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RESEARCH ARTICLE | *Translational Physiology*

Crucial roles of nitric oxide synthases in β-adrenoceptor-mediated bladder relaxation in mice

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Satake Y, Satoh K, Nogi M, Omura J, Godo S, Miyata S, Saito H, Tanaka S, Ikumi Y, Yamashita S, Kaiho Y, Tsutsui M, Arai Y, Shimokawa H. Crucial roles of nitric oxide synthases in β-adrenoceptor-mediated bladder relaxation in mice. *Am J Physiol Renal Physiol* 312: F33–F42, 2017. First published October 26, 2016; doi:10.1152/ajprenal.00137.2016.—The specific roles of nitric oxide (NO) synthases (NOSs) in bladder smooth muscle remain to be elucidated. We examined the roles of NOSs in β-adrenoceptor (AR)-mediated bladder relaxation. Male mice (C57BL6) deficient of neuronal NOS [nNOS-knockout (KO)], endothelial NOS (eNOS-KO), neuronal/endothelial NOS (n/eNOS-KO), neuronal/endothelial/inducible NOS (n/e/iNOS-KO), and their controls [wild-type (WT)] were used. Immunohistochemical analysis was performed in the bladder. Then the responses to relaxing agents and the effects of several inhibitors on the relaxing responses were examined in bladder strips precontracted with carbachol. Immunofluorescence staining showed expressions of nNOS and eNOS in the urothelium and smooth muscle of the bladder. Isoproterenol-induced relaxations were significantly reduced in nNOS-KO mice and were further reduced in n/eNOS-KO and n/e/iNOS-KO mice compared with WT mice. The relaxation in n/e/iNOS-KO mice was almost the same as in n/eNOS-KO mice. Inhibition of Ca^{2+}-activated K^+ (K_{Ca}) channel with charybdotoxin and apamin abolished isoproterenol-induced bladder relaxation in WT mice. Moreover, direct activation of K_{Ca} channel with NS1619 caused comparable extent of relaxations among WT, nNOS-KO, and n/eNOS-KO mice. In contrast, NONOate (a NO donor) or hydrogen peroxide (H_2O_2) (another possible relaxing factor from eNOS) caused minimal relaxations, and catalase (H_2O_2 scavenger) had no inhibitory effects on isoproterenol-induced relaxations. These results indicate that both nNOS and eNOS are substantially involved in β-AR-mediated bladder relaxations in a NO- or H_2O_2-independent manner through activation of K_{Ca} channels.

bladder; nitric oxide; nitric oxide synthase; smooth muscle relaxation

BLADDER IS A UNIQUE ORGAN that stores and excretes urine by smooth muscle relaxation and contraction, respectively (39). The physiological contraction of the bladder is principally caused by acetylcholine released from parasympathetic nerve terminal (4). Adenosine triphosphate, bradykinin, and 5-HT are also associated with bladder contractions in physiological or pathological conditions (27, 48, 59). It has also been demonstrated that bladder relaxation is caused by noradrenaline-induced activation of β-adrenoceptors (AR) (34, 39). The balance of contraction and relaxation is regulated by many mechanisms in the bladder (8), including nitric oxide (NO) (20). NO is synthesized by three distinct NO synthase (NOS) isoforms [neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)] (18). nNOS and eNOS are constitutively expressed in various organs and tissues (18). Major functions of nNOS are synaptic plasticity, blood pressure regulation, and neurotransmission, such as penile erection (18), whereas those of eNOS include vasodilatation, prevention of atherosclerosis, and erection (10). In contrast, iNOS is upregulated in response to inflammation, such as urinary tract infection (5).

In the lower urinary tract, all of the NOS isoforms are expressed, playing crucial physiological roles in smooth muscle relaxation of the urethra (35). nNOS and NO mediate urethral relaxation in mice (12), and neuronally released NO also acts on the urethral sphincter and prostatic smooth muscle cells (35). Reduced NOS/NO promotes prostatic smooth muscle proliferation and enhance urinary tract symptoms in patients with benign prostatic hyperplasia (38). Recently, phosphodiesterase type 5 inhibitors with NO-mediated relaxing effects are used for men with lower urinary tract symptoms (5, 15, 20). However, direct relaxing effects of NO in bladder smooth muscle (detrusor muscle) may be minimal in animals and humans (58). Detrusor relaxation to a NO donor may also be trivial in mice (12) and humans (54). Although NOS/NO functions in the detrusor muscle have not been fully understood, they may be involved in the bladder functions because phosphodiesterase type 5 inhibitors improve not only the excretion symptoms, but also the storage symptoms (15). The major role of NOSs in the bladder is considered to be mediated by modulation of afferent nerve signals (5). Indeed, it has been demonstrated that NO modulates N-type Ca^{2+} channels in bladder afferent neurons (60) or inhibits afferent nerve from the bladder (2, 14). NO is also produced by eNOS and is released from the urothelium in response to noradrenaline or β-AR agonist (9). Urothelium-derived NO may modulate the afferent nerve activities (56). NO donor was shown to decrease the amplitude of spontaneous and carbachol (CCh)-enhanced contrac-

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tions via cGMP and protein kinase G (7), suggesting that NOS/NO is associated with bladder smooth muscle activity. In this way, there is a long time question as to whether NO has direct effects on the smooth muscle cells of the bladder (31, 43). Furthermore, the specific roles and regulations of NOS isoforms in bladder smooth muscle relaxation remain to be fully elucidated. In most of the previous studies, physiological roles of NOS in the urinary tract were examined by pharmacological intervention with NOS inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (6, 41) and N^{G}-

