Pharmacological Characterization of ASP6432, a Novel Type 1 Lysophosphatidic Acid Receptor Antagonist, in Lower Urinary Tract Functions

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Kazuyuki SAKAMOTO

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Kazuyuki SAKAMOTO

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Abstract

Benign prostatic hyperplasia (BPH) is one of the most common chronic urological diseases among elderly men. Proliferation of the prostate and contraction of prostate and urethral smooth muscles cause bladder outlet obstruction (BOO) and lower urinary tract symptoms (LUTS). Current pharmacotherapies such as alpha1-adrenoceptor antagonists (α_1-blockers), steroid 5α-reductase inhibitors, and type 5 phosphodiesterase inhibitors for alleviating BOO, LUTS, particularly bladder storage symptoms, and prostate stromal cell proliferation are in need of improvement.

Lysophosphatidic acid (LPA) is a simple phospholipid with diverse biological actions. However, the role of LPA and LPA receptors in the lower urinary tract remain poorly understood. This study investigated the role of LPA and the type 1 LPA receptor (LPA_1), the most abundantly expressed LPA receptor subtype, in the regulation of the lower urinary tract using ASP6432 (potassium 1-(2-{[3,5-dimethoxy-4-methyl-*N*-(3-phenylpropyl)benzamido]methyl}-1,3-thiazole-4-carbonyl)-3-ethyl-2,2-dioxo-2λ^6-diazathian-1-ide), a novel LPA_1 antagonist.

ASP6432 exhibited potent and selective antagonistic activity against LPA_1. ASP6432 dose-dependently inhibited LPA-induced urethral and prostate contractions. In anesthetized rats, ASP6432 decreased the urethral perfusion pressure (UPP) in the absence of exogenous LPA stimulation more potently than the α_1-blocker tamsulosin. ASP6432 also suppressed LPA-induced human prostate stroma cell proliferation. These results suggest a pivotal role for LPA_1 in the regulation of urethral tonus and prostate cell proliferation.

Because the reduction in UPP by ASP6432 suggests the importance of LPA_1 in maintaining urethral tonus, the effect of ASP6432 on UPP at the bladder filling phase (UPP_{base}) and the minimum UPP at the urine voiding phase (UPP_{nadir}) was measured in anesthetized rats to evaluate the role of LPA_1 during urine voiding. ASP6432 dose-dependently decreased UPP_{base} and UPP_{nadir}. While tamsulosin reduced UPP_{base}, it did not change UPP_{nadir}. To further investigate the potential of an LPA_1 antagonist during voiding, the effect of ASP6432 on voiding dysfunction induced by the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was evaluated. ASP6432 dose-dependently suppressed the L-NAME-induced increase in post-void residual urine (PVR) and the decreased voiding efficiency (VE), neither of which were altered by tamsulosin. These results suggest that LPA_1 may play a significant role in regulating urethral tonus during urine voiding, and that ASP6432 has the potential to improve voiding dysfunction.

One significant unmet need of current pharmacotherapies for BPH is in the improvement of urine storage symptoms. As the role of LPA and LPA_1 in the regulation of bladder function is unknown *in vivo*, the effect of LPA and ASP6432 on the micturition reflex was investigated. Intravenous infusion of LPA caused urinary frequency characterized by decreases in the micturition interval (MI) and threshold pressure (TP). ASP6432 dose-dependently inhibited the LPA-induced decrease in MI. To explore the potential of improving urinary frequency by suppressing LPA_1, the effect of ASP6432 on urinary frequency induced by L-NAME was evaluated. ASP6432 reversed the L-NAME-induced decrease in MI. These findings suggest that LPA is capable of modulating urinary frequency *in vivo* via LPA_1, and that LPA_1 antagonists can improve urinary frequency.

The present study indicates that ASP6432 had potent and selective antagonistic activity against LPA_1, and suppressed not only LPA-induced urethral contractions but also urethral pressure in the absence of external LPA stimulation to a greater extent than tamsulosin. In addition, ASP6432 suppressed urethral pressure during urine voiding and L-NAME-induced voiding dysfunction, neither of which were affected by tamsulosin. Moreover, ASP6432 inhibited the LPA-induced proliferation of human prostate stromal cells. Further, ASP6432 improved both LPA- and L-NAME-induced urinary frequency. These findings demonstrate for the first time a significant role for LPA_1 in the regulation of the lower urinary tract, and the potential of ASP6432 as a novel therapy for the treatment of BPH by relaxing the urethra, ameliorating bladder overactivity, and suppressing prostate stromal cell proliferation.

Abbreviations

AUC	area under the curve
BOO	benign outlet obstruction
BP	baseline pressure
BPE	benign prostatic enlargement
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CMG	cystometry
HFOs	high frequency oscillations
IUP	intraurethral pressure
iv	intravenous
IVP	intravesical pressure
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LPA	lysophosphatidic acid
LPA_1	type 1 lysophosphatidic acid receptor
LUTS	lower urinary tract symptoms
max IVP	maximum intravesical pressure
MI	micturition interval
NO	nitric oxide
NOS	nitric oxide synthase
PDE	phosphodiesterase
PPAR	peroxisome proliferator activated receptor
PVR	post-void residual urine
TP	threshold pressure

TRP	transient receptor potential
UAB	underactive bladder
UPP	urethral perfusion pressure
UPP_{base}	urethral perfusion pressure at the bladder filling phase
UPP_{nadir}	the minimum urethral perfusion pressure at the urine voiding phase
VE	voiding efficiency

General introduction

While few people pay attention to maintaining continence and urinating at the appropriate time and in a controlled manner, these functions are fundamental for leading a normal daily life. The storage and periodic elimination of urine are coordinated between the bladder and a bladder outlet component such as the prostate or urethra. The reciprocal functional relationship between these components is controlled by a complex neural system (de Groat et al., 2015). During bladder filling, the sympathetic pathway releases noradrenaline and activates α_1-adrenoceptors to contract the proximal part of the urethra. Noradrenaline also activates β_3-adrenoceptors expressed in the bladder to relax the detrusor smooth muscle for proper reservoir function of the bladder. When voiding urine, the parasympathetic pathway is activated to contract the bladder and relax the urethra by releasing acetylcholine and nitric oxide (NO), respectively, and adrenergic excitatory inputs are removed (Fowler et al., 2008).

The continuing increase in average life expectancy and aging population in a growing number of countries is being accompanied by an increasing number of patients with urological disorders that affect their quality of life. Benign prostatic hyperplasia (BPH) is one of the most common chronic urological diseases among elderly men, with histological BPH being detected in 60% to 70% of men over 70 years of age (Berry et al., 1984). Although the exact pathogenesis of histological BPH remains poorly understood, its clinical pathology is characterized by a hypertrophic nodule comprised of proliferative prostate epithelial cells and stromal cells around the prostatic part of the urethra (benign prostatic enlargement; BPE), mechanical and functional constriction of the prostate and

urethra causing bladder outlet obstruction (BOO), and lower urinary tract symptoms (LUTS). LUTS can be divided into two subgroups, voiding symptoms (problems in voiding urine) such as slow stream, straining, hesitancy, and terminal dribbling, and storage symptoms (problems in storing urine) such as urgency, daytime urinary frequency, and nocturia (Roehrborn, 2008). Although the traditional concept of BPH included all three of these elements, in many cases LUTS is not always correlated with clear evidence of BPE or BOO. Research suggests that functional abnormality of the prostate and urethra due to excess sympathetic inputs (McVary et al., 2005) and/or impaired nitrergic inputs (Klotz et al., 1999) may be involved in the voiding symptoms of LUTS. Meanwhile, dysregulation of bladder function is thought to contribute to storage symptoms (Chapple and Roehrborn, 2006), in addition to a decrease in functional bladder capacity due to post-void residual (PVR) urine resulting from BOO.

Since it is generally assumed that the clinical manifestations of BPH are the result of BOO, pharmacological treatment strategy has been directed toward reducing BOO (McNicholas, 2012). α_1-adrenoceptor antagonists (α_1-blockers) are prescribed as the first-line pharmacotherapy for LUTS associated with BPH (LUTS/BPH). α_1-blockers improve BOO by suppressing prostate and urethral smooth muscle contractions induced by norepinephrine released from sympathetic nerves (McNicholas, 2012). However, α_1-blockers only moderately improve clinical symptoms (40% to 60%) compared to surgical intervention (60% to 80%) (Speakman, 2001), suggesting that further reduction of the obstruction can lead to further improvements in symptoms. In addition, the efficacy of α_1-blockers on storage symptoms, which are sometimes considered more bothersome than

voiding symptoms (Chapple et al., 2014), is not always satisfactory (van Kerrebroeck et al., 2013). Furthermore, α_1-blockers have little effect on prostate hyperplasia itself (Roehrborn, 2006), indicating its ineffectiveness for treating a fundamental component of BPH. Steroid 5α-reductase inhibitors, another class of pharmacotherapies, reduce the size of the enlarged prostate and improve symptoms caused by mechanical obstruction. However, 5α-reductase inhibitors have a slower onset of efficacy and are slightly less effective at improving LUTS than α_1-blockers (Djavan, 2003). In addition, 5α-reductase inhibitors have little effect on stromal cell hyperplasia(Marks et al., 1997), a key component of human BPH (Bartsch et al., 1979). The type 5 phosphodiesterase (PDE5) inhibitor tadalafil has recently become clinically available for the treatment of LUTS associated with BPH, and has demonstrated additional potential benefits such as improving blood flow, anti-inflammatory effects, and afferent inhibitory effects (Andersson et al., 2011) . However, their positions in the treatment algorithm has not been fully established. Therefore, the unmet needs of current pharmacotherapies for BPH are as follows: i) potent relaxation of the bladder outlet component (prostate and urethra), ii) further improvement in storage symptoms, and iii) suppression of stromal cell proliferation. An agent that induces more potent urethral relaxation during voiding, suppresses stromal hyperplasia, and/or further improves bladder dysfunction is expected to improve treatment for LUTS/BPH patients.

Lysophosphatidic acid (LPA) is a simple glycerophospholipid produced in various parts of the body with diverse biological actions, including smooth muscle contraction, cell proliferation, and afferent nerve stimulation (Aikawa et al., 2015;

Yung et al., 2015). In the lower urinary tract, LPA and autotaxin, an LPA synthesizing enzyme, are present in the seminal plasma (Tanaka et al., 2004). Acylglycerol kinase, another LPA synthesizing enzyme, is overexpressed in the hyperplastic prostate nodule (Zeng et al., 2009). LPA reportedly induces urethral smooth muscle contraction (Saga et al., 2014) and cell proliferation, such as that of prostatic smooth muscle cells isolated from BPH patients (Adolfsson et al., 2002). In the bladder, LPA induces contraction of isolated detrusor smooth muscle cells (Kropp et al., 1999). These known functions of LPA suggest that it may play a physiological and pathophysiological role in mechanical (hyperplasia) and functional (contraction) BOO as well as bladder dysfunction (storage symptoms) observed in BPH. Therefore, suppressing LPA may contribute to improving LUTS/BPH, although its exact role in the lower urinary tract and its receptor subtype have not been fully elucidated.

The effects of LPA are mediated by at least six G protein-coupled receptors, LPA receptors 1 to 6 ($LPA_{1\text{-}6}$). Although these LPA receptors are broadly expressed, they vary significantly in their tissue distribution, and appear to have both distinct and overlapping biological roles (Choi et al., 2010). The type 1 LPA receptor (LPA_1) was the first identified LPA receptor subtype. LPA_1 couples with $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ to initiate downstream signaling cascades through phospholipase C, mitogen-activated protein kinase (MAPK), Akt, and RhoA. LPA_1 activation induces a range of cellular responses, including cell proliferation, cell migration and cytoskeletal changes, Ca^{2+} mobilization, and adenylyl cyclase inhibition (Yung et al., 2014). Prior studies using human samples have demonstrated more abundant expression of LPA_1 than other LPA receptor

subtypes in the prostate with increased expression in the hyperplastic stroma compared to surrounding benign glands (Zeng et al., 2009) and in cultured bladder smooth muscle cells (Kawashima et al., 2015) with increased expression in muscle-invasive bladder cancer specimens (Kataoka et al., 2015). LPA_1 mediates LPA-induced upregulation of CYR61 (Wu et al., 2014), which is overexpressed in BPH and is possibly linked to its progression (Sakamoto et al., 2004), suggesting that LPA_1 may be associated with BPH. A recent study demonstrated that LPA_1 antagonists modulate urethral pressure (Terakado et al., 2016). In addition, a study suggested that LPA may be involved in stretch-induced cellular activation, possibly via LPA_1 (Kawashima et al., 2015). These published findings suggest that LPA_1 may have roles in the functional regulation of the lower urinary tract. However, the integrated functional role of LPA_1 in the lower urinary tract has not been fully clarified.

In this study, I investigated the functional role of LPA and LPA_1 in the regulation of the lower urinary tract and the therapeutic potential of LPA_1 antagonism in the treatment of LUTS/BPH using ASP6432 (potassium 1-(2-{[3,5-dimethoxy-4-methyl-*N*-(3-phenylpropyl)benzamido]methyl}-1,3-thiazole-4-carbonyl)-3-ethyl-2,2-dioxo-2λ^6-diazathian-1-ide; Figure 1-1), a novel LPA_1 antagonist discovered by Astellas Pharma Inc. In Chapter 1, the aim was to understand the function of LPA and LPA_1 in the urethra and prostate by investigating the effects of LPA and ASP6432 on urethral and prostatic contractile function and prostate stromal cell proliferation. In Chapter 2, the role of LPA_1 in the regulation of urethral tonus during urine voiding was pharmacologically examined by evaluating the effects of ASP6432 and tamsulosin, an α_1-

adrenoceptor antagonist, on urethral perfusion pressure (UPP) at the filling and voiding phases in anesthetized rats and on voiding dysfunction induced by N^{ω}-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, in conscious rats. In Chapter 3, the effects of LPA and LPA_1 on bladder functions were studied in conscious rats using continuous cystometry. In addition, the effect of ASP6432 on urinary frequency induced by L-NAME was assessed in conscious rats.

Chapter 1: Effect of ASP6432, a Novel Type 1 Lysophosphatidic Acid Receptor Antagonist, on Urethral Function and Prostate Cell Proliferation

Introduction

BPH is one of the most common chronic urological diseases among elderly men. The proliferation of periurethral prostate stromal cells and contraction of prostate and urethral smooth muscles contribute to the development of BOO and LUTS (Roehrborn, 2008). Alpha$_1$-blockers are prescribed as the first-line pharmacotherapy for LUTS associated with BPH (LUTS/BPH). Alpha$_1$-blockers improve BOO by suppressing prostate and urethral smooth muscle contractions induced by norepinephrine released from sympathetic nerves. However, improvement of clinical symptoms with α_1-blockers is moderate (40% to 60%) compared to surgical intervention (60% to 80%) (Speakman, 2001). In addition, α_1-blockers are suggested to have little effect on prostate hyperplasia itself (Roehrborn, 2006). Steroid 5α-reductase inhibitors, another class of pharmacotherapies, reduce the size of the enlarged prostate and improve symptoms caused by mechanical obstruction. However, 5α-reductase inhibitors have a slower onset of efficacy and are slightly less effective at improving LUTS than α_1-blockers (Djavan, 2003). In addition, 5α-reductase inhibitors have little effect on stromal cell hyperplasia (Marks et al., 1997), a key component of human BPH (Bartsch et al., 1979). Therefore, an agent that induces more potent urethral

relaxation and suppression of stromal hyperplasia would improve treatment for LUTS/BPH patients.

LPA is a simple glycerophospholipid produced in various parts of the body. In the lower urinary tract, LPA and autotaxin, an LPA synthesizing enzyme, are present in the seminal plasma (Tanaka et al., 2004). In the hyperplastic prostate nodule, acylglycerol kinase, another LPA synthesizing enzyme, is overexpressed (Zeng et al., 2009). LPA has diverse biological effects including smooth muscle contraction (Tokumura et al., 1980), as shown by its induction of urethral smooth muscle contraction (Saga et al., 2014), and cell proliferation (Daaka, 2002), such as of prostatic smooth muscle cells isolated from BPH patients. These functions suggest that LPA may play a physiological and pathophysiological role in the mechanical and functional BOO observed in BPH.

The functional roles of LPA are mediated by at least six G protein-coupled receptors ($LPA_{1\text{-}6}$). Although these LPA receptors are broadly expressed, they vary significantly in their tissue distribution, and appear to have both distinct and overlapping biological roles(Choi et al., 2010). LPA receptors modulate various intracellular signaling pathways by activating multiple heterotrimeric G proteins. LPA_1 was the first identified LPA receptor subtype. LPA_1 couples with $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ to initiate downstream signaling cascades through phospholipase C, mitogen-activated protein kinase, Akt, and RhoA. LPA_1 activation induces a range of cellular responses, including cell proliferation, cell migration and cytoskeletal changes, Ca^{2+} mobilization, and adenylyl cyclase inhibition (Yung et al., 2014). An investigation using surgically-obtained human prostate tissue samples demonstrated LPA_1 expression in both the stroma and epithelia, and increased

expression in the stroma of hyperplastic glands compared to that in surrounding benign glands (Zeng et al., 2009). LPA_1 mediates LPA-induced induction of CYR61 (Wu et al., 2014), a molecule overexpressed in BPH and possibly linked to its progression (Sakamoto et al., 2004), suggesting that LPA_1 may be associated with BPH. A recent study demonstrated that LPA_1 antagonists modulate urethral pressure (Terakado et al., 2016), suggesting that LPA_1 may also have a role in urethral contraction. However, the integrated role of LPA_1 in urethral and prostate function has not been fully clarified.

