairment of detrusor contractility was documented in a model of chronic bladder ischemia, and was suggested to be due to fibrosis of the bladder muscle layer and reduced innervation [33]. Thus, bladder ischemia as evidenced by the increased expression of a marker of hypoxia may not be severe enough in our model to cause distinct histological changes or nerve degeneration in detrusor muscle.

There are several limitations of the current experiments. Although the occurrence of bladder ischemia in the L-NAME group was suggested by increased expression of HIF-1α at the transcriptional and protein levels, we did not evaluate actual bladder flow in vivo. Additionally, it remains to be determined whether NO deficiency is present in bladder and urethra of L-NAME-treated rats, and whether the altered expressions of key molecules by NOS inhibition are found in a histochemical method.

Bladder obstruction models based on partial ligation of the urethra have frequently been utilized in pathophysiological and pharmacological studies, since this model has been shown to possess the features of human partial BOO, including increased bladder mass, bladder overactivity, and residual urine. However, this model has several disadvantages, such as marked variability in the extent of obstruction, a relatively high mortality rate, denervation injury around the bladder neck, and complications of laparotomy. Moreover, outlet obstruction is acute and does not allow bladder adaptation. In contrast to the fixed partial ligation model, the rat model presented in this study does not require surgery and probably leads to a gradual increase in bladder outlet resistance. We found that administration of L-NAME for 4 weeks did not affect bladder weight, despite changes of in vivo bladder function. Unlike the standard animal model, men with BOO due to benign prostate hyperplasia develop gradual and progressive obstruction, and their bladder mass is reportedly related to the duration of BOO and the intrinsic bladder response to obstruction, rather than solely depending on the severity of bladder outlet narrowing [34]. L-NAME inhibits all three NOS isozymes, including endothelial NOS, and this

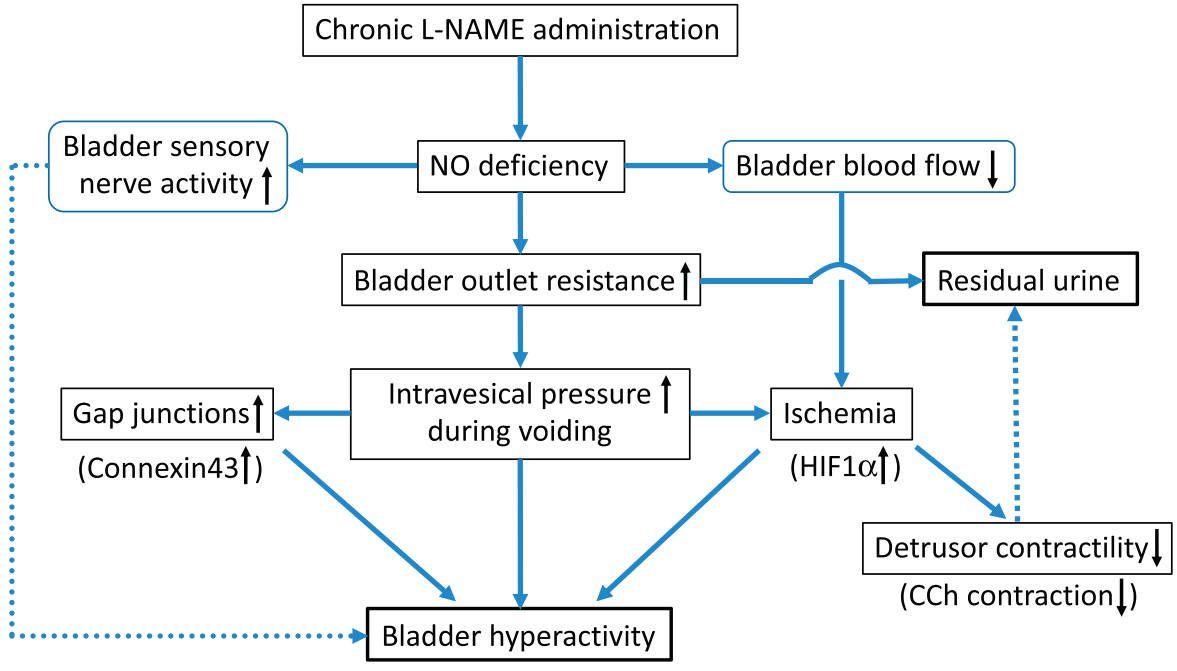

Fig. 6. Diagram of the mechanisms possibly involved in chronic L-NAME-induced bladder dysfunction.

effect can lead to impairment of endothelial cell function. The effects of aging, sex hormone imbalance, and development of atherosclerosis are closely associated with endothelial cell dysfunction [35]. In this respect, chronic L-NAME administration may potentially replicate the disease state associated with aging and hypertension.