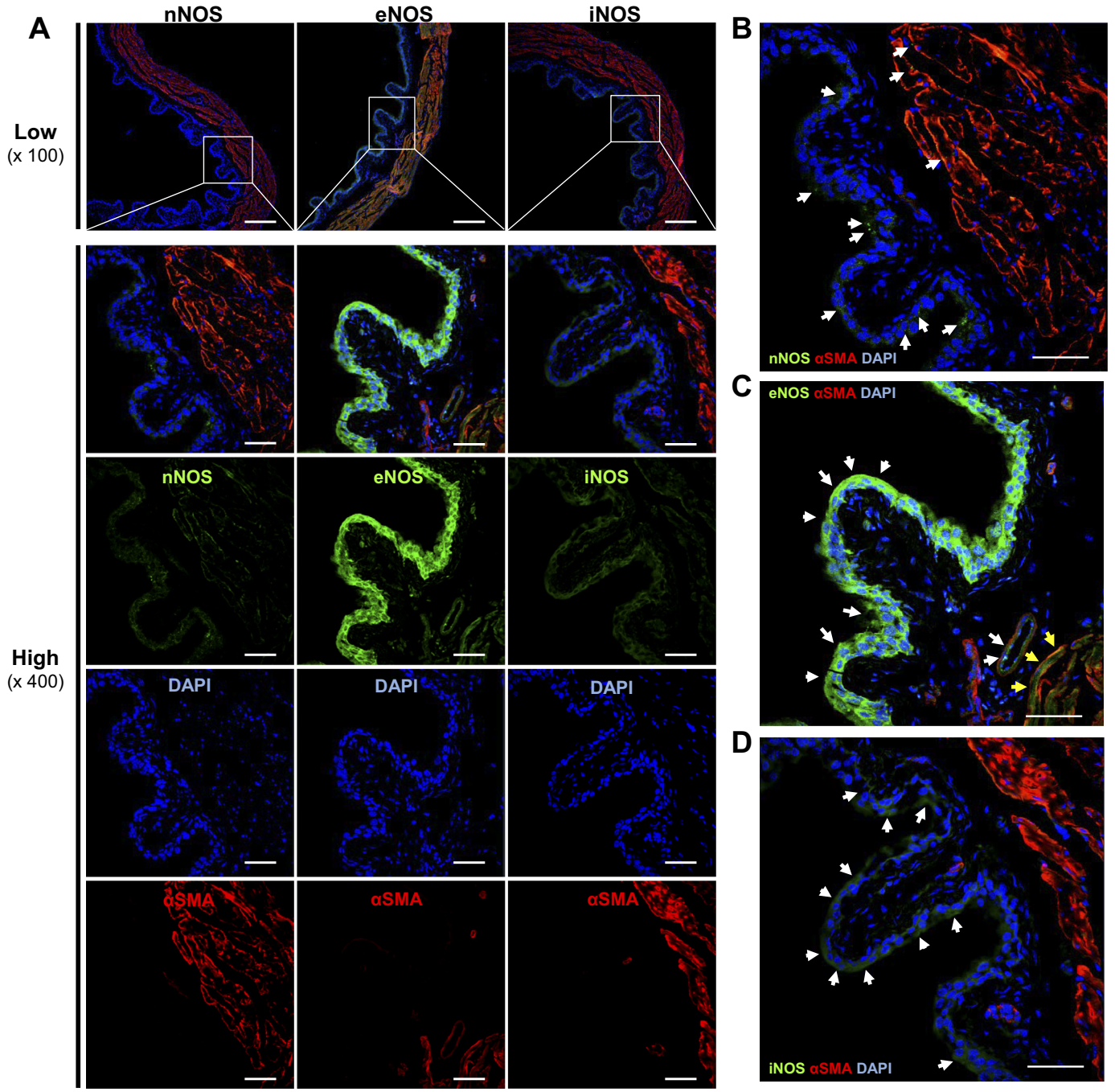

Fig. 1. Expression of three NOS isoforms in the bladder. *A*: representative immunofluorescence staining showing neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) in the bladder of wild-type mice. Bladder smooth muscle cells and vascular smooth muscle cells are stained with α-smooth muscle actin (α-SMA, Cy3, red). Three NOS isoforms (nNOS, eNOS, and iNOS) are shown in green (Alexa Fluor-488) in the urothelium and bladder smooth muscle cells. Nuclei are shown in blue [4′-6-diamidino-2- phenylindole (DAPI)]. Scale bars in low-power fields, 200 μm, and in high-power fields, 50 μm. *B*: representative immunofluorescence staining of nNOS in the bladder. nNOS is expressed in the urothelium and bladder smooth muscle cells, with spotty patterns (white arrows). Scale bars, 50 μm. *C*: representative immunofluorescence staining of eNOS in the bladder. eNOS is expressed in all layers of the urothelium, suburothelial microvascular endothelium (white arrows), and bladder smooth muscle cells (yellow arrows). Scale bars, 50 μm. *D*: representative immunofluorescence staining of iNOS in the bladder. iNOS is expressed to the limited extent in the urothelium (white arrows) and smooth muscle cells. Scale bars, 50 μm.

monomethyl-L-arginine (36). However, those NOS inhibitors possess nonspecific actions (13). Although some studies reported the role of nNOS in the bladder using nNOS-knockout (KO) mice (12, 51), the results were contradictory, probably due to possible compensatory mechanisms among the NOS isoforms (50, 55).

Thus, in the present study, we examined the roles of eNOS and nNOS in modulation of bladder smooth muscle relaxation, contraction, and urinary tract function, using eNOS, nNOS, n/eNOS double-KO mice, and n/e/iNOS triple-KO mice.

METHODS

Animals. This study was approved by the Committee on Ethics of Animal Experiments of Tohoku University (no. 2014-Kodo-006), in accordance with the "Guidelines for Proper Conduct of Animal Experiments, Japan" and the "Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academies, USA." nNOS-KO (23), eNOS-KO (24), n/eNOS-KO (42), and n/e/iNOS-KO mice (42) (11–14 wk old males, C57BL6 background) and their controls [wild type (WT)] were used. We generated n/eNOS-KO and n/e/iNOS-KO mice by crossing nNOS-KO, eNOS-KO mice, and iNOS-KO mice (42). All animals were fed a standard chow and maintained on 12:12-h light-dark cycles.