ASP6432 (Figure 1-1) is a novel LPA_1 antagonist discovered by Astellas Pharma Inc. To elucidate the function of LPA and LPA_1 in the urethra and prostate, I investigated the effect of ASP6432 on urethral and prostatic contractile function and prostate stromal cell proliferation.

Materials and Methods

Test substances

LPA was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), Enzo Life Sciences International Inc. (Plymouth Meeting, PA, USA) and Cayman Chemical (Ann Arbor, MI, USA). Based on published literature (Saga et al., 2014), 1-linolenoyl LPA was used for tissue contraction experiments, while 1-oleoyl LPA, the most commonly used form of LPA(Castilla-Ortega et al., 2014), was used for all other studies. ASP6432 and tamsulosin were synthesized at Astellas Pharma Inc. (Tokyo, Japan). Concentrations were calculated using the molecular weight of the free form.

Cells/recombinant expression

Cells expressing LPA receptors were generated according to a previously reported method (Murai et al., 2017). Human and rat LPA_1, human LPA_2, and human LPA_5 were stably expressed in Chinese hamster ovary (CHO) cells and cultured in Minimum Essential Medium-alpha containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 100 nmol/L methotrexate. Human LPA_3 was stably expressed in hepatoma tissue culture-4 (HTC4) cells and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 70 μmol/L Zeocin™. Human LPA_4 was stably expressed in human embryonic kidney (HEK) cells and cultured in DMEM containing 10% FBS and 0.4 mg/mL G418.

Measurement of intracellular Ca^{2+} concentration

The antagonistic effect of ASP6432 on human and rat LPA_1 and its selectivity for human LPA_1 over human LPA_2 to human LPA_4 were evaluated using Ca^{2+} flux assays previously used to investigate another LPA_1 antagonist (Swaney et al., 2010), with some modifications. Briefly, cells were seeded at a density of 15,000 (LPA_1 and LPA_2) or 20,000 (LPA_3) cells per well in 96-well plates and incubated in culture medium containing 1% FBS for one day. HEK 293 cells expressing human LPA_4 were seeded at a density of 15,000 cells per well in 384-well plates and incubated in culture medium for one day.

On the day the measurements were to be taken, the cells were loaded with Fluo-4-AM dissolved in assay buffer [Hank's Balanced Salt Solution containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 0.05% bovine serum albumin (BSA), and 2.5 mmol/L probenecid] for LPA_1 to LPA_3 or using the Fluo-4 NW Calcium Assay Kit (Invitrogen, Carlsbad, CA, USA) for LPA_4, and incubated for 3 hours at room temperature (LPA_1, LPA_2, and LPA_3) or 30 minutes at 37°C (LPA_4). The cells were incubated with test compounds for 2 (LPA_1) or 4 (LPA_2 and LPA_3) minutes after washing or 30 (LPA_4) minutes without washing, and LPA at a final concentration of 100 (LPA_1), 30 (LPA_2) or 800 (LPA_3) nmol/L was added. The final concentration of LPA was determined for each cell line to produce a submaximal reaction. After LPA treatment, the change in fluorescence (excitation wavelength: 470-495 nm, emission wavelength: 515-575 nm) was monitored using the fluorometric imaging plate reader FLIPR TETRA® (Molecular Devices Corporation Japan, Tokyo, Japan). For LPA_4, 2.8 μmol/L of LPA was used, and the change in fluorescence was measured using

the fluorometric imaging plate reader FLEX STATION-III (Molecular Devices, CA, USA) at an excitation wavelength of 494 nm and emission wavelength of 516 nm.

Measurement of intracellular cyclic AMP

The effect of ASP6432 on LPA-induced cyclic AMP (cAMP) production in cells expressing human LPA_5 was evaluated using a previously described method (Murai et al., 2017). Briefly, CHO cells expressing human LPA_5 were seeded at a density of 15,000 cells per well and cultured in 96-well plates. On the day the measurements were to be taken, ASP6432 or vehicle (dimethylsulfoxide: DMSO) was added to the cells with 1.3 mmol/L 3-isobutyl-1-methylxanthine (IBMX) and incubated for 6 minutes. LPA (final concentration: 1 μmol/L) was subsequently added and the cells were incubated for 20 minutes at room temperature. After incubation, 1.2% Triton X-100 solution was added to stop the reaction. The amount of cAMP in the cell lysate was determined by the homogenous time resolved fluorescence (HTRF) assay using a cAMP kit (cAMP femto 2 bulk kit, Cisbio, Codolet, France).

Measurement of isolated smooth muscle contraction

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc.

Male Wistar rats (Charles River Laboratories Japan Inc., Kanagawa, Japan) were sacrificed by exsanguination under pentobarbital anesthesia. The ventral lobes of the prostate were immediately removed and divided into four longitudinal strips approximately 5 mm in length and 2 mm in width. The urethra located next to the bladder neck was cut open in a circular orientation to form a rectangular strip

approximately 5 mm in length and 2 mm in width. The tissue strips were suspended in 10 mL organ baths at 37°C containing Krebs-Henseleit solution consisting of the following (mmol/L): NaCl, 118.4; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; and glucose, 11.1. Each bath was gassed with a mixture of 95% O_2 and 5% CO_2. After loading with 0.5 g of initial resting tension, the force was measured using an isometric force displacement transducer (TB-611T, Nihon Kohden, Tokyo, Japan), a pressure amplifier (AP-621G, Nihon Kohden) and a recorder (SR6211, SR6221 or SR6335; Graphtec Corporation, Kanagawa, Japan).

Each tissue strip was repeatedly contracted with 60 mmol/L KCl until a stable response was obtained. Subsequently, 100 μmol/L LPA was applied and the maximum contractile response was recorded as the initial LPA response. The concentration of LPA was selected according to a previous study (Saga et al., 2014) and our preliminary study in which we aimed to generate the maximum contraction under experimentally feasible conditions. In our preliminary study, LPA-induced contraction was observed from 1 μmol/L but did not reach maximum even at 100 μmol/L (Figure 1-2). However, at 300 μmol/L, the solution became cloudy, making it difficult to continue with the experiment (data not shown). We therefore selected 100 μmol/L LPA as the test concentration.

After washing, the strip was incubated with ASP6432 (0.01 to 10 μmol/L for urethra, 0.001 to 10 μmol/L for prostate) or vehicle (DMSO, 0.1%) for 30 minutes before the addition of LPA (100 μmol/L). The LPA-induced contractile response following treatment with ASP6432 or vehicle was recorded and expressed as a percentage of the initial LPA response (pre-value). For urethral strips, multiple concentrations (0.01 to 10 μmol/L) of ASP6432 were tested in an incremental

manner on the same strip because repeated treatment with DMSO and LPA did not affect the amplitude of the contractile response (data not shown). In contrast, only one concentration of ASP6432 was tested on each prostate strip due to the attenuation of LPA-induced contractile responses after multiple treatments (data not shown).

Measurement of intraurethral pressure (IUP) in rats

Male Wistar rats (Charles River Laboratories Japan Inc.) were anesthetized with an intraperitoneal administration of urethane (1.2 g/kg). A midline incision was made in the abdominal wall, and a 3.5 F sensor-tip transducer catheter (SPR-524, Millar Instruments. Inc., Houston, TX, USA) was inserted into the urethra through a small incision at the superior aspect of the bladder. IUP was measured using a pressure amplifier (AP-601G, Nihon Kohden) and analyzed using a digital acquisition and analysis system (PowerLab 8/30, AD Instruments, Colorado Springs, CO, USA). For intravenous (iv) administration, a polyethylene catheter (PE-50) filled with physiological saline containing sodium heparin was inserted into the jugular vein. Following a stabilization period of at least 30 minutes, vehicle (physiological saline with 5% dimethylformamide) or ASP6432 (0.03, 0.1, 0.3, and 1 mg/kg with incremental dosing at 15-minute intervals) was intravenously injected through the catheter. LPA (3 mg/kg iv) was administered 5 minutes after vehicle or ASP6432 administration. The dose of LPA that induced an IUP increase comparable to that induced by the α_1-blocker phenylephrine (30 μg/kg iv) reported in another study (Akiyama et al., 1999) was used to evaluate the effect of ASP6432. The area under the curve (AUC) of IUP at one minute before vehicle injection was defined as the

pre-value. The change in IUP induced by LPA following either vehicle or ASP6432 treatment was defined by the AUC at one minute after LPA injection.

Measurement of urethral perfusion pressure (UPP)

UPP was measured according to a previously described method(Kurihara et al., 2016). Briefly, female Wistar rats (Charles River Laboratories Japan Inc.) were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) and fixed in the supine position. For drug administration, a polyethylene catheter (PE-50) was inserted into the left femoral vein. For measurement of UPP, a midline incision was made in the abdominal wall and a double-lumen polyethylene catheter (PE-190 and PE-50) was inserted through a small incision at the bladder apex, and the tip was ligated to the bladder neck. Female rats were used because it was easier and less invasive to fix the position of the tip of the catheter at the bladder neck compared to that in male rats as there is no need to remove the ventral lobe of the prostate. Physiological saline was perfused into the urethra through the outer lumen of the catheter using an infusion pump (TE-331, Terumo, Tokyo, Japan) at 4.5 mL/h. UPP was recorded through the inner lumen of the catheter, which was connected to a pressure transducer with an amplifier (AP-601G or AP-621G, Nihon Kohden) and recorder (WT-688G, Nihon Kohden).

After a stabilization period of over 30 minutes, rats with a UPP lower than 10 mmHg were excluded from further evaluation. Vehicle (physiological saline for tamsulosin, physiological saline with 5% dimethylformamide for ASP6432), tamsulosin (0.003, 0.01, and 0.03 mg/kg) or ASP6432 (0.1, 0.3, 1 and 3 mg/kg) was administered intravenously, and the change in UPP was measured for 15 minutes.

The change between UPP before administration (UPP_{pre}) and the minimum UPP during the observation period (UPP_{min}) was calculated as follows:

$$\text{UPP (\% change from pre)}: -100\times(UPP_{pre} - UPP_{min})/UPP_{pre}$$

Cell proliferation assay

Primary cultured normal human prostate stroma cells (Lonza Walkersville Inc., Walkersville, MD, USA) were cultured in Stromal Cell Growth Medium (SCGM; Lonza Walkersville Inc.). Cells were suspended in SCGM diluted 10 times with stromal cell basal medium (Lonza Walkersville Inc.) containing 0.1% BSA, seeded at a density of 5,000 cells per well in 96-well plates and incubated overnight. ASP6432 (final concentration: 0.1 to 10 μmol/L) and LPA (final concentration: 10 μmol/L) were added the next day and incubated for 24 hours. The final concentration of LPA was selected according to a previous study (Adolfsson et al., 2002). Incorporation of bromodeoxyuridine (BrdU) into cells was measured using an ELISA kit (Cell Proliferation ELISA, BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim, Germany). Optical densities at 450 nm and 690 nm were measured using a spectrophotometer (Spectramax M2, Molecular Devices Japan KK, Tokyo, Japan) and the difference in values at these wavelengths was used to indicate the extent of BrdU incorporation. The result was expressed as a percentage of the normal (LPA non-treated, DMSO-treated) group.

Data analysis

The results are presented as mean ± standard error of the mean (SEM). Half maximal inhibitory concentration (IC_{50}) values were calculated using sigmoid-

E_{max} non-linear regression analysis and expressed as the geometric mean with 95% confidence interval for LPA_1. In the tissue contraction study, Williams' multiple comparisons test was used, and a probability value (p value) less than 0.025 indicated statistical significance. In the *in vivo* studies, Dunnett's multiple comparisons test and Student's t-test were used, and $p<0.05$ was considered statistically significant. In the cell proliferation assay, paired t-test and Williams' multiple comparisons test were used, and $p<0.05$ and $p<0.025$ were considered statistically significant, respectively.

Results

Antagonistic effect of ASP6432 on the LPA_1 receptor and its receptor subtype selectivity

The potency of ASP6432 for LPA_1 and selectivity for the different LPA receptors was determined by Ca^{2+} flux assays (for LPA_1 to LPA_4) and a cAMP assay (for LPA_5) using cells over-expressing human LPA_1 to LPA_5. ASP6432 concentration-dependently inhibited the LPA-stimulated increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in cells expressing human LPA_1 with an IC_{50} (95% confidence interval) value of 11 (6.8 to 18) nmol/L. ASP6432 also inhibited the LPA-induced $[Ca^{2+}]_i$ increase in cells expressing rat LPA_1 with an IC_{50} of 30 (19 to 45) nmol/L. ASP6432 inhibited the LPA-induced $[Ca^{2+}]_i$ increase in cells expressing human LPA_4 with an IC_{50} of 114 nmol/L. In contrast, ASP6432 at concentrations up to 10,000 nmol/L did not inhibit the LPA-induced increase in $[Ca^{2+}]_i$ by 50% or more in cells expressing human LPA_2 or LPA_3. Likewise, ASP6432 at concentrations up to 30,000 nmol/L did not affect the LPA-induced increase in cAMP in cells expressing human LPA_5. These results are summarized in Table 1-1.

The affinity of ASP6432 for a total of 57 receptors, ion-channels, transporters, and enzymes were also evaluated. ASP6432 at 10,000 nmol/L did not exhibit significant (>50%) effects on any of the targets examined, except for the human neurokinin 1 receptor with a K_i value of 1400 nmol/L (data not shown).

Inhibition of LPA-induced contractions of urethral and prostate strips isolated

from rats by ASP6432

Application of LPA (0.1 to 100 μmol/L) induced contractions in urethral and prostate tissue strips (N=5-7) (Figure 1-2). Pretreatment with ASP6432 (0.001 or 0.01 to 10 μmol/L) inhibited these LPA (100 μmol/L)-induced contractions in both tissues in a concentration-dependent manner (N=5). This effect was statistically significant at ASP6432 concentrations of 0.1 μmol/L and above, with almost complete inhibition observed at 10 μmol/L (Figure 1-3).

Inhibition of LPA-induced IUP elevation in anesthetized rats by ASP6432

Intravenous administration of LPA (3 mg/kg iv) increased the IUP (Figure 1-3) by 759 mmHg·min (17.2 cmH_2O·sec). This was comparable to the effects of phenylephrine shown in a previous study (approximately 12 cmH_2O at 30 μg/kg iv in rats with no urethral ligation) (Akiyama et al., 1999). ASP6432 (0.03 to 1 mg/kg iv) dose-dependently inhibited the LPA-induced IUP elevation (N=6). This effect was statistically significant at ASP6432 doses of 0.3 mg/kg and above, with almost complete inhibition observed at 1 mg/kg (Figure 1-4). The mean plasma concentration of ASP6432 in rats at 6 and 30 minutes after single intravenous administration at 1 mg/kg was 477.28 and 77.65 ng/mL (851.24 and 138.49 nmol/L, calculated from the free form molecular weight of 560.69), respectively (N=3) (Table 1-2).

Decrease in UPP by tamsulosin and ASP6432 in anesthetized rats

Intravenous administration of tamsulosin (0.003 to 0.03 mg/kg) decreased the UPP (N=7 to 15). This effect was statistically significant at a dose of 0.01 mg/kg iv,

maximally decreasing the UPP by 21.6% from baseline (Figure 1-5A). Intravenous administration of ASP6432 (0.1 to 3 mg/kg) decreased the UPP in a dose-dependent manner (N=10). This effect was statistically significant at ASP6432 doses of 0.3 mg/kg iv and above. The decrease in UPP with ASP6432 reached a maximum of 42.5% at 3 mg/kg iv (Figure 1-5B).

Effect of ASP6432 on LPA-induced proliferation of primary cultured human prostate stromal cells

LPA (10 μmol/L) significantly enhanced the incorporation of BrdU into human prostate stromal cells (172.7% of the normal group). Treatment with ASP6432 (0.1 to 10 μmol/L) suppressed LPA-induced BrdU incorporation in a concentration-dependent manner. This effect was statistically significant at ASP6432 concentrations of 0.3 μmol/L and above, with almost complete suppression observed at 10 μmol/L (Figure 1-6).

Discussion

In this chapter, I showed that ASP6432 had potent and selective antagonistic activity against LPA_1, and suppressed not only LPA-induced urethral and prostatic contractions but also urethral pressure in the absence of external LPA stimulation. These results suggest that LPA_1 activation plays an important role in the physiological regulation of urethral tonus. In addition, ASP6432 inhibited the LPA-induced proliferation of human prostate stromal cells. To my knowledge, this is the first study to demonstrate that an LPA_1 antagonist can modulate both LPA-induced urethral tonus and prostate stromal cell proliferation.

In the *in vitro* studies, ASP6432 exhibited a potent antagonistic effect against LPA_1, and was more selective for LPA_1 by more than ten-fold over all other LPA receptor subtypes (Table 1-1) and other receptors, ion-channels, transporters, and enzymes tested. Although ASP6432 showed some antagonistic activity against LPA_4 and its effect on LPA_6 was not investigated, ASP6432 appears to be one of the most potent LPA_1 antagonists among currently reported LPA receptor modulators (Llona-Minguez et al., 2015).

LPA was previously reported to induce contractions in rat urethral tissue strips at a magnitude comparable to that of phenylephrine, an α_1-adrenoceptor agonist (Saga et al., 2014). However, the receptor subtype responsible for this effect was not identified. I showed that LPA induced contractions in rat urethral and prostate strips (Figure 1-2) and ASP6432 concentration/dose-dependently inhibited LPA-induced contractions (Figure 1-3) and IUP elevation in anesthetized rats (Figure 1-4) with near-complete inhibition at the highest concentration/dose tested. These results indicate that LPA_1 regulates LPA-induced urethra and prostate contractions.