In conclusion, chronic administration of L-NAME to rats caused frequency and residual urine together with increased urethral pressure. It is possible that long-term NO deficiency may be a useful model of lower urinary tract symptoms associated with bladder obstruction, especially at the early stage of obstruction.

Acknowledgment

None

Declaration of competing interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116772.

References

[1] D.E. Irwin, I. Milsom, S. Hunskaar, K. Reilly, Z. Kopp, S. Herschorn, K. Coyne, C. Kelleher, C. Hampel, W. Artibani, P. Abrams, Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study, Eur. Urol. 50 (2006) 1306–1314 (discussion 1314-1315).
[2] K.E. Andersson, D.B. Boedtkjer, A. Forman, The link between vascular dysfunction, bladder ischemia, and aging bladder dysfunction, Ther. Adv. Urol. 9 (2017) 11–27.
[3] R.M. Levin, P. Chichester, M.A. Hass, J.A. Gosling, R. Buttyan, Obstructive bladder dysfunction: morphological, biochemical and molecular changes, Eur. Urol. Suppl. 1 (2002) 14–20.
[4] A. Mattiasson, B. Uvelius, Changes in contractile properties in hypertrophic rat urinary bladder, J. Urol. 128 (1982) 1340–1342.
[5] M.O. Ribeiro, E. Antunes, G. de Nucci, S.M. Lovisolo, R. Zatz, Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension, Hypertension 20 (1992) 298–303.
[6] F.Z. Monica, R. Reges, D. Cohen, F.H. Silva, G. De Nucci, C.A. D'Ancona, E. Antunes, Long-term administration of BAY 41-2272 prevents bladder dysfunction in nitric oxide-deficient rats, Neurourol. Urodyn. 30 (2011) 456–460.
[7] R. Reges, C. D'Ancona, F. Monica, E. Antunes, Effect of acute administration of sildenafil to rats with detrusor overactivity induced by chronic deficiency of nitric oxide, Int. Braz. J. Urol. 39 (2013) 268–275.
[8] R.P. Regadas, R. Reges, J.B. Cerqueira, D.G. Sucupira, F.V. Jamacaru, M.O. Moraes, L.F. Gonzaga-Silva, Effects of chronic administration of tamsulosin and tadalafil, alone or in combination, in rats with bladder outlet obstruction induced by chronic nitric oxide deficiency, Int. Braz. J. Urol. 40 (2014) 546–552.
[9] I. Ehren, H. Iversen, O. Jansson, J. Adolfsson, N.P. Wiklund, Localization of nitric oxide synthase activity in the human lower urinary tract and its correlation with neuroeffector responses, Urology 44 (1994) 683–687.
[10] B.C. Bennett, M.N. Kruse, J.R. Roppolo, H.D. Flood, M. Fraser, W.C. de Groat, Neural control of urethral outlet activity in vivo: role of nitric oxide, J. Urol. 153 (1995) 2004–2009.
[11] K. Sugaya, S. Nishijima, K. Kadekawa, K. Ashitomi, T. Ueda, H. Yamamoto, Pelvic venous congestion with castration causes chronic prostatitis in rats, Int. J. Urol. 23 (2016) 431–435.
[12] K. Sugaya, S. Nishijima, K. Kadekawa, K. Ashitomi, T. Ueda, H. Yamamoto, Effects of silodosin on bladder activity in rats with frequent urination induced by pelvic venous congestion, Int. J. Urol. 23 (2016) 881–887.
[13] Y. Igawa, S. Kumano, N. Aizawa, Y. Saito, H. Ito, S. Watanabe, N. Takahashi, M. Tajimi, H. Nishimatsu, Y. Homma, Changes in the function and expression of T-type and N-type calcium channels in the rat bladder after bladder outlet obstruction, J. Urol. 191 (2014) 1159–1167.
[14] C.E. Bryant, G.H. Allcock, T.D. Warner, Comparison of effects of chronic and acute administration of NG-nitro-L-arginine methyl ester to the rat on inhibition of nitric oxide-mediated responses, Br. J. Pharmacol. 114 (1995) 1673–1679.
[15] A.L. Burnett, Nitric oxide control of lower genitourinary tract functions: a review, Urology 45 (1995) 1071–1083.
[16] K. Persson, Y. Igawa, A. Mattiasson, K.E. Andersson, Effects of inhibition of the L-arginine/nitric oxide pathway in the rat lower urinary tract in vivo and in vitro, Br. J. Pharmacol. 107 (1992) 178–184.