Immunohistochemistry. Mice were anesthetized with isoflurane (1.5%) and perfused with calcium- and magnesium-free phosphate-buffered saline and 4% paraformaldehyde via the inferior vena cava. Bladders were removed and fixed with 4% paraformaldehyde and dehydrated in phosphate-buffered saline containing 10, 20, and 30% sucrose, were then embedded in optimal cutting temperature compound, and frozen sections were prepared. Tissues were incubated in Tris-buffered saline with 3% bovine serum albumin containing 0.2% Triton X-100 for 1 h at room temperature and incubated with primary antibody overnight at 4°C. Primary antibodies used were rabbit polyclonal nNOS antibody (610310, BD Transduction Laboratories, Lexington, KY), rabbit polyclonal eNOS antibody (ab66127, Abcam, Cambridge, UK), rabbit polyclonal iNOS antibody (ab3523, Abcam, Cambridge, UK), and mouse monoclonal anti-α-smooth muscle actin-Cy3 antibody (C6198, Sigma Aldrich, St. Lois, MO). All primary antibodies were used at a dilution of 1:400, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG (H^+L; A11008, Invitrogen Molecular Probes) for 1.5 h. Tissues were mounted using mounting solution contained with 4′-6-diamidino-2-phenylindole. Slides were viewed with a LSM 780 confocal microscope (Carl Zeiss, Jena, Germany).

Tissue preparations. Mice were killed by intraperitoneal injection of pentobarbital, and bladders were immediately removed via a lower midabdominal incision. Transverse circular strips from the middle of the bladder excluding bladder neck were dissected in 3-mm widths. In some strips, the urothelium was gently removed by peeling the lamina propria with a cotton swab under a microscope. The strips were mounted in the 5-ml organ bath system (myograph, 620M, Danish Myo Technology, Aarhus, Denmark) containing Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2 at 37°C. The strips were stretched to a tension of 5 mN, the tension was adjusted to 5 mN every 2–5 min for 1 h, and the buffer solution was refreshed every 15 min. The strips were equilibrated for 30 min. Following the equilibration, the strips were contracted with 60 mM KCl. After washout and 30 min later, the experiments were performed. The responses were monitored by a computer-based analysis system in LabChart 7.0 software.

Relaxation and contraction effects. Strips were precontracted with 1 μM CCh, and, after ~25–35 min when the strips had reached a stable tension, cumulative concentration of relaxing agents was administered. The relaxation responses were calculated as a percentage of precontraction level induced by CCh (45). To verify the contributions of adenylyl cyclase, prostaglandins, NO, hydrogen peroxide (H_2O_2), and Ca^{2+}-activated K^+ (K_{Ca}) channel and for β-AR-mediated relaxations, we used each inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, 10^{-4} M), indomethacin (10^{-4} M), N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} M), catalase (1,250 U/ml), iberiotoxin [large-conductance Ca^{2+}-activated K^+ channel (BK) inhibitor, 10^{-7} M], a combination of charybdotoxin [BK channel and intermediate-conductance Ca^{2+}-activated K^+ (IK) channel inhibitor, 10^{-7} M], and apamin [small-conductance Ca^{2+}-activated K^+ (SK) channel inhibitor, 10^{-7} M], respectively. Catalase was applied 60 min before, and other inhibitors were applied 30 min before, precontraction with CCh. We also assessed relaxation responses to cumulative concentration of forskolin (adenylyl cyclase activator), 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl) −2H-benzimidazol-2-one (NS1619, K_{Ca} channel opener), sodium nitroprusside (SNP, NO donor), 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (DETA NONOate, NO donor), and H_2O_2. Detrusor contractions were examined in response to cumulative concentration of CCh and were calculated as a percentage of the contractions by 60 mM KCl (45).

Drugs and solution. SQ22536 was purchased from Abcam (Cambridge, UK), iberiotoxin, and charybdotoxin from Peptide Institute (Osaka, Japan), SNP from Maruishi Seiyaku (Osaka, Japan), and all other drugs from Sigma Aldrich (St. Lois, MO). The composition of Krebs-Henseleit solution was as follows (mM): Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24, Cl^- 129.7, and glucose 5.5 (314 mosmol/kgH_2O). To achieve the contractions by 60 mM KCl, we added the solutions prepared by mixture of KCl, distilled water, and Krebs-Hensleit solution (277 mosmol/kgH_2O).

Statistical analysis. Results are expressed as means ± SD. Concentration-response curve was analyzed by two-way ANOVA, followed by Tukey's honestly significant difference for multiple comparisons. Statistical analysis was performed using JMP Pro version 11 software (SAS Institute, Cary; NC) and R version 3.3.1. P value <

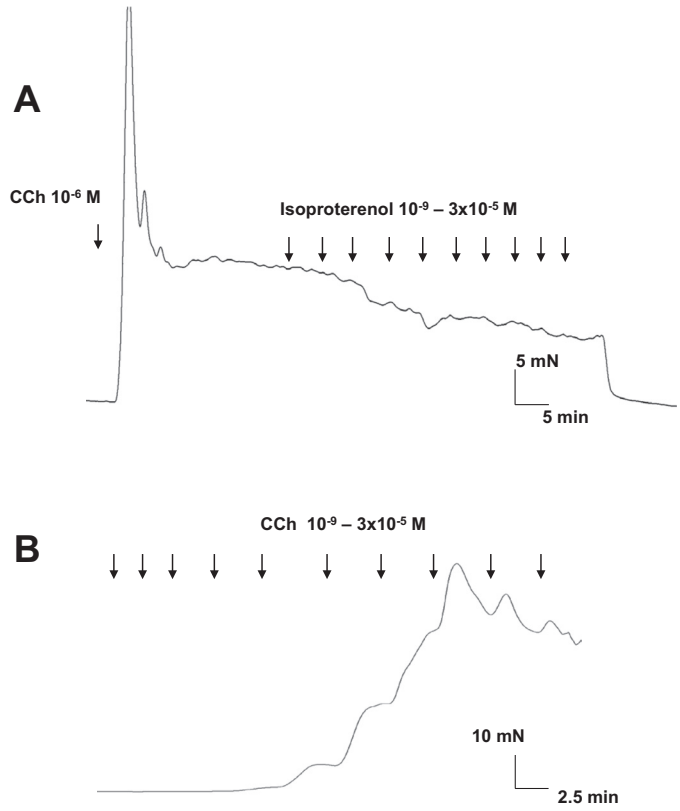

Fig. 2. Isoproterenol-induced relaxation and carbachol (CCh)-induced contraction of the bladder. *A*: representative recording of CCh-induced precontraction and isoproterenol-induced relaxation. *B*: representative recording of contraction in response to cumulative concentration of CCh.