One notable finding in this study was that ASP6432 not only inhibited LPA-induced contractions, but also reduced the UPP in the absence of exogenous LPA stimulation in anesthetized rats (Figure 1-5B). ASP6432 maximally decreased the UPP by 42.5% from baseline, which is more pronounced than that induced by tamsulosin in this study (maximum 21.6% decrease, Figure 1-5A) at doses sufficient to inhibit phenylephrine-induced urethral pressure elevation (Ohtake et al., 2006). Female rats were used in our study because of the similar efficacy of tamslosin when compared to male rats, the experimental benefit, and the potentially limited advantage of male rats as a model for urethral tonus in humans from an anatomical point of view. The tamsulosin-induced suppression of the UPP was similar to that demonstrated in a previous study in male rats (around 20% decrease) (Saga et al., 2014). Tamsulosin decreased the urethral pressure in male and female dogs at a similar dose range(Ohtake et al., 2004; Sudoh et al., 1996), and in healthy women at a dose approved for men with BPH (0.4 mg) (Reitz et al., 2004). These findings support the notion that the sympathetic nervous system and α_1-adrenoceptors contribute to regulating physiological urethral tonus (Fry et al., 2010) in both males and females. Another reason for using female animals was the experimental benefit for evaluating the perfusion pressure. In female rats, access to the bladder neck and subsequent securing of the catheter tip at the intended position was much easier and less invasive than that in male rats, which helped ensure stable pressure measurements. Further, the use of male rats might not significantly increase the clinical relevance of the UPP compared with that of female rats, because the rat prostate is anatomically different from the human prostate in that it does not completely surround the urethra, and therefore may not cause the

mechanical and/or functional urethral obstruction like the prostate of a BPH patient does. These points indicate that the experimental conditions of this study were appropriate for examining the effect of a drug that potentially modulates the urethral contraction *in vivo* compared with a drug acting on the sympathetic nervous system.

LPA induced contraction of urethral strips at 1 μmol/L and above in a previous study (Saga et al., 2014) and in the present study (Figure 1-2). The fact that the rat plasma LPA concentration reported in the previous study was in the micromolar order (around 1 μmol/L) (Saga et al., 2014) suggests that LPA induces urethral contraction at a concentration similar to the endogenous concentration. In addition, previous studies have demonstrated that an inhibitor of autotaxin and another LPA_1 antagonist decrease the IUP in the absence of exogenous LPA stimulation in rats (Saga et al., 2014; Terakado et al., 2016) to a similar extent to that observed with ASP6432 in this study. Moreover, pharmacokinetics data of ASP6432 in rats (Table 1-2) support my hypothesis that ASP6432 reduces UPP by suppressing LPA_1. The plasma concentration of ASP6432 at the efficacious dose for urethral pressure (0.3 mg/kg iv, Figure 1-5) is estimated to be around 40 to 250 nmol/L, which is comparable to the IC_{50} value of ASP6432 on rat LPA_1 (30 nmol/L, Table 1-1). Taken together, the results of this study suggest that endogenous LPA constantly activates LPA_1 and plays a significant role in the regulation of urethral tonus, which is suppressed by ASP6432, at least in rats.

LPA induces proliferation of human prostate stromal cells, similar to that observed for cells isolated from BPH patients(Adolfsson et al., 2002). ASP6432 suppressed this proliferation (Figure 1-6). These results suggest that LPA, in addition to its role in inducing urethral and prostate contractions, also regulates

stromal cell proliferation via LPA_1 and contributes to the development of the two major components, mechanical and functional obstruction, of BOO. Because there is currently no pharmacotherapy that is simultaneously efficacious for both of these components, the present findings indicate that LPA_1 antagonists may represent a novel therapy with dual mechanisms for improving BOO. A similar concept was proposed using inhibitors of Rac, a small monomeric GTPase (Wang et al., 2015). Given that LPA_1 may also potentially activate Rac (Van Leeuwen et al., 2003), it would be interesting to determine the relationship between LPA_1 and Rac in lower urinary tract functions.

Since autotaxin is one of the primary enzymes responsible for LPA production, autotaxin inhibitors may theoretically have similar efficacy to LPA_1 antagonists, such as in reducing the IUP as shown in a previous study(Saga et al., 2014). Various autotaxin inhibitors have been studied and some are in clinical development. While inhibition of LPA production may efficiently suppress the LPA-LPA receptor signaling axis, the risk of toxicity may be high due to the suppression of functions mediated by other LPA receptor subtypes. Indeed, autotaxin knockout mice show a more severe phenotype (lethal around embryonic day 10.5 due to defects in blood vessel formation) than LPA receptor knock-out mice(Tanaka et al., 2006). Therefore, specific targeting of LPA_1 may be more suitable for the treatment of non-life-threatening diseases like BPH, which require a high safety profile.

The present study results provide various insights for further investigations into the role of LPA and LPA_1. The physiological and pathophysiological role of the LPA-LPA_1 signaling axis in the modulation of urethral pressure has not been extensively investigated. Currently, no study has directly measured the tissue

concentration of LPA in the urethra or prostate, even though the presence of LPA in seminal plasma (Tanaka et al., 2004)(Tanaka *et al.*, 2004) and LPA-producing enzymes in the prostate (Zeng et al., 2009) suggest that it may be produced locally in these organs. The mechanism underlying LPA_1 activation-induced urethral and prostate smooth muscle contractions has not been fully clarified. In mouse aorta, LPA_1-mediated thromboxane A2 release has been suggested as a potential mechanism underlying smooth muscle contraction(Dancs et al., 2017). However, studies showing that an autotaxin inhibitor (Saga et al., 2014) and LPA_1 antagonist (Terakado et al., 2016) decrease the urethral pressure without affecting blood pressure suggest the presence of an alternative mechanism for regulating urethral smooth muscle contraction. Further studies are required to unravel the mechanistic details. In addition, the role of LPA_1 on urethral pressure during urine voiding needs to be determined. Since the sympathetic and parasympathetic nervous system regulate the on-off of the bladder and the urethral outlet functions in an antagonistic fashion for proper urine storage and voiding(Fowler et al., 2008), it would be worthwhile to investigate how the activity of LPA_1 is regulated at bladder filling and urine voiding. The effect of LPA_1 on bladder function represents another area of interest. While the effect on pathological prostate proliferation should ideally be assessed using *in vivo* models, fully validated animal models for stromal proliferation are currently lacking. Future studies on these aspects will allow for a more extensive characterization of the therapeutic potential of LPA_1 antagonists in the treatment of BPH and associated LUTS.

In conclusion, I demonstrated the roles of LPA and LPA_1 in urethral and prostate contraction and prostate stromal cell proliferation using ASP6432, a selective

LPA_1 antagonist. ASP6432 induced potent urethral relaxation compared to tamsulosin and inhibited prostate stromal cell growth, indicating the potential of an LPA_1 antagonist as a novel therapy for LUTS/BPH.

Table 1-1. Antagonistic activity of ASP6432 on lysophosphatidic acid (LPA)-induced cellular responses in cells expressing LPA_1 to LPA_5 receptors

	LPA_1 (Human)	LPA_1 (Rat)	LPA_2 (Human)	LPA_3 (Human)	LPA_4 (Human)	LPA_5 (Human)
IC_{50} (nmol/L)	11	30	>10,000	>10,000	114	>30,000

Effects of ASP6432 on the LPA-induced increase in intracellular Ca^{2+} (LPA_1 to LPA_4) or cyclic AMP (LPA_5) were measured, and IC_{50} values were calculated using sigmoid-E_{max} non-linear regression analysis and expressed as the geometric mean of two to four independent experiments.

Table 1-2. Plasma concentrations of ASP6432 in rats after single intravenous (1 mg/kg) administration

Time after dosing (h)	Plasma concentration (ng/mL)					
	0.1	**0.25**	**0.5**	**1**	**2**	**4**
Mean	477.28	168.84	77.65	40.18	22.41	10.49
SD	41.85	5.04	5.33	6.77	2.68	1.74

Male F344/DuCrlCrlj rats (7 weeks) were used. ASP6432 was dissolved in a polyethylene glycol 400/water (1:1, v/v) solution and administered intravenously (1 mg/mL/kg).

Plasma samples were collected, and plasma concentration was determined using liquid chromatography-tandem mass spectrometry. Results were expressed as the mean and standard deviation (SD) of three animals.

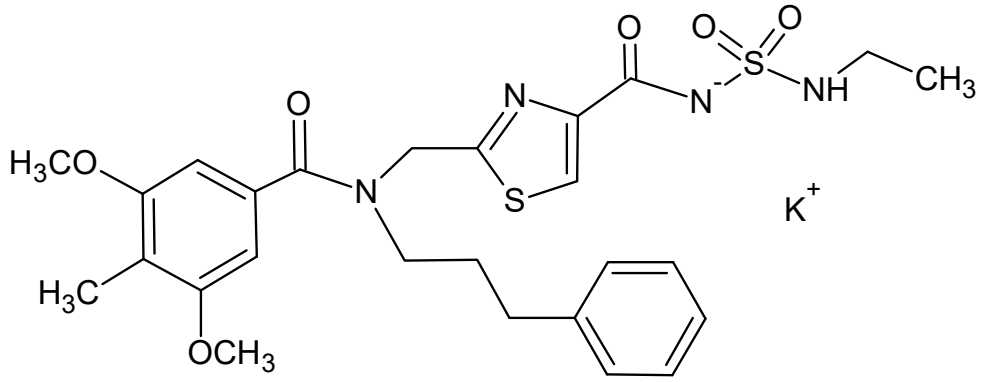


Figure 1-1. Chemical structure of ASP6432 (potassium 1-(2-{[3,5-dimethoxy-4-methyl-*N*-(3-phenylpropyl)benzamido]methyl}-1,3-thiazole-4-carbonyl)-3-ethyl-2,2-dioxo-2λ^6-diazathian-1-ide)

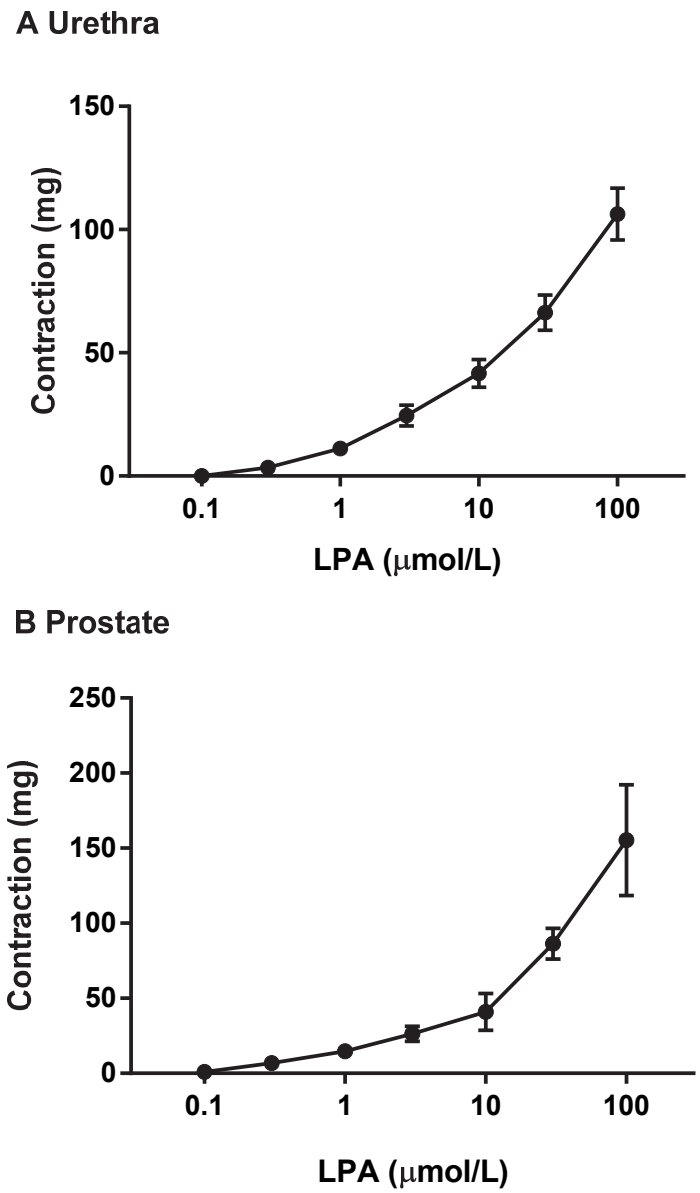
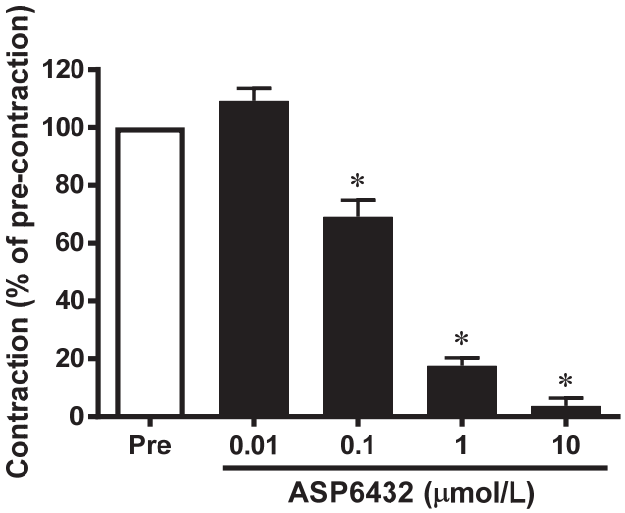


Figure 1-2. Contractile effects of lysophosphatidic acid (LPA) on isolated rat urethra (A) and prostate (B) tissue strips
Tissue strips were treated with 0.1 to 100 μmol/L LPA to elicit contractile responses. Each point represents the mean ± SEM of seven (urethra) or five to six (prostate) strips.

A Urethra



B Prostate

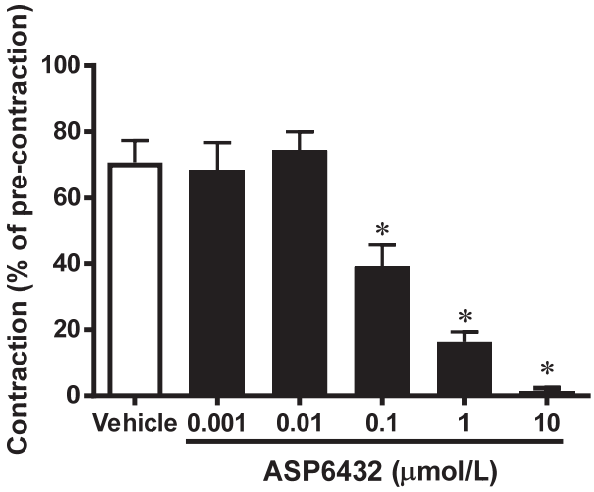


Figure 1-3. Effect of ASP6432 on lysophosphatidic acid (LPA)-induced contractions in isolated rat urethra (A) and prostate (B)

For the urethra (A), tissue strips were incubated with vehicle or various concentrations of ASP6432 for 30 minutes before 100 μmol/L LPA was added to elicit a contractile response. Each column represents the mean ± SEM of five strips. Pre: initial LPA-induced contraction response. *$p<0.025$ vs. pre (initial LPA contraction) (Williams' multiple comparisons test using within-subject error).

For the prostate (B), tissue strips were incubated with ASP6432 or vehicle (DMSO) for 30 minutes before 100 μmol/L LPA was added to elicit a contractile response. Each column represents the mean ± SEM of five strips. *$p<0.025$ vs. vehicle (Williams' multiple comparisons test).

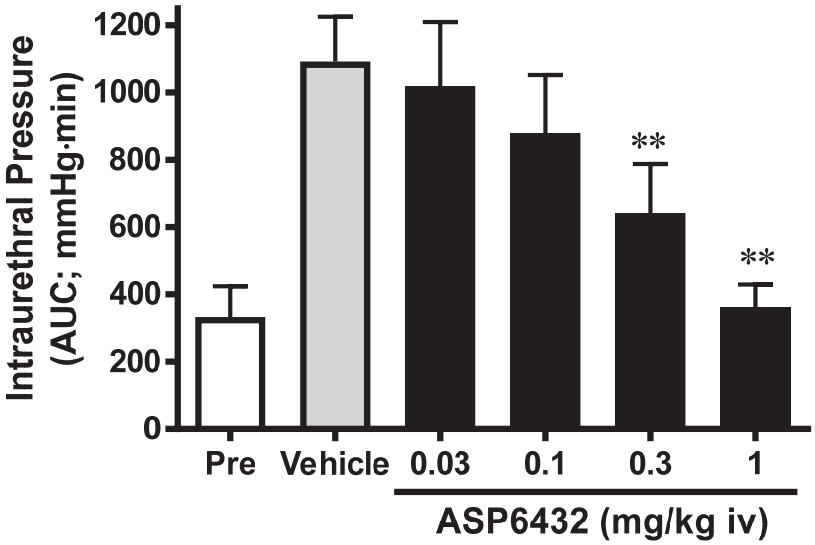
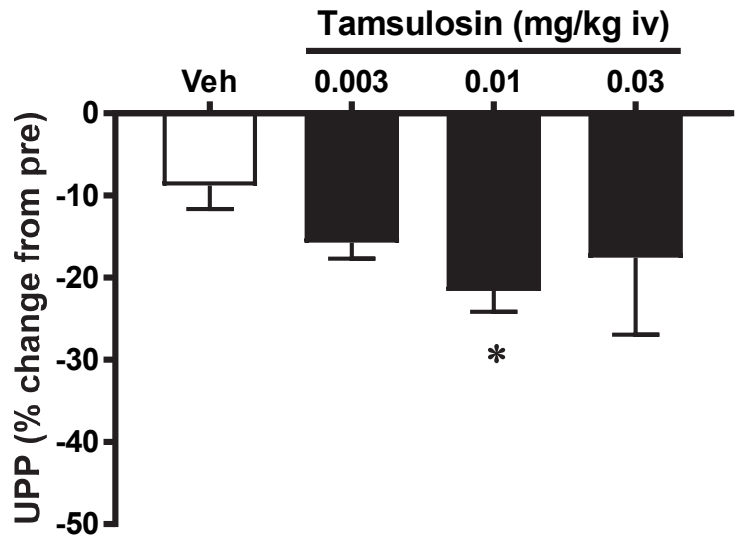


Figure 1-4. Effect of ASP6432 on lysophosphatidic acid (LPA)-induced intraurethral pressure (IUP) elevation in anesthetized rats

IUP was measured in male Wistar rats under urethane anesthesia. LPA (3 mg/kg iv) was administered five minutes after treatment with vehicle or ASP6432 (0.03 to 1 mg/kg iv), and the area under the curve (AUC) of IUP was recorded for one minute. The AUC at one minute before vehicle injection is represented as Pre-value. Each column represents the mean ± SEM of six animals.