[17] N. Aizawa, Y. Igawa, O. Nishizawa, J.J. Wyndaele, Effects of nitric oxide on the primary bladder afferent activities of the rat with and without intravesical acrolein treatment, Eur. Urol. 59 (2011) 264–271.
[18] Y. Yu, W.C. de Groat, Nitric oxide modulates bladder afferent nerve activity in the in vitro urinary bladder-pelvic nerve preparation from rats with cyclophosphamide induced cystitis, Brain Res. 1490 (2013) 83–94.
[19] J. Camoes, A. Coelho, D. Castro-Diaz, F. Cruz, Lower urinary tract symptoms and aging: the impact of chronic bladder ischemia on overactive bladder syndrome, Urol. Int. 95 (2015) 373–379.
[20] F.M. Chu, R. Dmochowski, Pathophysiology of overactive bladder, Am. J. Med. 119 (2006) 3–8.
[21] G.L. Semenza, Regulation of oxygen homeostasis by hypoxia-inducible factor 1, Physiology (Bethesda) 24 (2009) 97–106.
[22] M. Ekman, B. Uvelius, S. Albinsson, K. Sward, HIF-mediated metabolic switching in bladder outlet obstruction mitigates the relaxing effect of mitochondrial inhibition, Lab. Investig. 94 (2014) 557–568.
[23] M.A. Ghafar, A.G. Anastasiadis, L.E. Olsson, P. Chichester, S.A. Kaplan, R. Buttyan, R.M. Levin, Hypoxia and an angiogenic response in the partially obstructed rat bladder, Lab. Investig. 82 (2002) 903–909.
[24] N. Iguchi, A. Hou, H.K. Koul, D.T. Wilcox, Partial bladder outlet obstruction in mice may cause E-cadherin repression through hypoxia induced pathway, J. Urol. 192 (2014) 964–972.
[25] G.J. Christ, N.S. Day, M. Day, W. Zhao, K. Persson, R.K. Pandita, K.E. Andersson, Increased connexin43-mediated intercellular communication in a rat model of bladder overactivity in vivo, Am J Physiol Regul Integr Comp Physiol 284 (2003) R1241–R1248.
[26] A.M.J. Haferkamp, P.J. Bastian, A. Reitz, J. Dörsam, J. Pannek, S. Schumacher, B. Schurch, R. Büttner, S.C. Müller, Increased expression of connexin 43 in the overactive neurogenic detrusor, Eur. Urol. 46 (2004) 799–805.
[27] M. Miyazato, K. Sugaya, S. Nishijima, K. Kadekawa, N. Machida, Y. Oshiro, S. Saito, Changes of bladder activity and connexin 43-derived gap junctions after partial bladder-outlet obstruction in rats, Int. Urol. Nephrol. 41 (2009) 815–821.
[28] F.Z. Monica, A.A. Bricola, F.R. Bau, L.L. Freitas, S.A. Teixeira, M.N. Muscara, F.M. Abdalla, C.S. Porto, G. De Nucci, A. Zanesco, E. Antunes, Long-term nitric oxide deficiency causes muscarinic supersensitivity and reduces beta(3)-adrenoceptor-mediated relaxation, causing rat detrusor overactivity, Br. J. Pharmacol. 153 (2008) 1659–1668.
[29] M. Nomiya, O. Yamaguchi, H. Akaihata, J. Hata, N. Sawada, Y. Kojima, K.E. Andersson, Progressive vascular damage may lead to bladder underactivity in rats, J. Urol. 191 (2014) 1462–1469.
[30] W. Conners, C. Whitebeck, P. Chicester, R. Legget, A.D. Lin, A. Johnson, B. Kogan, R. Levin, A. Mannikarottu, L-NAME, a nitric oxide synthase inhibitor, diminishes oxidative damage in urinary bladder partial outlet obstruction, Am J Physiol Renal Physiol 290 (2006) F357–F363.
[31] J. Kopincova, A. Puzserova, I. Bernatova, L-NAME in the cardiovascular system - nitric oxide synthase activator? Pharmacol. Rep. 64 (2012) 511–520.
[32] J. Vasquez-Vivar, B. Kalyanaraman, P. Martasek, N. Hogg, B.S. Masters, H. Karoui, P. Tordo, K.A. Pritchard Jr., Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9220–9225.
[33] K. Sagawa, K. Aikawa, M. Nomiya, S. Ogawa, H. Akaihata, N. Takahashi, O. Yamaguchi, Y. Kojima, Impaired detrusor contractility in a rat model of chronic bladder ischemia, Urology 81 (2013) 1379 e1379–1314.
[34] F.G. Almeida, D.G. Freitas, H. Bruschini, Is the ultrasound-estimated bladder weight a reliable method for evaluating bladder outlet obstruction? BJU Int. 108 (2011) 864–867.
[35] M.A. Gimbrone Jr., G. Garcia-Cardena, Endothelial cell dysfunction and the pathobiology of atherosclerosis, Circ. Res. 118 (2016) 620–636.