0.05 was considered to be statistically significant. Since sample sizes had not been prespecified in the present study, *P* values were considered to be descriptive only and were not hypothesis-testing.

RESULTS

Expressions of the three NOS isoforms in the bladder. We performed immunofluorescence staining for the NOSs in the bladder of WT mice (Fig. 1). The expression pattern of nNOS was spotty in both the urothelium and bladder smooth muscle cells (Fig. 1*B*, arrows). The expression of eNOS was intense in all layers of the urothelium and suburothelial microvascular endothelium (Fig. 1*C*, white arrows), while eNOS was moderately expressed in bladder smooth muscle cells (Fig. 1*C*, yellow arrows). iNOS expression was weak in the urothelium and smooth muscle cells (Fig. 1*D*, arrows).

Role of the urothelium in bladder relaxation and contraction. CCh (1 μM) rapidly caused contraction in bladder strip, then it was decreased and reached a stable tension thereafter (Fig. 2*A*). Isoproterenol caused relaxation of bladder strip in a concentration-dependent manner (Fig. 2*A*). The contractile responses to CCh were concentration dependent until 3×10^{-5} M, but decreased with higher concentrations (Fig. 2*B*). Interestingly, isoproterenol-induced relaxations (Fig. 3*A*) were comparable between urothelium-denuded and urothelium-intact strips. Although significant difference was noted, CCh-induced contractions (Fig. 3*B*) were small between urothelium-denuded and urothelium-intact strips. These results suggest no involvement of the three NOS isoforms in the urothelium in β-AR-mediated relaxations or cholinergic contractions of the bladder. Thus we used urothelium-intact strips in the following experiments.

Both nNOS and eNOS regulate isoproterenol-induced bladder relaxation. To further examine the roles of NOSs in bladder relaxation and contraction, we used nNOS-KO, eNOS-KO, n/eNOS-KO, and n/e/iNOS-KO mice. Importantly, isoproterenol-induced relaxations of bladder strips were reduced in both nNOS-KO and eNOS-KO mice compared with WT mice and were further attenuated in n/eNOS-KO and n/e/iNOS-KO mice (Fig. 3*C*). The relaxations in n/eNOS-KO and n/e/iNOS-KO mice were comparable (Fig. 3*C*). These results suggest that both nNOS and eNOS play a crucial role in β-AR-mediated bladder relaxation, and iNOS is not related to the relaxation. In contrast, CCh-induced contraction of bladder strips was significantly reduced in nNOS-KO mice and n/eNOS-KO mice compared with WT mice. Contractions in nNOS-KO mice were mostly decreased. However, n/e/iNOS-KO mice were almost the