**$p<0.01$ vs. vehicle (Dunnett's multiple comparisons test).

A Tamsulosin



B ASP6432

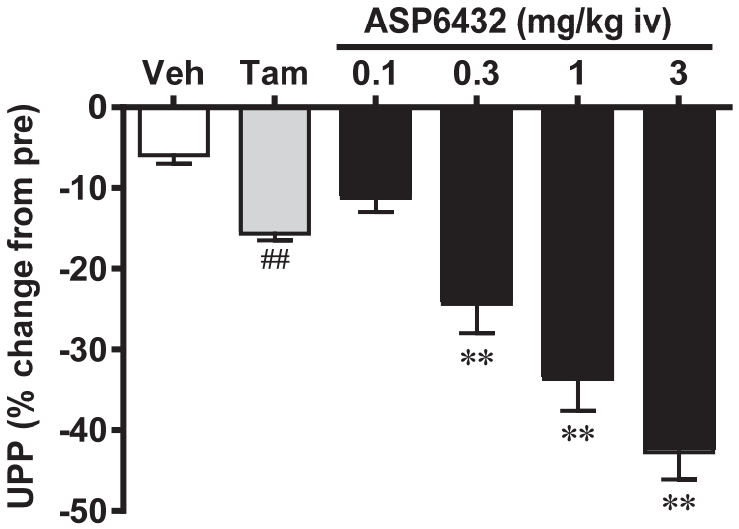


Figure 1-5. Effect of tamsulosin (A) and ASP6432 (B) on the urethral perfusion pressure (UPP) in anesthetized rats

UPP was measured in female Wistar rats under urethane anesthesia. Vehicle, tamsulosin (0.003 to 0.03 mg/kg iv) or ASP6432 (0.1 to 3 mg/kg iv) was administered, and the UPP was recorded for 15 minutes. The maximum change in UPP from baseline (%) was calculated for each animal. Each column represents the mean ± SEM of 7 to 15 animals.

Veh: vehicle, Tam: tamsulosin (0.01 mg/kg iv). ##$p<0.01$ vs. vehicle (Student's t-test); *$p<0.05$, **$p<0.01$ vs. vehicle (Dunnett's multiple comparisons test).

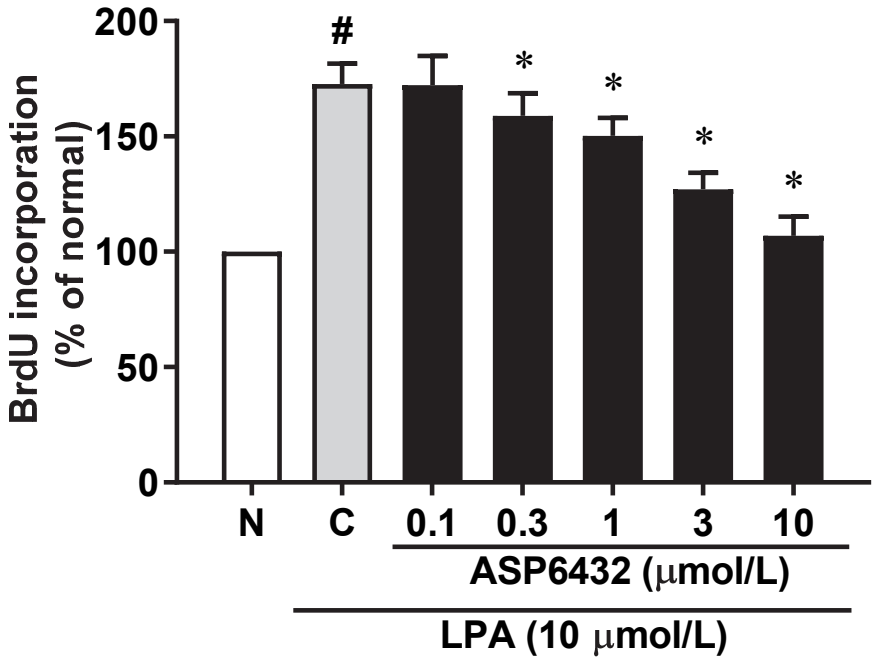


Figure 1-6. Effect of ASP6432 on lysophosphatidic acid (LPA)-induced bromodeoxyuridine incorporation into primary cultured human prostate stromal cells

The incorporation of bromodeoxyuridine (BrdU) into cells was measured in primary cultured normal human prostate stromal cells. The extent of BrdU incorporation was expressed as a percentage of that of the normal group (non LPA-treated). Each experiment was conducted in triplicate and each column represents the mean ± SEM of four experiments.

N: normal (non LPA-treated), C: control (treated with LPA and vehicle). #$p<0.05$ vs. normal (paired t-test), *$p<0.025$ vs. control (William's multiple comparisons test using within-subject error).

Chapter 2: Effect of ASP6432 on Urethral Function during Urine Voiding and Voiding Dysfunction

Introduction

The storage and periodic elimination of urine require coordination between the bladder and a bladder outlet component such as the urethra. The neural pathways controlling this coordination are organized as simple on-off circuits to maintain a reciprocal relationship. Throughout bladder filling, the sympathetic pathway releases noradrenaline, activates α_1-adrenoceptors, and contracts the proximal urethra. At the urine voiding phase, the parasympathetic pathway is activated to contract the bladder, and the urethra is relaxed by the release of nitric oxide (NO) and removal of adrenergic excitatory inputs (Fowler et al., 2008).

Functional dysregulation between the bladder and urethra causes voiding dysfunctions. Increased urethral tonus during voiding due to excessive sympathetic inputs (McVary et al., 2005) or impaired nitrergic inputs (Klotz et al., 1999) leads to incomplete bladder emptying and LUTS. Although pharmacotherapies for reducing urethral tonus such as α_1-adrenoceptor antagonists (α_1 blockers) are available for LUTS with BPH, their efficacy for other types of voiding dysfunctions such as female LUTS has not been established (Bae et al., 2005). Therefore, an agent that potently relaxes the urethra could improve treatment for voiding dysfunction and LUTS associated with BPH or other urology diseases.

LPA is a small ubiquitous glycerophospholipid found in vertebrate organisms that mediates diverse biological actions (Yung et al., 2014). The functional roles of LPA are primarily mediated by at least six G protein-coupled receptors, $LPA_{1–6}$.

Although these LPA receptors are broadly expressed, they vary significantly in their tissue distribution and appear to have both distinct and overlapping biological roles (Choi et al., 2010). LPA_1 was the first identified LPA receptor subtype. LPA_1 is broadly expressed in various tissues, including the normal and hyperplasic prostate (Zeng et al., 2009). LPA_1 activation induces a range of biological responses such as smooth muscle contraction and cell proliferation. Recent studies using pharmacological tools demonstrate that LPA and LPA_1 are involved in contraction of the urethra in rats (Saga et al., 2014; Terakado et al., 2016).

In Chapter 1, I demonstrated that ASP6432, a novel compound discovered by our group, demonstrated potent antagonistic activity against LPA_1 (IC_{50}=11 nmol/L) and was more than 10-fold selective for LPA_1 over other LPA receptor subtypes and other receptors, ion channels, transporters, and enzymes tested. ASP6432 suppressed not only LPA-induced urethral and prostatic contractions but also urethral perfusion pressure (UPP) in the absence of external LPA stimulation in rats, as shown in Chapter 1. However, whether and to what extent an LPA_1 antagonist can decrease the UPP at the voiding phase and improve voiding dysfunction has not been examined.

In this chapter, to elucidate the role of LPA_1 in the regulation of urethral tonus during urine voiding, I investigated the effects of ASP6432 and tamsulosin, an α_1 blocker, on UPP at the filling and voiding phases in anesthetized rats. In addition, the effects of ASP6432 and tamsulosin on voiding dysfunction induced by N^{ω}-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, was evaluated in conscious rats.

Materials and Methods

Test reagents

ASP6432 (Figure 1-1) and tamsulosin were synthesized at Astellas Pharma Inc. (Tokyo, Japan). For the study in anesthetized rats, ASP6432 and tamsulosin were dissolved and diluted in saline with 5% (v/v) dimethylformamide. For the experiment in conscious rats, tamsulosin was dissolved in distilled water and diluted with saline, and ASP6432 was dissolved and diluted in saline with 20% *N,N*-dimethylacetamide and 10% (v/v) Cremophor EL. The amount of these organic solvents and detergents used in this study was either below the clinical concentration, comparable to that used in a previous study, or much lower than the toxic exposure level. N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved and diluted in saline.

Experimental animals

In total, 32 male and 40 female animals were used. Female Wistar rats were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). Male Sprague-Dawley rats were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan). The number of animals per group was determined based on the expected effect size and standard deviation estimated from preliminary experiments. For the effect of tamsulosin on the L-NAME-induced model, the above estimation was not feasible since preliminary experiment showed that tamsulosin had no effect. Therefore, I terminated the experiment when we observed the same trend as that in our preliminary experiment, to keep the number of animals minimum. All animal

experimental procedures including the number of animals were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Furthermore, Astellas Pharma Inc. Tsukuba Research Center was awarded Accreditation Status by the AAALAC International.

Recording of UPP in anesthetized rats under isovolumetric conditions

UPP was measured according to a previously described method (Jung et al., 1999) with some modifications. Female Wistar rats were anesthetized with an intraperitoneal injection of urethane (1.0 g/kg) and fixed in the supine position. For drug administration, a polyethylene catheter (PE-50) was inserted into the left femoral vein. A midline incision was made in the abdominal wall, and a double-lumen polyethylene catheter (PE-160 and PE-50) was inserted through a small incision at the bladder apex, and the tip was positioned at the bladder neck. Two other polyethylene catheters (PE-50) were inserted into the bladder to measure intravesical pressure (IVP) and drain saline. Saline was perfused into the bladder dome through a catheter using an infusion pump (STC-525 or TE-331, Terumo, Tokyo, Japan; KDS100, Muromachi Kikai Co. Ltd., Tokyo, Japan or Neuroscience Inc., Tokyo, Japan) at 4.5 mL/h to induce isovolumetric rhythmic bladder contractions. Saline was also perfused into the urethra through the outer lumen of the double lumen-catheter at 4.5 mL/h to measure UPP. IVP and UPP were measured using a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan) connected to a pressure amplifier (AP-601G or AP-621G, Nihon Kohden).

After a stabilization period, vehicle (1 mL/kg), tamsulosin (0.01 mg/kg), or ASP6432 (0.1, 0.3, and 1 mg/kg) was administered intravenously (iv). The dose of tamsulosin used in this study (0.01 mg/kg iv) induced a maximum decrease in UPP

in the previous chapter. After drug administration, UPP at the bladder filling phase (UPP_{base}), the minimum UPP at the urine voiding phase (UPP_{nadir}), the duration of urethral relaxation, the duration of high frequency oscillations (HFOs) reflecting external urethral sphincter muscle contraction (Bennett et al., 1995) (Figure 1A) for 15 min, and maximum changes from baseline were recorded as % change from baseline.

Voiding dysfunction model induced by L-NAME in conscious rats

Voiding dysfunction, characterized by an increase in post-void residual urine (PVR) and a decrease in voiding efficiency (VE), was induced in conscious rats by intravenous administration of L-NAME (10 mg/kg). The dose of L-NAME was determined according to the preliminary experiment (data not shown).

Voiding parameters were measured according to the method descried by Kurihara et al. (Kurihara et al., 2016) in male Sprague-Dawley rats. Under isoflurane anesthesia, a PE-160 catheter was inserted into the bladder through an abdominal incision and a small incision at the center of the ventral side of the bladder. The other end of the catheter was exited through the abdominal incision in a vertical direction to infuse saline and collect residual urine. A PE-50 catheter was inserted into the jugular vein for drug administration. After surgery, the rat was placed in a Ballman cage (Natsume Seisakusho, Tokyo, Japan) and allowed to recover from anesthesia for at least 1 hour. The bladder catheter (PE-160) was connected to a pressure transducer (DX-100; Nihon Kohden) and an infusion pump (TE-331S, STC-525 or STC-528; Terumo) via a three-way stopcock. Intravesical pressure was monitored using an amplifier (AP-601G; Nihon Kohden) connected

to a pressure transducer.

Voiding parameters were measured by running single cystometry (CMG) twice (Figure 3A). The first CMG was to collect baseline values. Saline was infused into the bladder at 4.2 mL/h. The infusion was stopped immediately after initiation of the voiding reflex, and urine voided from the urethral orifice and PVR were collected and weighed using an electronic balance (GX-200; A and D, Tokyo, Japan). After the first series of CMGs, vehicle (1 mL/kg iv), ASP6432 (0.3, 1 or 3 mg/kg, iv), or tamsulosin (0.003 or 0.01 mg/kg iv) was administered. L-NAME was administered 5 min after drug administration. The second CMG was started 5 min after L-NAME administration, and the voided urine and PVR were measured after voiding, and changes in PVR (mL) and VE (%) from the respective baseline values were calculated. A weight of 1 g of urine was considered to be equivalent to a volume of 1 mL of urine. Voiding efficiency (VE) was calculated using the following formula:

VE (%)=100×(voided volume [mL])/(voided volume [mL]+PVR [mL])

Data analysis

PowerLab and LabChart (ADInstruments Japan, Aichi, Japan) were used to collect data. Statistical analyses were performed using SAS software (SAS Institute Japan, Tokyo, Japan) or Prism (GraphPad Software, San Diego, CA, USA). Results are presented as mean±SEM. Student's t-test, paired t-test, one-way analysis of variance (ANOVA), or ANOVA followed by Dunnett’s multiple comparisons test was used, and $p<0.05$ was considered statistically significant.

Results

ASP6432 but not tamsulosin decreased UPP during voiding in anesthetized rats under isovolumetric conditions

Under isovolumetric conditions, the bladder and urethra produced simultaneous and periodic contractions and relaxations (Figure 2-1A and 2-1B). There were no significant differences in baseline UPP_{base}, UPP_{nadir}, duration of urethral relaxation, or duration of HFOs at the voiding phase between groups (Table 2-1).

Intravenous administration of tamsulosin (0.01 mg/kg) significantly decreased the UPP_{base} compared to the vehicle-treated group, but did not significantly alter the UPP_{nadir} (Figure 2-1C, Figure 2-2A and 2B). Intravenous administration of ASP6432 (0.1 to 1 mg/kg) dose-dependently and statistically significantly decreased the UPP_{base} and UPP_{nadir} at 1 mg/kg iv and 0.3 mg/kg iv and above, respectively (Figure 2-1D, Figure 2-2A and 2B). Neither tamsulosin nor ASP6432 significantly changed the duration of HFOs or urethral relaxation at the urine voiding phase (Figure 2-1C and 1D, Figure 2C and 2D).

ASP6432 but not tamsulosin improved L-NAME-induced voiding dysfunction in conscious rats

Mean baseline values for PVR and VE were 0.04 to 0.06 mL and 94.1% to 96.2%, respectively (Table 2-2). No significant differences were observed between groups. In vehicle-treated groups, intravenous administration of L-NAME (10 mg/kg) increased PVR by 0.27 to 0.31 mL and decreased VE by 21.7% to 22.7%, with no significant changes in the time to voiding or maximum IVP (Figure 2-3B).

Treatment with tamsulosin (0.003 and 0.01 mg/kg iv) did not affect either the

increase in PVR or the decrease in VE induced by L-NAME (Figure 2-4A). On the other hand, ASP6432 significantly prevented the increase in PVR and decrease in VE elicited by L-NAME at 1 mg/kg iv and above compared to the vehicle-treated group (Figure 2-4B). Neither tamsulosin nor ASP6432 significantly changed the time to voiding or maximum IVP (data not shown).

Discussion

In this chapter, I demonstrated that the LPA_1 antagonist ASP6432 decreased UPP_{nadir}, while tamsulosin had no effect. ASP6432 also suppressed the increase in PVR and decrease in VE induced by L-NAME, while tamsulosin likewise had no effect. Although recent reports (Terakado et al., 2016) and the results in Chapter 1 have demonstrated a significant role for LPA_1 in maintaining urethral contraction, it has remained unclear whether LPA_1 antagonism decreases urethral pressure at the voiding phase and if it actually affects urine voiding. To my knowledge, this is the first study to show that an LPA_1 antagonist decreases urethral tonus during urine voiding and improves voiding dysfunction.