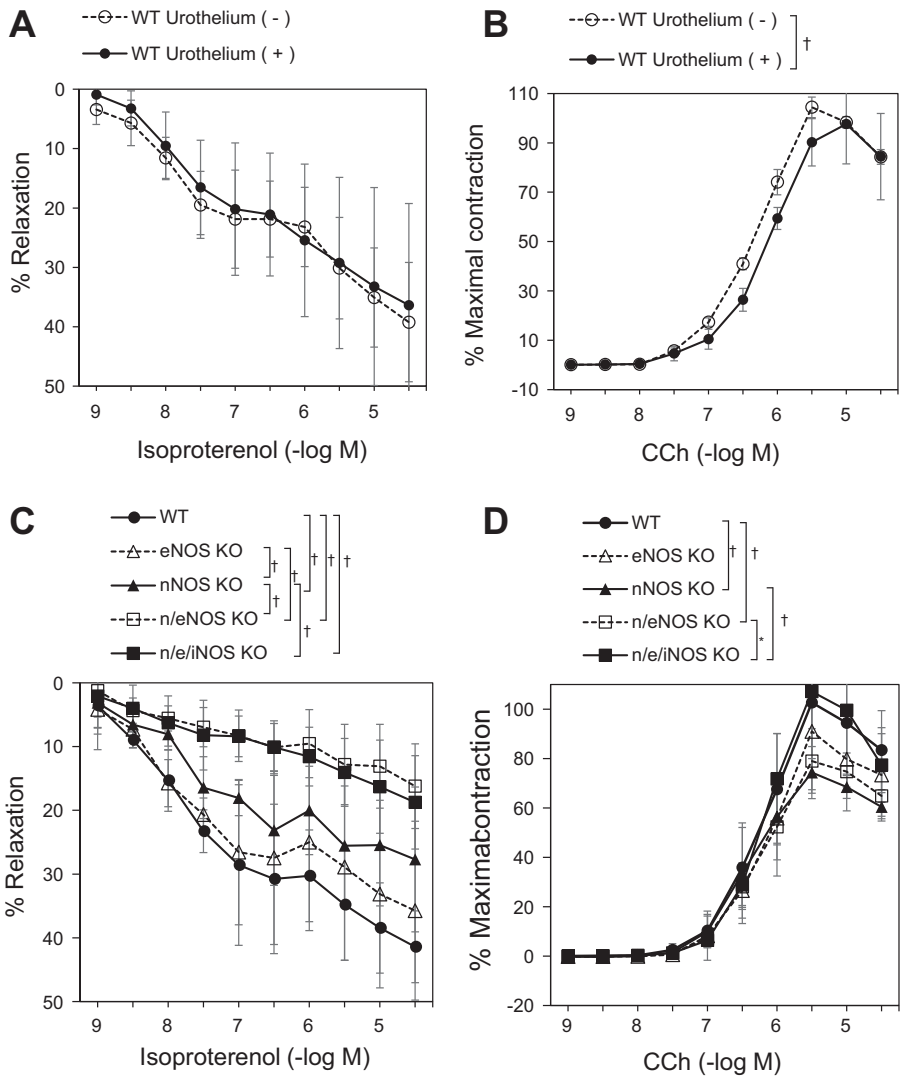

Fig. 3. Genetic disruption of nNOS and eNOS attenuates β-AR-mediated relaxation of the bladder. *A*: urothelium-denuded bladder strip and urothelium-intact strip in isoproterenol-induced relaxation ($n = 4$ each). $F = 0.57$. $P > 0.05$. *B*: urothelium-denuded bladder strip and urothelium-intact strip in CCh-induced contraction ($n = 3$ each). $F = 11.09$. †$P < 0.01$. *C*: isoproterenol-induced bladder relaxation in WT ($n = 13$), eNOS-KO ($n = 7$), nNOS-KO ($n = 4$), n/eNOS-KO ($n = 8$), and n/e/iNOS-KO mice ($n = 6$). $F = 81.63$. †$P < 0.01$. *D*: CCh-induced bladder contraction in WT ($n = 7$), eNOS-KO ($n = 2$), nNOS-KO ($n = 4$), n/eNOS-KO ($n = 4$), and n/e/iNOS-KO mice ($n = 5$). $F = 6.28$. †$P < 0.01$. *$P < 0.05$. Values are means ± SD.

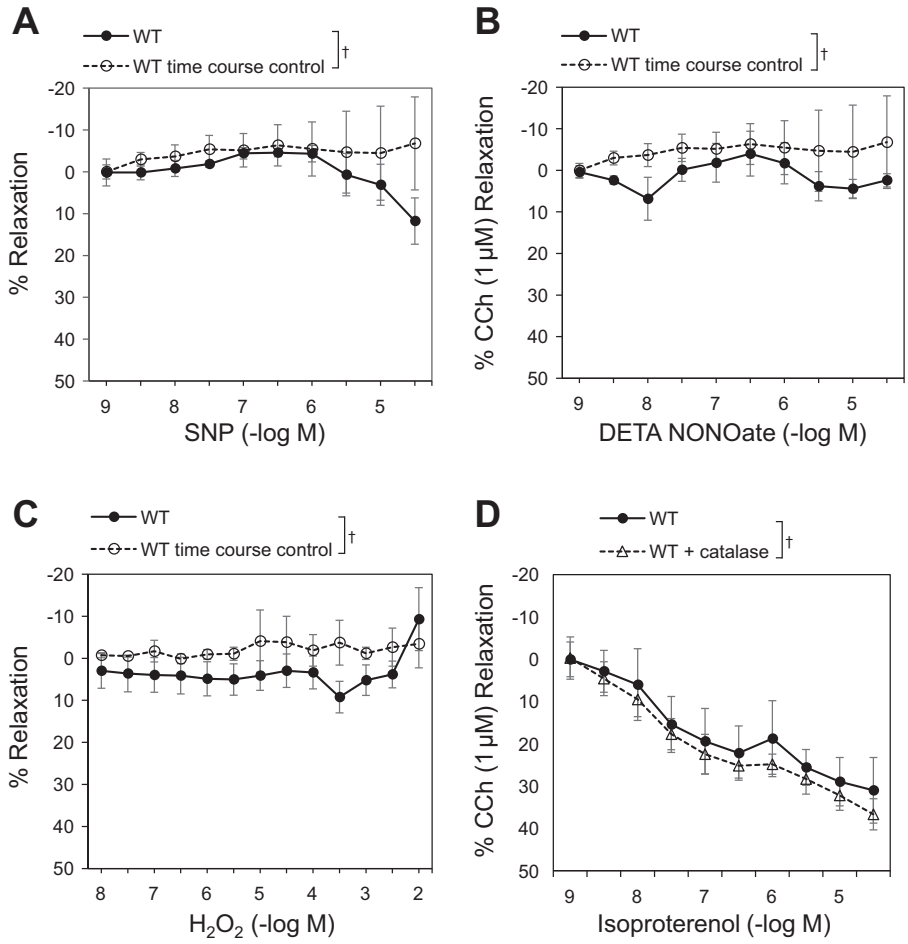

Fig. 4. Small effect of nitric oxide (NO) or hydrogen peroxide (H_2O_2) in bladder relaxation. *A*: NO donor (SNP) caused slight relaxation in WT mice ($n = 3$ each). $F = 10.78$. †$P < 0.01$. *B*: NO donor (DETA NONOate) caused small relaxation in WT mice ($n = 3$ each). $F = 16.99$. †$P < 0.01$. *C*: H_2O_2 caused small relaxation in WT mice ($n = 5$). $F = 39.99$. †$P < 0.01$. $n = 3$, WT time course control. *D*: isoproterenol-induced bladder relaxations were minimally affected in the presence of catalase. $n = 4$, WT; $n = 3$, WT with catalase. $F = 8.64$. †$P < 0.01$. Values are means ± SD.

same as WT mice in bladder contractions (Fig. 3*D*). The relations between the NOS genotype and the contractions are not clear.

Small relaxing effects of a NO donor or H_2O_2 in the bladder. To examine the role of NO and H_2O_2 in β-AR-mediated bladder relaxation, we used pharmacological approach. NO donors, SNP (Fig. 4*A*) and DETA NONOate (Fig. 4*B*), caused little relaxation in WT mice compared with time course control, indicating that NO has small direct relaxing effect in the bladder. Next, to examine the role of endogenous H_2O_2, which could be produced from eNOS in vascular wall (49), we used cumulative concentrations of H_2O_2 and catalase (scavenger of H_2O_2) for β-AR-mediated bladder relaxation. However, both H_2O_2 (Fig. 4*C*) and catalase (Fig. 4*D*) showed slight effects on bladder relaxation. These results suggest that involvement of NO and H_2O_2 in β-AR-mediated bladder relaxation is minimal in mice.