In anesthetized rats under isovolumetric conditions, both ASP6432 and tamsulosin reduced UPP_{base} (Figure 2-2A), as shown in Chapter 1. Interestingly, ASP6432 also decreased UPP_{nadir}, which was not significantly affected by tamsulosin (Figure 2-2B). The decrease in UPP_{nadir} by ASP6432 suggests that activated LPA_1 contributes to the regulation of urethral tonus at the voiding phase, in contrast to the notion that the sympathetic nervous system is not active at the voiding phase (Fowler et al., 2008). The decrease in UPP_{base} but not UPP_{nadir} by tamsulosin is also consistent with this notion and prior findings that α_1 blockers decrease the baseline UPP (Conley et al., 2001) but not UPP at the voiding phase (Bae et al., 2005). ASP6432 demonstrated high selectivity in an off-target assay that included α_1-adrenceptors (Chapter 1, data not shown). To my knowledge, no study has shown a direct relationship between LPA_1 and α_1-adrenceptors. Comparison of the present study results with the above-mentioned studies suggests that inhibition

of LPA_1 activation decreases urethral tonus at the voiding phase via a mechanism that is not directly related to α_1-adrenoceptor antagonism. However, detailed studies are needed to determine whether there is any crosstalk between the LPA-LPA_1 signaling axis and the adrenergic system.

To examine the benefit of decreasing UPP_{nadir} on voiding dysfunction, I investigated the effect of ASP6432 and tamsulosin in a L-NAME-induced voiding dysfunction model in conscious rats. I used this approach instead of the measurement of UPP_{nadir} in anesthetized rats under isovolumetric conditions, because in the preliminary experiment L-NAME frequently eliminated reflex urethral relaxation, which was also observed using another NOS inhibitor (Bennett et al., 1995), making it difficult to evaluate the effect of drugs on impaired urethral relaxation induced by L-NAME. Single CMG showed that L-NAME increased PVR and decreased VE without significantly altering bladder parameters (Figure 2-3B), consistent with a prior study demonstrating that L-NAME primarily inhibits urethral relaxation at the voiding phase and increases PVR (Persson et al., 1992).

ASP6432 prevented the L-NAME-induced decrease in VE and increase in PVR (Figure 2-4B). Given that ASP6432 decreased the UPP_{nadir} (Figure 2-2B), it is reasonable to assume that ASP6432 prevented voiding dysfunction by decreasing urethral tonus during urine voiding via LPA_1 antagonism, thereby functionally counteracting the impaired urethral relaxation induced by L-NAME. However, whether ASP6432 and L-NAME act independently or if there is interplay between them remains to be clarified. To my knowledge, only one study has investigated the relationship between LPA and NO-related signaling in smooth muscle tissue (the lower esophageal sphincter of cats) (Lee et al., 2011). While ASP6432 had no effect

on the duration of urethral relaxation and HFOs (Figure 2-2C and 2-2D), the non-selective phosphodiesterase inhibitor zaprinast and NO donors reportedly enhance the duration of urethral relaxation (Jung et al., 1999; Wibberley et al., 2002), suggesting that ASP6432 may modulate urethral functions differently to drugs that act on the nitrergic pathway. More research is needed to elucidate the relationship between the LPA_1 and nitrergic pathways.

Tamsulosin did not affect the L-NAME-induced voiding dysfunction (Figure 2-4A), even though the α_1 blocker prazosin was previously shown to potentiate the relaxation effect of NO released from nerve endings in isolated rabbit urethra strips (Seshita et al., 2000). The effect of α_1-blockers on NO-mediated urethral relaxation may be limited, or differ across experimental settings or species. Tadalafil, the only approved PDE5 inhibitor for the treatment of LUTS associated with BPH (Gacci et al., 2016), may suppress the effects of L-NAME. However, the effect of tadalafil was not evaluated in this study, because the L-NAME-induced model is not appropriate to compare the efficacy of the drug acting on the nitrergic pathway with those mainly acting on other pathways. In addition, PDE5 inhibitors enhance relaxation of the bladder (Filippi et al., 2007; Oger et al., 2010; Ribeiro et al., 2014) and tadalafil did not significantly improve maximum urine flow or PVR in the majority of clinical studies (Hatzimouratidis, 2014), suggesting that PDE5 inhibition may not be an ideal approach for improving uroflowmetric parameters such as PVR and VE.

The present study results provide various insights for further investigation into the role of LPA and LPA_1. Given that this study suggests that the effect of ASP6432 on urethral tonus is not directly related to α_1-adrenoceptor antagonism, I expect that

ASP6432 will at least have an additive effect when combined with tamsulosin. However, further investigations into potential interactions between LPA_1 and the adrenergic and nitrergic pathways are required to clarify the potential clinical benefit of the combined use of ASP6432 and α_1 blockers or PDE5 inhibitors, as well as the physiological role of LPA_1 in the lower urinary tract. The effects of LPA and LPA_1 in the regulation of bladder function should also be investigated to elucidate their overall roles in the lower urinary tract. When studying the effect of LPA on bladder functions, it should be noted that LPA binds and directly activates molecular targets other than LPA receptors such as transient receptor potential (TRP) channel TRPV1 (Nieto-Posadas et al., 2011), TRPA1 (Kittaka et al., 2017), and peroxisome proliferator-activated receptor γ (PPARγ) (McIntyre et al., 2003), since TRPV1 and TRPA1 play important roles as sensors of stretch or chemical irritation to activate bladder sensory signaling (Skryma et al., 2011). However, for urethral functions, it is unlikely that these molecules are responsible for LPA-induced urethral smooth muscle contractions, because other LPA_1 antagonists have been shown to inhibit LPA-induced contractions (Terakado et al., 2017; Terakado et al., 2016) and TRPA1 agonists inhibit the contraction of isolated human urethral strips in cooperation with TRPV1-mediated signals (Weinhold et al., 2010). Further, it is unlikely that relatively rapid biological responses like smooth muscle contraction can be explained by transactivation of nuclear receptors like PPARγ.

The finding that ASP6432 significantly reduced the UPP_{nadir} and suppressed L-NAME-induced voiding dysfunction, while tamsulosin did not, suggests that ASP6432 has the potential to improve voiding dysfunctions that are refractory to current pharmacotherapies in humans. Hypofunction of NO mechanisms and

urethral dysfunction in aged rats (Kimura et al., 2018), impaired NO synthesis with increasing age in humans (Schulze et al., 2005; Yoshida et al., 2003), and voiding dysfunction in mice deficient of neuronal NO synthase (Burnett et al., 1997) suggest that patients with age-related urethral dysfunction may benefit from treatment withASP6432. Underactive bladder (UAB), a recently proposed disease entity (Chapple et al., 2018), may be another potential target indication given that treatments that solely target the bladder have been reported to be ineffective (Barendrecht et al., 2007), suggesting the need for alternative approaches (Deruyver et al., 2018). ASP6432 may be effective for improving bladder emptying by potently relaxing the urethra during voiding and correcting the relative imbalance between bladder contraction and urethral relaxation.

Before investigating the therapeutic potential for ASP6432 in a clinical setting, extensive nonclinical profiling for pharmacokinetics and safety must be completed. Although the detailed data were not shown here, pharmacokinetic studies in rats and dogs demonstrated that the absolute bioavailability of ASP6432 ranged from 34.7% to 76.7%, and that oral administration of ASP6432 showed no effect on blood pressure, heart rate, or electrocardiogram findings in dogs at up to 100 mg/kg. Although it was not feasible to evaluate the effect of ASP6432 on urethral functions after oral administration due to technical limitations, comparison of these results with the effective dose after intravenous administration (0.3 to 1 mg/kg; Figure 2-2 and Figure 2-4) suggests that ASP6432 will have favorable oral absorption with expected efficacy after oral administration, and a low risk of cardiovascular side effects. Regarding toxicity, theoretical side effects assumed from findings in LPA_1-deficient mice, such as abnormalities in fetal development (Contos et al., 2000),

should be carefully assessed. However, the clinical study results of LPA_1 antagonists (Allanore et al., 2018; Palmer et al., 2018) suggest a low risk of mechanism-based serious toxicity precluding clinical application. Future studies on these aspects will provide a more extensive characterization of the therapeutic potential of ASP6432 in the treatment of voiding dysfunction.

In conclusion, I demonstrated that ASP6432 but not tamsulosin decreased urethral pressure during urine voiding and improved L-NAME-induced voiding dysfunction. The results in this chapter suggest that LPA_1 has a significant role in regulating urethral tonus during urine voiding, and highlight the potential of ASP6432 for improving voiding dysfunctions and LUTS associated with BPH and other lower urinary tract diseases.

Table 2-1. Baseline urethral perfusion pressure (UPP) parameters under isovolumetric conditions.

Parameter	Vehicle	ASP6432 (mg/kg iv)			Tamsulosin (mg/kg iv)
		0.1	**0.3**	**1**	**0.01**
UPP_{base} (mmHg)	23.23 ±2.31	24.57 ±2.59	27.40 ±2.45	26.13 ±2.06	23.23 ±2.31
UPP_{nadir} (mmHg)	9.51 ±0.97	10.36 ±1.75	10.22 ±1.19	8.88 ±0.96	9.09 ±1.09
Urethral relaxation (s)	26.5 ±4.9	35.6 ±4.2	36.9 ±2.3	29.3 ±5.7	31.4 ±5.3
HFOs(s)	13.8 ±2.1	14.7 ±2.0	13.2 ±1.7	15.6 ±3.2	14.9 ±1.7

UPP_{base}, baseline UPP at the bladder filling phase; UPP_{nadir}, minimum UPP at the urine voiding phase; HFOs, high frequency oscillations. Values indicate the mean±SEM of eight animals. There were no statistical differences in any parameters between groups (one-way ANOVA).

Table 2-2. Baseline cystometric parameters of each treatment group.

Parameter	ASP6432 (mg/kg iv)				Tamsulosin (mg/kg iv)		
	Veh.	0.3	1	3	Veh.	0.003	0.01
PVR (mL)	0.05 ±0.02	0.06 ±0.01	0.04 ±0.01	0.06 ±0.03	0.05 ±0.01	0.05 ±0.02	0.04 ±0.01
VE (%)	94.8 ±1.7	94.1 ±1.2	95.2 ±1.4	95.6 ±1.5	95.1 ±1.2	96.2 ±1.3	95.4 ±0.9
Time to voiding (min)	10.2 ±2.4	11.8 ±1.3	10.8 ±1.2	12.2 ±2.2	11.0 ±0.8	10.6 ±2.4	9.2 ±1.7
Maximum IVP (mmHg)	26.6 ±2.2	30.0 ±2.1	25.4 ±0.7	25.6 ±1.5	26.8 ±0.7	29.8 ±3.2	26.8 ±4.1

Veh., vehicle; PVR, post-void residual urine; VE, voiding efficiency; IVP, intravesical pressure. Values indicate the mean±SEM of five (ASP6432) or four (tamsulosin) animals. There were no statistical differences in any parameters between groups (one-way ANOVA).

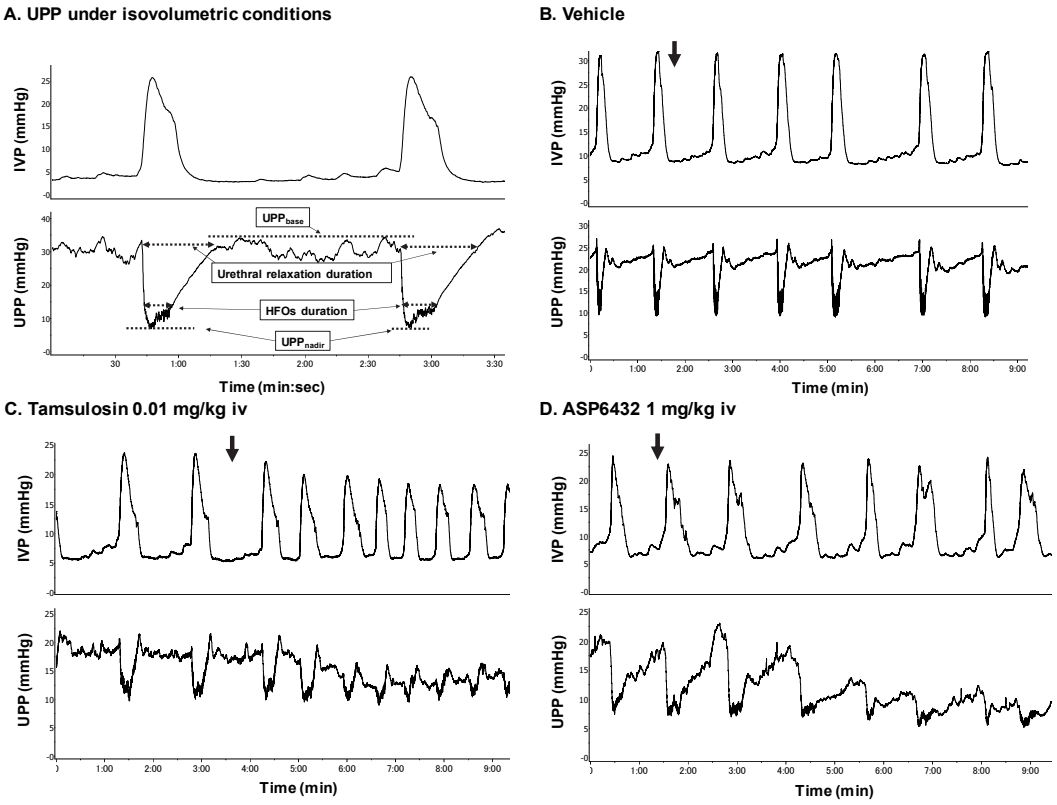


Figure 2-1. Representative traces of urethral perfusion pressure (UPP) in anesthetized rats under isovolumetric conditions (A) and changes after treatment with vehicle (B), tamsulosin (0.01 mg/kg iv) (C), and ASP6432 (1 mg/kg iv) (D).

UPP was measured under urethane anesthesia and isovolumetric conditions. Intravesical pressure (IVP) was monitored to confirm that reflex bladder contractions and urethral relaxations occur in a synchronized manner. Drugs were administered intravenously. Down arrows indicate the timing of administration. UPP_{base}, UPP at the bladder filling phase; UPP_{nadir}, the minimum UPP at the urine voiding phase; HFOs, high frequency oscillations.

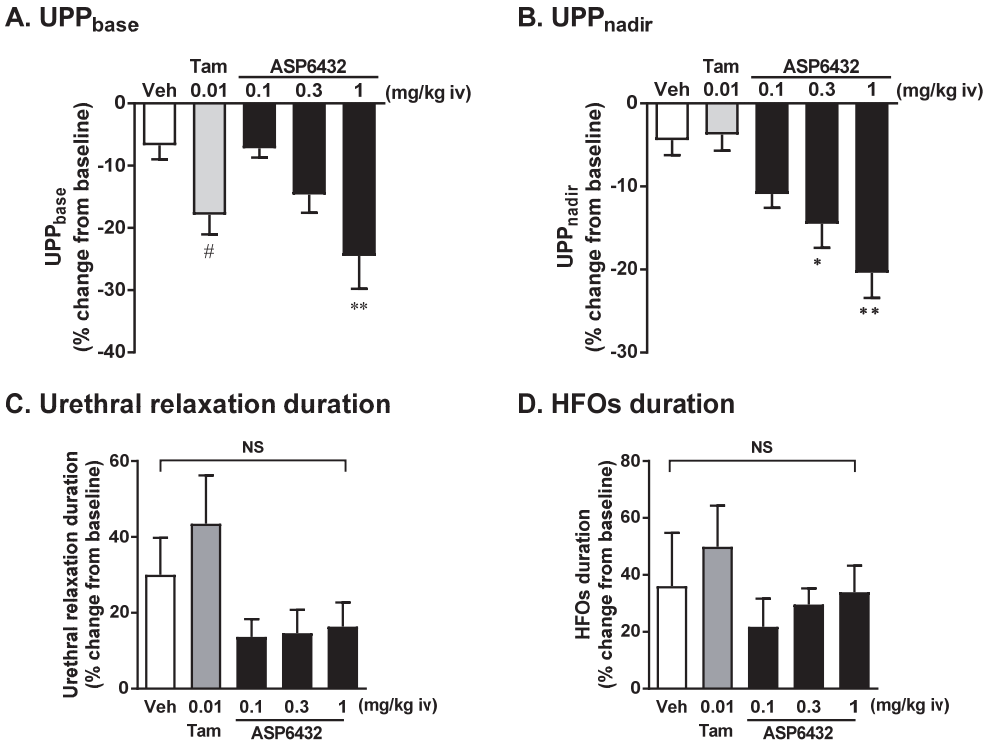


Figure 2-2. Effect of ASP6432 and tamsulosin on urethral perfusion pressure (UPP)-related parameters in anesthetized rats under isovolumetric conditions.

The maximum change in UPP at the bladder filling phase (UPP_{base}) (A), the minimum UPP at the urine voiding phase (UPP_{nadir}) (B), the duration of urethral relaxation at the urine voiding phase (C), and the duration of high frequency oscillations (HFOs) (D) in 15 min after treatment with vehicle (Veh), ASP6432 (0.1 to 1 mg/kg iv), or tamsulosin (Tam) (0.01 mg/kg iv) were calculated for each animal. Each column represents the mean±SEM of eight animals.

#$p<0.05$ compared with vehicle (Student's t-test); *$p<0.05$, **$p<0.01$ compared with vehicle (Dunnett's test using within subject error).

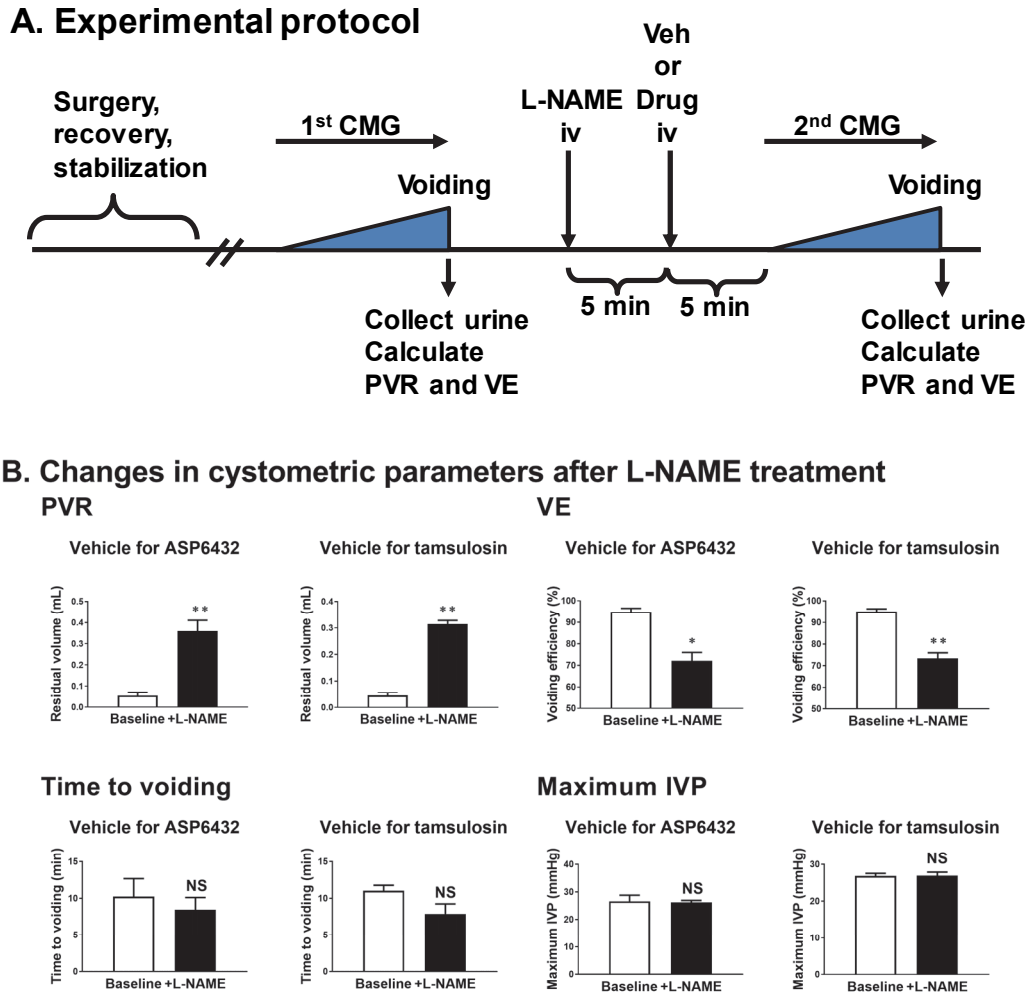


Figure 2-3. Schematic diagram of the experimental protocol (A) and changes in cystometric parameters after N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) treatment (B) in conscious rats.

In (B), changes in post-void residual urine (PVR), voiding efficiency (VE), time to voiding, and maximum intravesical pressure (IVP) from baseline after L-NAME treatment were calculated for each animal. Each column represents the mean±SEM of five (Vehicle for ASP6432) or four (Vehicle for tamsulosin) animals. *$p<0.05$, **$p<0.01$ compared with baseline (paired t-test). CMG, cystometry; NS, not significant; Veh, vehicle.

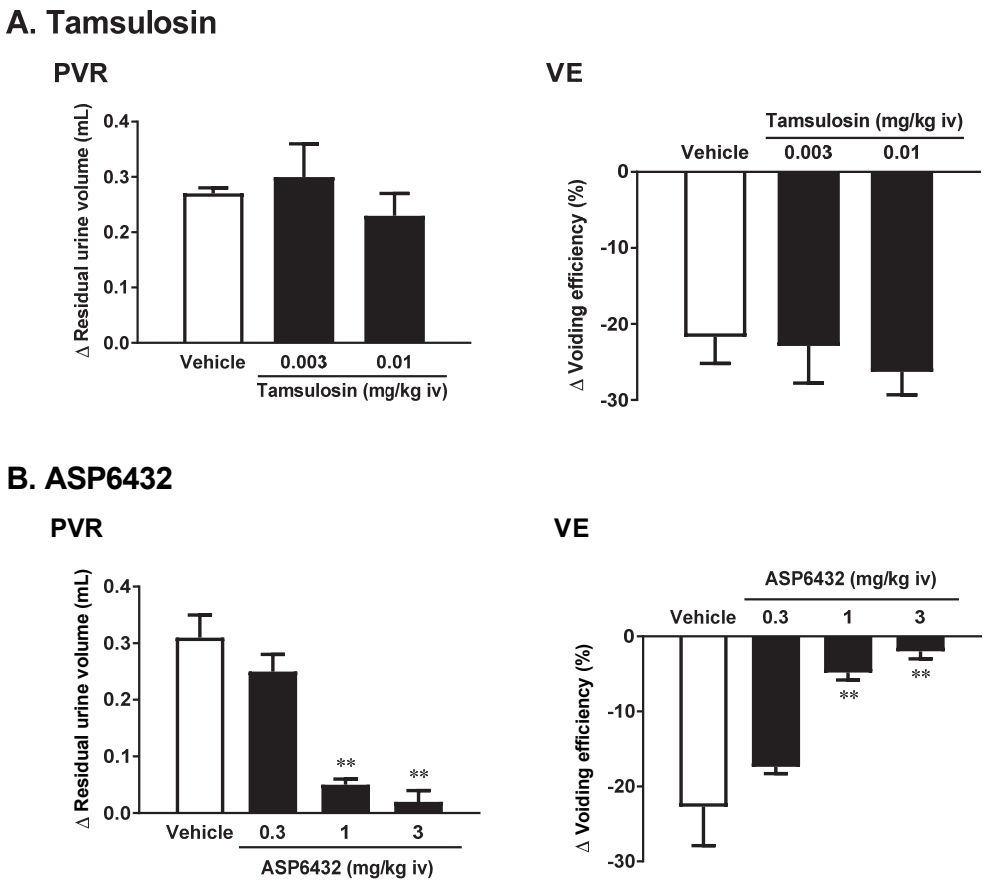


Figure 2-4. Effect of tamsulosin (A) and ASP6432 (B) on voiding dysfunctions induced by N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME).

Changes in post-void residual urine (PVR), voiding efficiency (VE), time to voiding, and maximum intravesical pressure (IVP) from baseline after treatment with vehicle, ASP6432 (0.3 to 3 mg/kg iv), or tamsulosin (0.003 to 0.01 mg/kg iv) were calculated for each animal. Each column represents the mean±SEM of five (ASP6432) or four (tamsulosin) animals.

**p<0.01 compared with vehicle (Dunnett's test using within subject error).

Chapter 3: Effect of Lysophosphatidic Acid (LPA) and the Type 1 LPA Receptor Antagonist ASP6432 on Urinary Frequency: Potential for LPA_1 antagonism in the Treatment of Bladder Overactivity

Introduction

Maintenance of continence and control of the timing of urination by proper storage of urine in the bladder are fundamental to leading a normal personal and social life. The micturition center located in the pons and the autonomic nervous system cooperatively regulate the normal reservoir function of the bladder. Noradrenaline released from sympathetic nerve endings activates β_3-adrenoceptors expressed in the bladder to distend the detrusor smooth muscle and α_1-adrenoceptors to contract the urethral smooth muscle (de Groat et al., 2015).

Dysregulation of bladder urine storage function causes storage symptoms, characterized by urgency, urinary frequency, nocturia, or incontinence (Abrams et al., 2002). Storage symptoms are commonly observed in patients with LUTS associated with BPH. Although patients typically find storage symptoms the most bothersome (Chapple et al., 2014), treatment of LUTS associated with BPH tends to focus on improving voiding symptoms (slow stream hesitancy, incomplete bladder emptying, and terminal dribbling). $Alpha_1$-blockers, currently first-choice pharmacotherapies, primarily relax the contractions of the prostatic part of the urethra and improve BOO, but do not always sufficiently alleviate storage symptoms (van Kerrebroeck et al., 2013). Therefore, an agent that potently

improves bladder dysfunction in addition to BOO may be a better treatment option for LUTS associated with BPH.

LPA is a small glycerophospholipid found ubiquitously in vertebrates that mediates diverse biological actions demonstrating medicinal relevance (Yung et al., 2014). In the lower urinary tract, LPA contracts urethral strips isolated from rats, and increases intraurethral pressure in rats and dogs via LPA_1 (Terakado et al., 2017; Terakado et al., 2016). In cultured bladder smooth muscle cells, LPA induces contraction (Kropp et al., 1999) and is suggested to mediate stretch-induced cellular activation possibly via LPA_1, the most prominently expressed LPA receptor subtype (Kawashima et al., 2015). To my knowledge, however, no study has yet investigated the effect of LPA on bladder function *in vivo*. In Chapter 1 and 2, I demonstrated that LPA_1 regulates the contraction and tonus of the prostate and urethra, using the potent and selective antagonist ASP6432. However, the effect of ASP6432 on bladder function remains to be elucidated.

To clarify the role of LPA and LPA_1 in the regulation of bladder function, I examined in this Chapter the effects of LPA and the LPA_1 antagonist ASP6432 on the micturition reflex in conscious rats using continuous cystometry. In addition, I evaluated the effect of ASP6432 on the decrease in micturition interval (MI, a cystometry parameter reflective of urinary frequency) induced by the NOS inhibitor L-NAME.

Materials and Methods

Test reagents

ASP6432 (Figure 1-1) was synthesized at Astellas Pharma Inc. (Tokyo, Japan). ASP6432 was dissolved and serially diluted either with distilled water containing 0.025 mol/L NaOH or with saline containing 5% *N,N*-dimethylformamide. LPA (1-linolenoyl-2-hydroxy-sn-glycero-3-phosphate) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) was dissolved in saline containing 0.1% BSA. L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved and diluted in distilled water.

Experimental animals

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Astellas Pharma Inc. Tsukuba Research Center was awarded Accreditation Status by the AAALAC International. Male Wistar rats and female Sprague Dawley rats were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). The number of animals per group was determined based on the effect size and standard deviation estimated from preliminary experiments.

Surgical procedures

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and fixed in the supine position. For saline infusion into the bladder and measurement of IVP, a midline incision was first made in the abdominal wall, and a polyethylene catheter (PE-50; Becton, Dickinson & Co., Franklin Lakes, NJ,

USA) was then inserted through a small incision made at the bladder apex and the tip was placed inside the bladder dome. For drug administration, a polyethylene catheter (PE-50) filled with saline containing sodium heparin was inserted into the jugular vein. These catheters were tunneled subcutaneously and anchored to the skin of the back of the neck using silk ligatures. To evaluate the effect of LPA and the effect of ASP6432 on changes induced by LPA, animals were administered ampicillin sodium (20 mg/kg, sc) and housed for another three days with free access to food and water in individual cages. To evaluate the effect of ASP6432 on L-NAME-induced changes, cystometry experiments were conducted on the same day as the surgery, after the animals had recovered from the anesthesia in Ballman's cages (Natsume Seisakusho Co., Ltd., Tokyo, Japan).

Cystometry experiments under conscious conditions

Animals were placed in Ballman's cages, and saline was continuously infused into the bladder through the inserted catheter using an infusion pump (STC-525 or TE-331, Terumo, Tokyo, Japan; KDS100, Muromachi Kikai Co. Ltd., Tokyo, Japan or Neuroscience Inc., Tokyo, Japan) at 4.2 mL/h to induce the micturition reflex. IVP was measured using a pressure transducer (TP-400T, Nihon Kohden, Tokyo, Japan) connected to a pressure amplifier (AP-601G or AP-621G, Nihon Kohden).

To evaluate the effect of LPA and ASP6432, female Sprague Dawley rats were used. The effect of LPA (1, 5, 10, and 15 mg/kg/h) on bladder parameters was evaluated in four groups. Before LPA administration, vehicle for LPA (saline containing 0.1% BSA) was intravenously infused at 3 mL/kg/h until the micturition reflex was stabilized, and the average MI, maximum IVP, BP, and TP of two

consecutive micturition cycles were measured as baseline. After determining baseline values, intravenous infusion was switched to LPA and cystometry was continued. After the changes by LPA were stabilized, the average MI, max IVP, BP, and TP of two consecutive micturition cycles were measured. To evaluate the effect of ASP6432, a separate experiment was performed. In animals showing greater than a 10% decrease in MI from baseline after LPA (5 mg/kg/h) infusion, ASP6432 (0.3, 1, 3 and 10 mg/kg) was intravenously injected at the end of each micturition cycle with incremental dosing and the effect was evaluated at the next micturition cycle. Changes following drug treatment were expressed as a percentage of baseline values.

To evaluate the effects of ASP6432 on L-NAME-induced changes, male Wister rats were used. L-NAME (10 mg/kg) was intravenously injected after the micturition reflex was stabilized. Animals showing greater than a 10% decrease in MI at around 30 min after L-NAME administration were used to evaluate the effect of ASP6432. ASP6432 (0.3 or 1 mg/kg) or vehicle (saline with 5% DMF) was administered intravenously. Cystometry measurement was continued for another 30 min, and changes in MI, max IVP, BP and TP were calculated.

Data analysis

PowerLab and LabChart (ADInstruments Japan, Aichi, Japan) were used to collect data. Statistical analyses were performed using SAS software (SAS Institute Japan, Tokyo, Japan) or Prism (GraphPad Software, San Diego, CA, USA). Results are presented as mean ±SEM. The number of animals per group is indicated in either the graph or legend of the respective figure. Paired t-test was

used to compare changes before and after drug treatment. One-way analysis of variance (ANOVA) or ANOVA followed by Dunnett's multiple comparisons test was used to compare the difference between groups. A p value<0.05 was considered statistically significant.

Results

LPA caused bladder overactivity in conscious rats

Under intravenous infusion of vehicle for LPA (saline containing 0.1% BSA), there were no differences in baseline MI, max IVP, BP, or TP between the groups. After switching the infusion from vehicle to LPA (1, 5, 10, or 15 mg/kg/h), MI dose-dependently decreased compared to baseline at 5 mg/kg/h and greater. TP also statistically significantly decreased compared to baseline at 10 mg/kg/h. No significant changes were observed in BP or max IVP (Figure 3-1).

LPA-induced urinary frequency was reversed by the LPA_1 antagonist ASP6432

Based on the above findings, we evaluated the effect of ASP6432 on LPA (5 mg/kg/h)-induced cystometric changes. Intravenous infusion of LPA significantly decreased MI and TP compared to baseline by 47% and 38%, respectively. ASP6432 (0.3 to 10 mg/kg iv) dose-dependently inhibited the LPA-induced decrease in MI, with statistically significant inhibition observed at doses of 1 mg/kg and greater compared to the vehicle-treated group (Figure 3-2 and 3-3). In contrast, ASP6432 did not inhibit the LPA-induced decrease in TP up to 10 mg/kg iv. Neither LPA nor ASP6432 had an effect on BP or max IVP (Figure 3-3).

ASP6432 improved L-NAME-induced urinary frequency

Intravenous injection of L-NAME (10 mg/kg) significantly decreased MI compared to baseline without significant changes in max IVP, BP, or TP (Figure 3-4 and 3-5).

Treatment with ASP6432 (0.3 and 1 mg/kg iv) dose-dependently and significantly reversed the L-NAME-induced decrease in MI compared to the vehicle-treated group (Figure 3-4 and 3-6). ASP6432 had no effect on the other cystometric parameters tested (Figure 3-6).

Discussion

In this chapter, I demonstrated that LPA decreased MI and TP in conscious rats, and that the LPA_1 antagonist ASP6432 inhibited the LPA- and L-NAME-induced decreases in MI. Despite recent studies demonstrating a significant role for the LPA-LPA_1 signaling axis in urethral contraction (Saga et al., 2014; Terakado et al., 2017)(Terakado et al., 2016), the effect of LPA and LPA_1 in the bladder has not been clarified *in vivo*. To my knowledge, this is the first study to show that i) LPA causes bladder overactivity characterized by decreases in MI and TP, ii) the LPA_1 antagonist ASP6432 reverses the LPA-induced decrease in MI, and iii) ASP6432 improves urinary frequency induced by the NOS inhibitor L-NAME.

In the continuous cystometry study in conscious rats, intravenous infusion of LPA decreased MI and TP (Figure 3-1). Although I did not measure the plasma LPA concentration, the amount of LPA administered in this study (1 to 15 mg/kg/h, approximately 0.04 to 0.6 μmol/kg/min calculated from the free form molecular weight of around 450) appeared to be within the physiological level, since blood LPA concentrations range from 0.1 to 10 μmol/L (Yung et al., 2014) and LPA is rapidly eliminated from the plasma (Salous et al., 2013). Given that LPA is a multifactorial bioactive phospholipid (Yung et al., 2014), it is likely that these LPA-induced changes result from multiple mechanisms. One simple

possible mechanism is that LPA induces contraction of the detrusor smooth muscle, since LPA contracts cultured bladder smooth muscle cells (Kropp et al., 1999). However, intravenous LPA infusion did not significantly increase bladder contractility parameters such as BP and max IVP up to 10 mg/kg/h, while MI and TP decreased at 10 mg/kg/h or less (Figure 3-1), suggesting a minimal, if any, contribution of direct detrusor contraction by LPA. Alternatively, urethral contraction by LPA shown in Chapter 1 may have reduced the functional capacity of the bladder by causing BOO and accumulating post-void residual urine (PVR) in the bladder(Vesely et al., 2003). However, intravenous LPA infusion did not increase max IVP (Figure 3-1) indicative of a compensatory increase in bladder contractility, suggesting that LPA-induced significant BOO is not the cause of decrease in MI, although we did not measure PVR due to technical difficulties under the present study conditions.