Both nNOS and eNOS regulate activation of K_{Ca} channel. To further examine the roles of NOSs in β-AR-mediated bladder relaxation, we performed several pharmacological experiments. Isoproterenol-induced relaxations of bladder strips were not altered by inhibition of BK channel (iberiotoxin) (Fig. 5*A*). Adenylyl cyclase inhibitor SQ22536 showed small inhibitory effect on isoproterenol-induced relaxation (Fig. 5*A*). Additionally, L-NNA slightly reduced the relaxations, and indomethacin did not affect the relaxations (Fig. 5*B*). These results suggest that β-AR-medicated bladder relaxations are not regulated by BK channel nor prostaglandins and are slightly affected by adenylyl cyclase or NO. In contrast, inhibition of the SK channel with apamin reduced β-AR-mediated bladder relaxation (Fig. 5*C*), and, moreover, inhibition of all types of K_{Ca} channels (BK, IK, and SK) with charybdotxin and apamin abolished the relaxation (Fig. 5, *B* and *C*), suggesting that Kc_a channels (mainly SK channel) play a crucial role in β-AR-mediated bladder relaxation in mice.

Although little difference was found between nNOS-KO and eNOS-KO mice, bladder strips of NOS KO-mice caused comparable β-AR-mediated relaxations when treated with forskolin (adenylyl cyclase activator) (Fig. 6*A*). In addition, NS1619 (K_{Ca} channel opener) also caused similar relaxations in the bladder of NOS-KO mice (Fig. 6*B*). These results suggest that the mechanistic involvement of nNOS and eNOS in β-AR-mediated bladder relaxation is not in the downstream of adenylyl cyclase or K_{Ca} channel signaling.

DISCUSSION

The major findings of the present study were that *1*) nNOS and eNOS are expressed in both the urothelium and bladder smooth muscle cells in WT mice; *2*) NO donors or H_2O_2 caused slight bladder relaxation; *3*) nNOS and eNOS played a cumulative role in β-AR-mediated bladder relaxation; and *4*) both nNOS and eNOS mainly regulated activation of K_{Ca} channel or partially regulated adenylyl cyclase. Based on these findings, we propose that both nNOS and eNOS in bladder

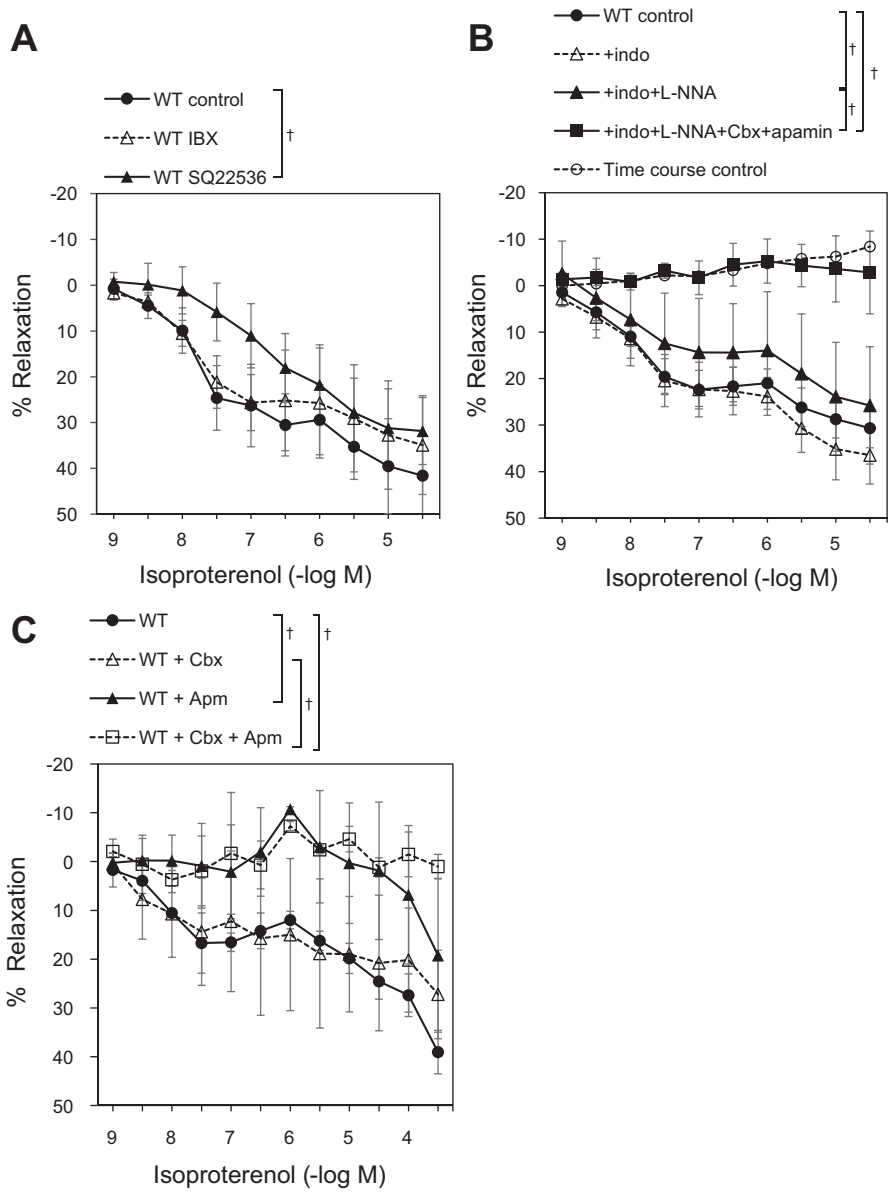

Fig. 5. K_{Ca} channel mediates isoproterenol-induced relaxation of the bladder. *A*: effects of inhibition of adenylyl cyclase (SQ22536) or BK channel (iberiotoxin) on isoproterenol-induced bladder relaxation in WT mice ($n = 3$ each). $F = 11.71$. †$P < 0.01$. *B*: effects of indomethacin (indo), L-NNA, charybdotoxin (Cbx), and apamin on isoproterenol-induced bladder relaxation in WT mice. $n = 5$, WT control; $n = 5$, with indo; $n = 5$, with indo and L-NNA; $n = 4$, with indo, L-NNA, Cbx, and apamin; $n = 2$, time course control. $F = 109.76$. †$P < 0.01$. *C*: effects of charybdotoxin and apamin on isoproterenol-induced bladder relaxation in WT mice ($n = 3$~4 each). $F = 46.81$. †$P < 0.01$. Values are means ± SD.

smooth muscle cells cause β-AR-mediated bladder relaxations through activation of K_{Ca} channels. To the best of our knowledge, this is the first study demonstrating the novel mechanism for bladder relaxation.

Small effects of NO or H_2O_2 in bladder relaxation. In the present study, NO donors (e.g., SNP and DETA NONOate) slightly relaxed bladder strip and L-NNA reduced isoproterenol-induced relaxations to a small extent. Thus the contribution of NO for bladder relaxation may be minimal in β-AR-mediated relaxation. Consistently, previous reports demonstrated that NO had little effects on isoproterenol-induced bladder relaxation in rats (19) or humans (11). Additionally, detrusor muscle has been shown to be less sensitive to NO in mice (12) and humans (44). However, the present study demonstrated that nNOS and eNOS cause β-AR-mediated bladder relaxation. H_2O_2 or catalase showed minor effects in isoproterenol-induced bladder relaxation. Thus NO and H_2O_2 may not be involved in the β-AR-mediated bladder relaxation.

Crucial roles of nNOS and eNOS in β-AR-mediated bladder relaxation. All of the three NOS isoforms are expressed in various tissues of the lower urinary tract (35). For example, expression of eNOS has been shown in the urothelium (9, 16, 26) and bladder smooth muscle cells (9, 17). In addition, expression of nNOS has been shown in nerves in interstitial tissues (12, 17) and bladder smooth muscle cells (9, 17, 28). However, the localization of eNOS and nNOS varies among the species and the reports (5). Although many studies demonstrated that NO is produced in the urothelium in response to β-AR stimulation (9), it has long been a question whether urothelium-derived NO has a direct effect on bladder smooth muscle cells. In the present study, we identified all of the NOS isoforms in both the urothelium and bladder smooth muscle cells. However, there was no significant difference in isoproterenol-induced relaxation between bladder strips with and without urothelium, and removal of the urothelium caused minor effects in CCh-induced contraction. These results indi-

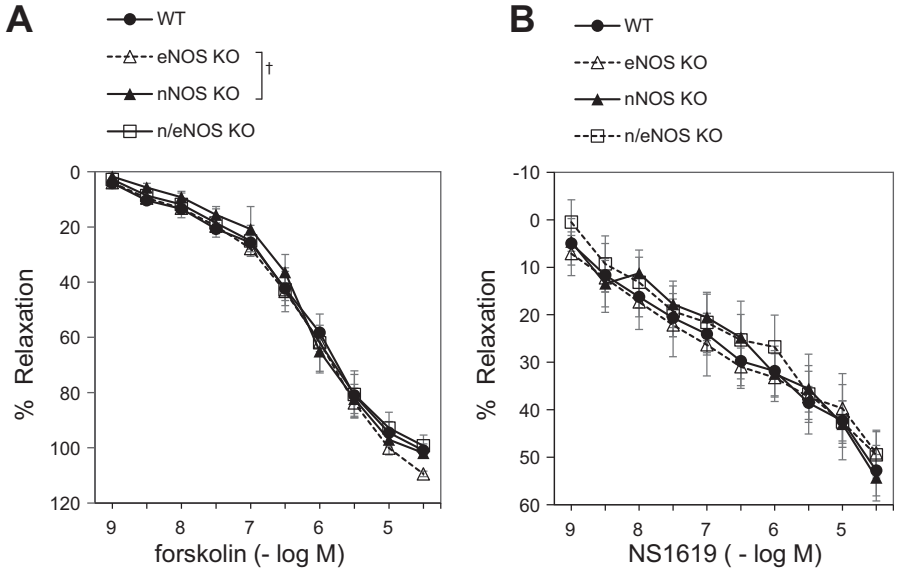

Fig. 6. Genetic disruption of nNOS and/or eNOS does not affect the bladder relaxation in response to activation of adenylyl cyclase or K_{Ca} channel. *A*: relaxation responses to cumulative concentration of forskolin (adenylyl cyclase activator) in eNOS-KO ($n = 3$), nNOS-KO ($n = 3$), n/eNOS-KO ($n = 8$), and WT mice ($n = 5$). $F = 3.01$. †$P < 0.01$. *B*: relaxation responses to cumulative concentration of NS1619 (K_{Ca} channel opener) in eNOS-KO ($n = 2$), nNOS-KO ($n = 3$), n/eNOS-KO ($n = 5$), and WT mice ($n = 4$). $F = 0.78$. Values are means ± SD.

cate that NO produced in the urothelium, if any, does not cause bladder relaxation and might affect bladder contraction.

Next, we examined isoproterenol-induced relaxation of bladder strips in nNOS-KO, eNOS-KO, n/eNOS-KO, and n/e/iNOS-KO mice. Interestingly, the relaxations were attenuated in nNOS-KO mice compared with WT mice and were further reduced in n/eNOS-KO and n/e/iNOS-KO mice. These results suggest that nNOS and eNOS in bladder smooth muscle cells play a crucial role in β-AR-mediated bladder relaxation. The relaxation in n/e/iNOS-KO mice was almost the same with n/eNOS-KO mice, indicating that iNOS is not involved in the β-AR-mediated bladder relaxation. However, we showed that NO donors minimally relaxed the bladder smooth muscle, and the inhibitory effects of L-NNA in isoproterenol-induced relaxations were by far smaller than those of n/eNOS disruption. Taken together with these results, nNOS and eNOS are associated with β-AR-mediated bladder relaxation in a NO-independent manner. Genetic disruption of n/e double NOS or n/e/i triple NOS have several benefits compared with the pharmacological inhibition. Indeed, pharmacological NOS inhibitors possess many kinds of nonspecific actions, such as muscarinic receptors antagonism or generation of superoxide anions (55). Moreover, they cannot inhibit generation of H_2O_2 from eNOS (37) or may not inhibit unknown actions of NOSs. Thus multiple NOS deficiency, including n/eNOS-KO and n/e/iNOS-KO mice, may solve the problems of compensatory mechanism (22, 29) by other preserved NOS isoforms in single NOS-KO mice. CCh-induced contraction of bladder strips showed some difference among nNOS-KO, n/eNOS-KO, n/e/iNOS-KO, and WT mice. However, regularity was not found in association with the number or the types of genetic NOS disruption.