Another possible mechanism underlying the LPA-induced decrease in MI and TP is the excitation of bladder sensory neurons by LPA. Afferent Aδ-fibers transduce normal sensations of bladder filling and C-fibers are activated under pathological conditions (Fowler et al., 2008; Yoshimura et al., 2002). Stimulation or suppression of these afferent fibers leads to decreases or increases in MI and TP, respectively (Du et al., 2007; Nagabukuro et al., 2010; Strittmatter et al.,

2012). In addition, LPA_1 mediates the peripheral LPA-induced activation of primary afferent and spinal cord neurons (Elmes et al., 2004; Renback et al., 2000). Further, LPA_1 modulates the activity of the tetrodotoxin-resistant sodium channel Nav1.8 in dorsal root ganglion neurons (Pan et al., 2016), which is implicated in the activation of C-fibers after chemical irritation of the bladder (Yoshimura et al., 2001). These prior studies suggest that activation of LPA_1 expressed in afferent neurons can enhance the sensory signaling from the bladder and decrease MI and TP.

Intravenous administration of the LPA_1 antagonist ASP6432 significantly but not completely inhibited the LPA-induced decrease in MI (Figure3-2, 3-3), indicating that LPA_1 plays a major role in the LPA-induced decrease in bladder capacity. However, the LPA-induced decrease in TP was not affected by ASP6432 (Figure 3-3), despite my initial hypothesis. The present study result suggests that the LPA-induced decrease in TP is mediated by other LPA receptor subtypes or other LPA-binding proteins that regulate sensory signaling. LPA_3 and LPA_5 receptors are implicated in the process of allodynia (Murai et al., 2017; Uchida et al., 2014), although it is unknown whether there is any mechanistic similarity between allodynia and bladder sensation. LPA can directly bind and activate transient receptor potential (TPR) channels TRPV1 and TRPA1 (Kittaka et al.,

2017; Nieto-Posadas et al., 2011), which are expressed in afferent fibers and are involved in bladder hypersensitivity by acting as sensors for stretch and/or chemical irritation (Andersson, 2016). Direct activation of these channels by LPA may activate bladder afferent fibers and contribute to decreasing the TP and MI, which were not reversed by ASP6432. Another possibility is that peripherally administered LPA acts in the central nervous system (CNS), which ASP6432 cannot affect due to its low brain permeability (data not shown), although it is unknown whether and to what extent LPA penetrates the brain blood barrier. Further studies are needed to identify the mechanistic basis of the effect of LPA and LPA_1 on bladder function, including the mechanism governing the LPA-induced decrease in TP and its implication for the micturition complex.

To explore the consequence of suppressing LPA_1 on bladder function, I evaluated the effect of ASP6432 on urinary frequency elicited by L-NAME in conscious rats. Previous studies suggest that L-NAME affects bladder urine storage function via multiple mechanisms. Bladder perfusion of L-NAME (10 mg/mL) enhances the decrease in TP by capsaicin (Caremel et al., 2010) and increases the activity of bladder Aδ- and C-fibers in anesthetized rats (Aizawa et al., 2011). The intravenous L-NAME administration (20 mg/kg) increases max IVP and decreases MI in conscious rats, while MI is increased in anesthetized rats

(Masuda et al., 2007). In the present study in conscious rats, intravenous administration of L-NAME (10 mg/kg) decreased MI without significantly affecting max IVP, BP, or TP (Figure 3-4 and 3-5), which is consistent with findings using intraarterial L-NAME administration (10 mg/kg) in conscious rats (Persson et al., 1992). Results of the present and previous studies suggest that systemic administration of L-NAME at 10 mg/kg decreases MI in conscious rats as a result of a combination of minor effects on multiple mechanisms regulating the micturition reflex.

ASP6432 significantly reversed the L-NAME-induced decrease in MI (Figure 3-4 and 3-6), indicating that inhibition of LPA_1 by ASP6432 can ameliorate urinary frequency resulting from impaired NO production. However, to my knowledge, no studies have investigated the correlation between impaired NO production and LPA_1 activation. Given that both L-NAME and ASP6432 altered MI without affecting other cystometoric parameters (Figure 3-5 and 3-6), the mechanism underlying the effect of ASP6432 on L-NAME-induced decrease in MI may be multifactorial, as suggested by the effect of L-NAME at 10 mg/kg iv on MI. Another hypothesis is that urethral relaxation by ASP6432 demonstrated in Chapter 1 and 2 may have suppressed the sensory signaling from the urethra that facilitates bladder contraction (Danziger and Grill, 2017; Yokoyama et al., 2007).

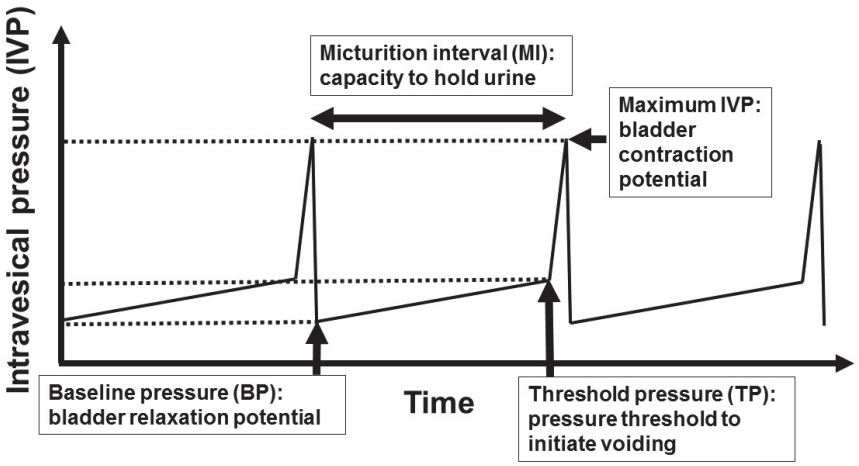
However, it is unknown whether and how L-NAME or ASP6432 affects the afferent signaling from the urethra and modulates MI. Further investigation is required to elucidate the exact mechanism of action of ASP6432 on L-NAME-induced changes in bladder function.

The present finding that ASP6432 significantly reversed the LPA- and L-NAME-induced decreases in MI suggests that ASP6432 has the potential to ameliorate urinary frequency and storage symptoms in BPH patients. This potential on top of potent urethral relaxation shown in previous chapters may provide ASP6432 with a significant advantage over existing pharmacotherapies, given that i) patients with symptomatic BPH normally have storage symptoms in addition to voiding symptoms (Gacci et al., 2018); ii) α_1-blockers, the current first-choice pharmacotherapies, do not always satisfactorily improve storage symptoms (van Kerrebroeck et al., 2013); and iii) tadalafil, the only approved PDE5 inhibitor for the treatment of LUTS associated with BPH, did not significantly improve maximum urine flow or PVR in the majority of clinical studies(Hatzimouratidis, 2014). Further preclinical and clinical investigations will contribute to determining the optimal therapeutic option for treatment of the diverse symptoms of LUTS associated with BPH.

The present study results provide various insights for further investigations into the role of LPA_1. Given that our results are limited to rodents, the expression and function of LPA and LPA_1 should be confirmed in the human bladder, particularly in pathological conditions. As mentioned above, detailed investigation is required to fully elucidate the exact mechanism of action of LPA_1 on MI, and the possible involvement of other LPA receptor subtypes or molecular targets on the LPA-induced change in TP. In addition, potential interaction between LPA_1 and NO or other neurotransmitters should be clarified. Future studies on these aspects will lead to a more extensive characterization of the therapeutic potential of ASP6432 in the treatment of lower urinary tract dysfunctions.

In conclusion, I demonstrated for the first time that LPA is a bioactive phospholipid capable of causing bladder overactivity characterized by decreases in MI and TP, and that the LPA_1 antagonist ASP6432 reversed the LPA- and L-NAME-induced decreases in MI. The results in this chapter suggest a significant role for LPA_1 in regulating the functional capacity of the bladder, and the potential of ASP6432 as a novel therapy for the treatment of bladder dysfunction caused by lower urinary tract diseases like storage symptoms in LUTS associated with BPH.

A.



B.

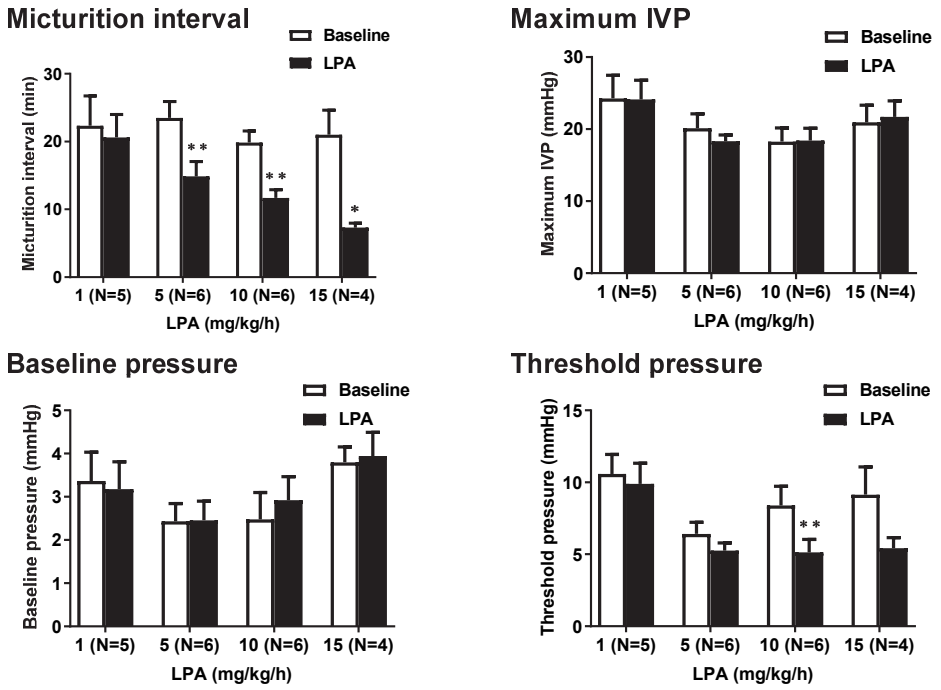


Figure 3-1. Cystometry parameters measured (A) and the effect of lysophosphatidic acid (LPA) on cystometric parameters in conscious rats (B). Micturition interval, maximum intravesical pressure (IVP), baseline pressure, and threshold pressure at baseline and after LPA infusion (1, 5, 10, and 15 mg/kg/h) were measured. Each column represents the mean ± SEM of four to six animals. * $p<0.05$, ** $p<0.01$ vs. baseline (paired t test).

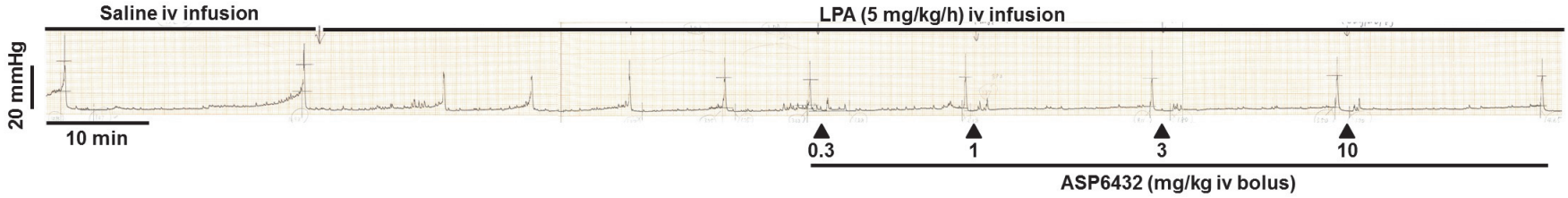


Figure 3-2. Representative trace of bladder pressure after treatment with lysophosphatidic acid (LPA) followed by ASP6432 during continuous cystometry in conscious rats.

Intravenous infusion of LPA (5 mg/kg/h) was started after the baseline micturition reflex under intravenous infusion of vehicle for LPA (saline containing 0.1% BSA, 3 mL/kg/h) was stabilized. After the decrease in micturition interval by LPA (5 mg/3 mL/kg/h) was stabilized, ASP6432 (0.3, 1, 3 and 10 mg/mL/kg) was intravenously injected after each micturition cycle (injected at the arrows) with incremental dosing.

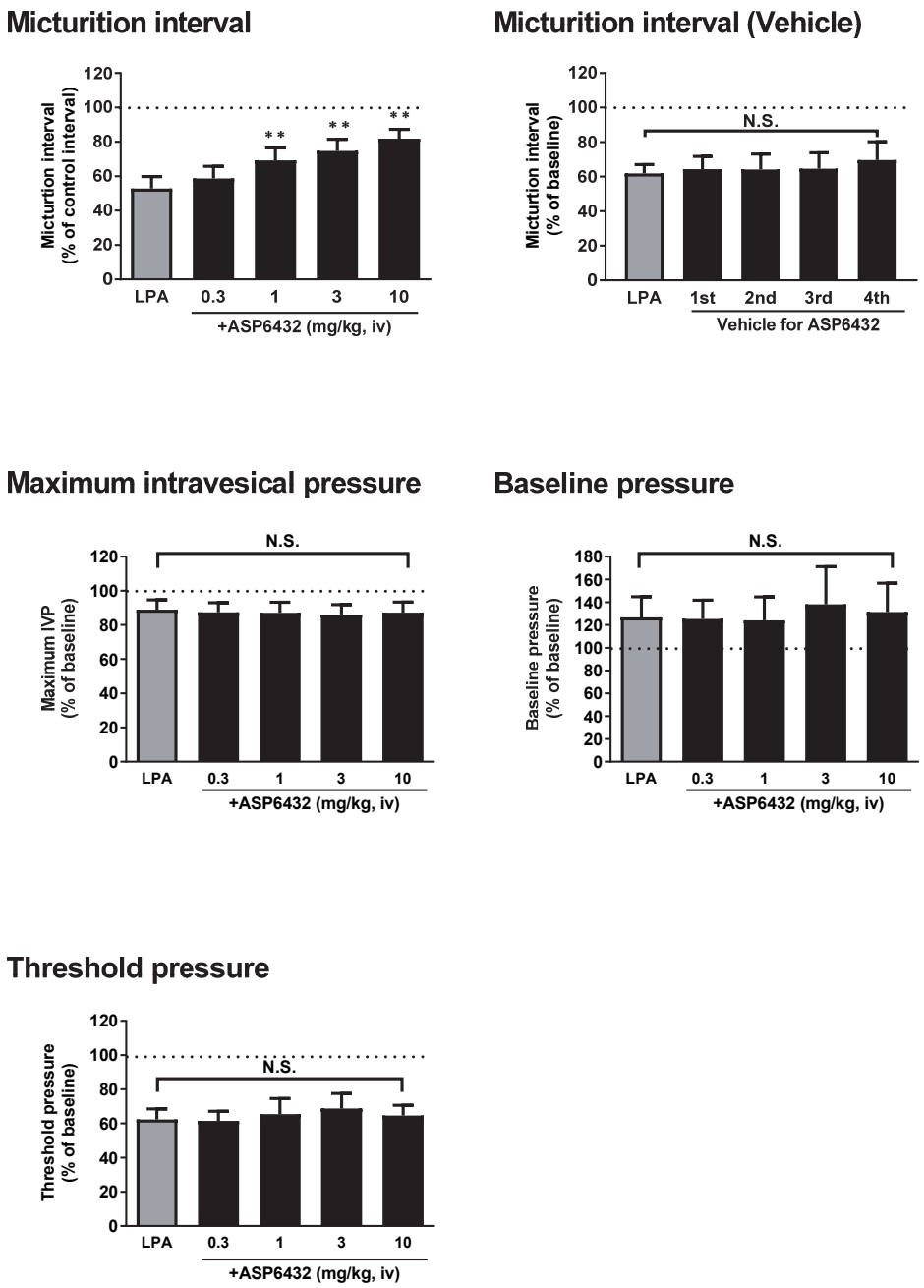


Figure 3-3. Effect of ASP6432 on lysophosphatidic acid (LPA)-induced changes in cystometric parameters in conscious rats.

Changes in micturition interval, maximum intravesical pressure (IVP), baseline pressure and threshold pressure after ASP6432 or vehicle (distilled water containing 0.025 mol/L NaOH) administration were measured. Each parameter was expressed as a percentage of baseline values. Data are presented as the mean ± SEM of eight (ASP6432) or six (vehicle) animals. ## $p<0.01$ vs. baseline (paired t test), ** $p<0.01$ vs. LPA (Dunnett's test using within subject error).BL, baseline; N.S., not significant.