We further performed mechanistic experiments in isoproterenol-induced bladder relaxation in WT mice, demonstrating that β-AR-mediated relaxations were not mediated by BK channel, prostaglandins. Inhibition of adenylyl cyclase slightly reduced the relaxations, indicating that adenylyl cyclase is partially associated with the β-AR-mediated bladder relaxation (19, 46). In contrast, the results with charybdotoxin (BK channel and IK channel inhibitor) and apamin (SK channel inhibitor) showed the involvement of K_{Ca} channels. Particularly, apamin treatment also reduced the relaxation, suggesting that SK channel is mainly involved in the response. These results are not consistent with the previous reports that showed the roles of BK channels in β-AR-mediated bladder relaxation (11, 19, 46). Since SK channels are expressed and involved in human bladder smooth muscle contractility (1) and associated with purinergic relaxation in the bladder (30), we consider that the channels may have important roles in bladder smooth muscle (47). However, many functions of SK channels in the bladder remain to be fully elucidated, including the role in β-AR-mediated relaxation. The present study may suggest a novel role of SK channels in the bladder. We consider that all types of K_{Ca} channels, including SK channels, are associated with β-AR-mediated bladder relaxation when we consider our present finding that the combination of charybdotoxin and apamin completely abolished the relaxation.

Although isoproterenol-induced bladder relaxation was significantly reduced in nNOS-KO and n/eNOS-KO mice, relaxations to forskolin (adenylyl cyclase activator) or NS1619 (K_{Ca} channel opener) were comparable between nNOS-KO, eNOS-KO, and n/eNOS-KO mice compared with WT mice. These results suggest that mechanistic involvement of NOSs is not in the downstream of adenylyl cyclase or K_{Ca} channels, but is in the pathway between β-AR and K_{Ca} channels. K_{Ca} channels may play a crucial role in β-AR-mediated bladder relaxation, in which eNOS and nNOS may regulate the calcium sensitivity of these channels or their associated proteins, such as calmodulin, that interact with NOS (53) and binds to the cytoplasmic COOH-terminus region of the peptide called the calmodulin binding domain in K_{Ca} channels (33). Alternatively, unknown agent produced by NOS, except NO or H_2O_2, may mediate the activation of K_{Ca} channels.

Study limitations. Several limitations should be mentioned for the present study. First, we used systemic NOS-KO mice in the present study. Systemic NOS deficiency during the development may cause neural, vascular, or urethral dysfunction. However, NOS-KO mice showed similar relaxation in re-

sponse to forskolin or NS1619 compared with WT mice, suggesting that potential relaxant ability of the bladder smooth muscle is maintained in NOS-KO mice. Second, NOS deficiency may have indirect effects on bladder relaxation. Therefore, tissue-specific KO mice will be useful for clear demonstration of each NOS isoform in bladder function. Third, in contrast to genetic disruption of eNOS and iNOS, alternative splice variants of nNOS remain in nNOS-KO mice (3). Although nNOS-β and -γ are expressed in nNOS-KO mice, NOS activity of the brain has been reported to be markedly decreased in nNOS-KO mice (23), and the expressions of nNOS and nNOS-β of the heart have been reported to be abolished in nNOS-KO mice (25). However, there is no study that showed the expressions of nNOS splice variants in the bladder. Thus this is one of the limitations in the present study. Fourth, NOS inhibitors possess many kinds of nonspecific actions, such as muscarinic receptors antagonism or generation of superoxide anions (55), and cannot inhibit generation of H_2O_2 from eNOS (37). On the other hand, genetic NOS deficiency does not involve pharmacological problems and can inhibit the H_2O_2 production by eNOS (52). Moreover, multiple NOS deficiency, including n/eNOS-KO and n/e/iNOS-KO mice, would resolve the problem of compensatory mechanism by other NOS isoforms. However, in the present study, we were unable to determine whether NOS isoform can compensate each other in the bladder of single NOS-KO mice. NOS activity and NOx production have been reported to be well preserved in single NOS-KO mice (42), and the compensatory mechanism of nNOS in response to eNOS deficiency has been shown in the vasculature (22, 29). Thus similar changes may also occur in the bladder. Fifth, in the present study, we only used male mice, and possible sex differences in NOS functions in the bladder remain to be examined. Finally, since the sample size was relatively small in some experiments, there is a possibility of failure to detect the small effects of the inhibitors or NOS deficiencies.

Clinical implications. β-AR-mediated detrusor relaxation is one of the most important mechanisms in urine storage. Bladder smooth muscle relaxation occurs via β_2-AR in mice, in contrast via β_3-AR in humans and rats (57, 58). The experiments using rats are more important in terms of clinical implication. However, NOS-KO rats are not available, and NOS inhibitor cannot inhibit all of the isoforms of NOS. Although there is a limitation as to the use of mice in the present study, genetic disruption of all isoforms of NOS may provide a new insight for better understanding of urinary function. The prevalence of storage disorders, such as overactive bladder, has been increasing along with rapid aging of the society (21). It has been shown that NOS activities are reduced in the bladder with aging in animal experiments, which may implicate the involvement of NOSs in bladder dysfunction in elderly patients (32, 40).

Conclusions. In the present study, we were able to demonstrate that both nNOS and eNOS mediate isoproterenol-induced bladder relaxation in a NO-independent manner, and that the mechanistic site of action of nNOS and eNOS may exist in the intracellular signaling between β-AR and K_{Ca} channels. Further detailed analyses will contribute to the development of novel therapy for lower urinary tract disturbances.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Y.S., M.T., Y.A., and H. Shimokawa conception and design of research; Y.S., M.N., J.O., S.G., S.T., Y.I., and S.Y. performed experiments; Y.S., K.S., S.G., S.M., and H. Saito analyzed data; Y.S., K.S., Y.K., and H. Shimokawa interpreted results of experiments; Y.S., K.S., and H. Shimokawa prepared figures; Y.S., K.S., and H. Shimokawa drafted manuscript; Y.S., K.S., and H. Shimokawa edited and revised manuscript; H. Shimokawa approved final version of manuscript.

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