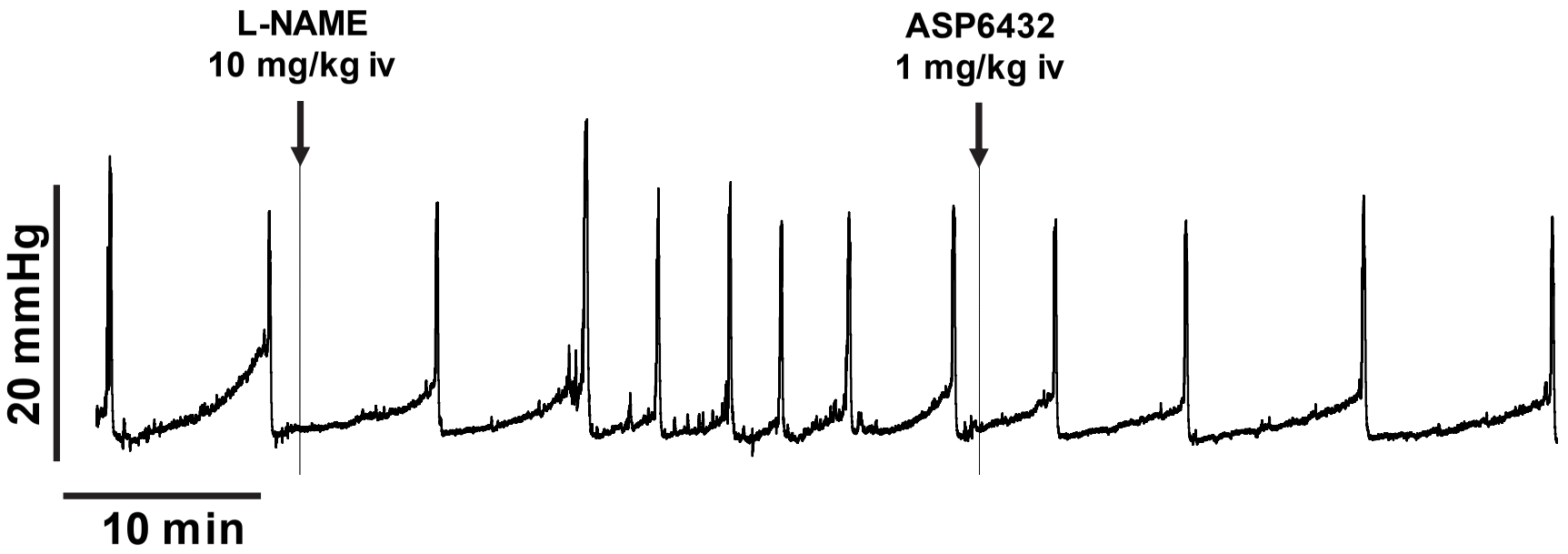


Figure 3-4. Representative traces of bladder pressure after treatment with N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) followed by ASP6432 during continuous cystometry in conscious rats.
L-NAME (10 mg/kg) was intravenously administered after the baseline micturition reflex was stabilized. Around 30 minutes after L-NAME administration was stabilized, ASP6432 (1 mg/kg) was intravenously injected.

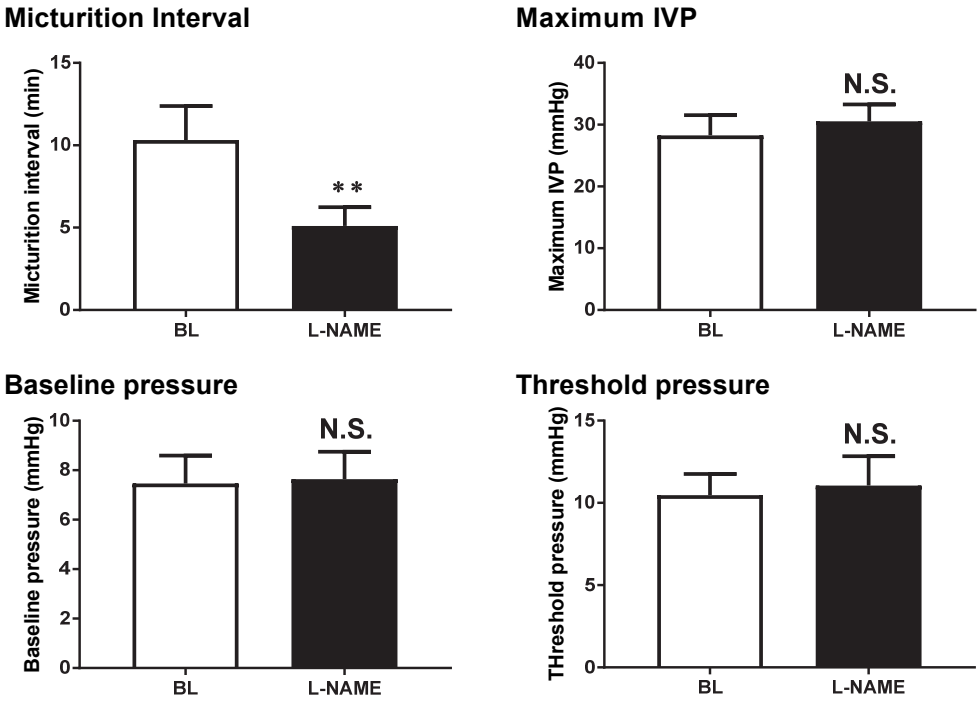


Figure 3-5. Effect of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on cystometric parameters in conscious rats.
Micturition interval, maximum intravesical pressure (IVP), baseline pressure, and threshold pressure at baseline and after L-NAME administration were measured. Each column represents the mean ± SEM of eight animals.
** $p<0.01$ vs. control interval (paired t test).
BL, baseline; N.S., not significant.

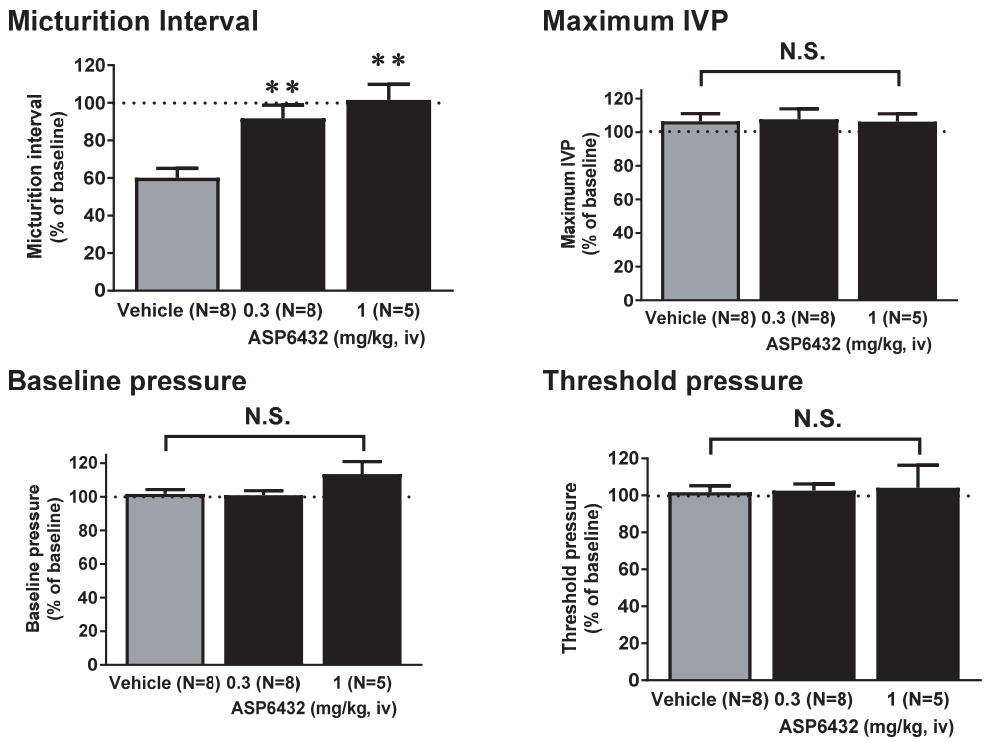


Figure 3-6. Effect of ASP6432 on the N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME)-induced decrease in micturition interval (MI) in conscious rats.
Changes in the micturition interval, maximum intravesical pressure (IVP), baseline pressure, and threshold pressure after treatment with L-NAME (10 mg/kg iv) followed by ASP6432 (0.3 and 1 mg/kg iv) or vehicle are shown. Each column represents the mean ± SEM of five to eight animals.
** $p<0.01$ compared to vehicle (Dunnett's multiple comparisons test).
BL, baseline; N.S., not significant.

General discussion

LUTS associated with BPH is one of the most common chronic urological diseases among elderly men, and the number of patients is expected to increase as the average life expectancy and aging population continue to rise (Roehrborn, 2005). Despite recent progress in medical research, a number of remaining unmet needs of current BPH therapies, such as potent relaxation of the bladder outlet component, further improvement in storage symptoms, and suppression of stromal cell proliferation, indicate that pharmacotherapies for LUTS/BPH require further improvement. There is currently no single pharmacotherapeutic agent that substantially suppresses the pathological progression of BPH and improves all elements of LUTS. Considering the heterogeneous and multifactorial pathological nature of LUTS/BPH, novel treatment strategies will have to improve multiple pathophysiological features to achieve a significant treatment effect. While this is challenging, it is exciting from a drug discovery perspective to identify a mechanism that can simultaneously induce more potent urethral relaxation during voiding, suppress stromal hyperplasia, and further improve bladder dysfunction because such an agent will significantly improve treatment for LUTS/BPH by meeting all major unmet needs of current pharmacotherapies.

The present study investigated the role of LPA, a pleiotropic lipid mediator, and its receptor LPA_1 in the lower urinary tract by conducting pharmacological characterization of the novel LPA_1 antagonist ASP6432. It was important and necessary to elucidate these characteristics in this study because the exact role of the LPA-LPA_1 signaling axis in the lower urinary tract including its potential as a

therapeutic target remained unclear, particularly *in vivo*, despite its diverse biological actions such as smooth muscle contraction (Saga et al., 2014), cell proliferation (Adolfsson et al., 2002), and effects on cultured smooth muscle cells, and the potential pathophysiological relevance in BPH (Zeng et al., 2009).

In Chapter 1, the pharmacological profile of ASP6432 and its effect on urethral/prostatic contractile function and prostate cell proliferation were characterized. ASP6432 exhibited potent antagonistic activity against human and rat LPA_1 with selectivity over all other receptors, ion-channels, transporters, and enzymes tested (Table 1-1). In isolated rat tissue strips and anesthetized rats, ASP6432 concentration/dose-dependently inhibited LPA-induced urethral and prostate contractions (Figure 1-3), demonstrating that LPA_1 was responsible for these actions. Interestingly, ASP6432 decreased the UPP in the absence of exogenous LPA stimulation in anesthetized rats, and the maximum reduction was greater than that induced by the α_1-blocker tamsulosin. The effect of LPA on smooth muscle contraction (Figure 1-2), reported LPA concentration in rat plasma (around 1 μmol/L) (Saga et al., 2014), antagonist activity of ASP6432 on rat LPA_1 (Table 1-1), and pharmacokinetic profile of ASP6432 in rats after intravenous administration (Table 1-2) support the hypothesis that endogenous LPA constantly activates LPA_1 and plays a significant role in the regulation of urethral tonus, which is suppressed by ASP6432, at least in rats. In addition, ASP6432 suppressed LPA-induced proliferation of human prostate stroma cells (Figure 1-6). Given that there is currently no pharmacotherapy that is simultaneously efficacious for both of these components, the present findings indicate that ASP6432 may represent a novel therapy with dual mechanisms for improving BOO. More specifically, in the short

term, ASP6432 may be more efficacious than α_1-blockers for improving voiding dysfunction and associated symptoms by potently relaxing the urethra. In the long term, ASP6432 may suppress the progression of stromal hyperplasia, which is not significantly improved by 5α reductase inhibitors (Marks et al., 1997), although this parameter is particularly difficult to examine in a preclinical setting due to the lack of appropriate animal models that mimic prostate stromal proliferation observed in the human condition (Hieble, 2011). Nevertheless, the findings in Chapter 1 suggest a pivotal role for LPA_1 in both urethral/prostatic contraction and cell proliferation, and the potential of ASP6432 as a novel therapy for LUTS/BPH with a greater urethral relaxation effect and possible suppression of stromal hyperplasia.

One notable finding in Chapter 1 was that ASP6432 significantly reduced the UPP (Figure 1-5), suggesting that endogenous LPA activates LPA_1 to induce contraction of the urethra to contribute to maintaining urethral tonus. However, the role of LPA_1 in the regulation of urethral tonus during urine voiding, which is critical for determining the efficiency of voiding and comparing the treatment effect with α_1-adrenoceptor antagonists, was unclear. Therefore, in Chapter 2, I generated an animal model that mimicked the status of the bladder and urethra during bladder filling and at urine voiding, and evaluated the effect of ASP6432 and tamsulosin on UPP_{base} and UPP_{nadir}.

In anesthetized rats under isovolumetric conditions, ASP6432 dose-dependently decreased UPP_{base} and UPP_{nadir}. In contrast, the α_1-adrenoceptor antagonist tamsulosin reduced UPP_{base} but did not change UPP_{nadir} (Figure 2-2A and 2-2B). To my knowledge, this is the first study to show that an LPA_1 antagonist decreases urethral tonus during urine voiding, suggesting a significant

role for LPA_1 in controlling urethral tonus during urine voiding. The lack of an effect by tamsulosin on UPP_{nadir} is consistent with the notion that the sympathetic nervous system is active at the filling phase but not at the voiding phase under normal conditions (Fowler et al., 2008). This study also suggests that the effect of ASP6432 on urethral tonus is not related to α_1-adrenoceptor antagonism because ASP6432 induced a greater decrease in UPP_{base} than tamsulosin (Figure 2-2A), reduced the UPP_{nadir} (Figure 2-2B), and showed good selectivity in an off-target screen that included α_1 receptors (data not shown). While drugs acting on the NO pathway reportedly modulate the duration of urethral pressure at the voiding phase (Jung et al., 1999; Wibberley et al., 2002), ASP6432 did not alter the duration of urethral relaxation or HFOs (Figure 2-2D), suggesting that LPA_1 and the NO pathway may act differently to modulate UPP. However, further investigations into potential interactions between LPA_1 and the adrenergic or nitrergic pathways are required to clarify the physiological role of LPA_1 in the lower urinary tract as well as the potential clinical benefits of the combined use of ASP6432 and α_1-blockers or PDE5 inhibitors.

To further assess the benefits of antagonizing LPA_1 for reducing urethral tonus during urine voiding and improving voiding dysfunction, another animal model was generated by suppressing the synthesis of NO using L-NAME. This model is based on evidence that NO is a neurotransmitter responsible for urethral relaxation during urine voiding (de Groat et al., 2015) and L-NAME inhibits urethral relaxation at the voiding phase (Bennett et al., 1995; Masuda et al., 2007) and increases PVR (Persson et al., 1992). As expected, L-NAME increased PVR and decreased VE (Figure 2-3B). ASP6432 dose-dependently suppressed the

increase in PVR and the decrease in VE induced by L-NAME (Figure 2-4B). In contrast, tamsulosin did not improve the L-NAME-induced changes in PVR or VE (Figure 2-4A). The effect of ASP6432 in the L-NAME model confirms a significant role for LPA_1 in regulating urethral tonus during urine voiding in male as well as female animals, which were used for UPP measurements for experimental reasons (see Discussion in Chapter 1).

The present study results suggest that ASP6432 has the potential to improve voiding dysfunctions that are not improved by current pharmacotherapies, given that pharmacotherapies approved for the treatment of LUTS with BPH are not necessarily effective for LUTS associated with other urological disorders. For example, the efficacy of α_1-blockers for female LUTS has not been established (Bae et al., 2005). Voiding dysfunctions associated with impaired urethral relaxation demonstrated in mice with targeted deletion of neuronal NO synthesis (Burnett et al., 1997) may be another candidate indication. Underactive bladder (Chapple et al., 2015), which may be caused by incomplete bladder emptying as a result of an imbalance between the bladder and the urethra, is one possible example. Potent urethral relaxation by ASP6432 may overcome the limited efficacy of currently available drugs for UAB (Andersson, 2017).

One significant unmet need of current pharmacotherapies for BPH is in the improvement of storage symptoms (Chapple et al., 2014; van Kerrebroeck et al., 2013). Although LPA_1 is predominantly expressed in cultured bladder smooth cells and is implicated in stretch-induced cellular activation (Kawashima et al., 2015), the role of LPA and LPA_1 in the regulation of bladder function has not been

investigated *in vivo*. In Chapter 3, the effect of LPA and ASP6432 on the micturition reflex was evaluated in conscious rats using continuous cystometry.

Intravenous infusion of LPA caused bladder overactivity, as characterized by the decrease in MI and TP (Figure 3-2 and 3-3). ASP6432 inhibited the LPA-induced decrease in MI in a dose-dependent manner (Figure 3-1 and 3-3). To my knowledge, this is the first study to show that LPA is a biological substance capable of inducing urinary frequency *in vivo* via LPA_1. Although the detailed mechanisms of the LPA-induced decrease in MI via LPA_1 have not been clarified, the lack of change in BP and max IVP by LPA (Figure 3-3) suggests a minor, if any, contribution by LPA-induced bladder smooth muscle contraction (which could negatively affect bladder distention) and LPA-induced urethral obstruction (which could reduce functional bladder capacity by causing incomplete bladder emptying) to the LPA-induced decrease in MI. One possible mechanism underlying the LPA-induced decrease in MP and TP is the activation of sensory neurons (Elmes et al., 2004; Renback et al., 2000). However, the expression and function of LPA_1 in bladder afferent neurons has not been closely examined. In addition, ASP6432 did not affect the LPA-induced decrease in TP (Figure 3-3), suggesting that another LPA receptor subtype or molecular target may be involved in the LPA-induced decrease in TP. Further investigation into the modulation of the bladder afferent pathway by LPA and its responsible receptor will help unravel the mechanism of LPA-induced changes in bladder function.

To explore the potential of improving urinary frequency by suppressing the LPA-LPA_1 signaling axis, I examined the effects of ASP6432 on urinary frequency induced by L-NAME in conscious rats using continuous cystometry. L-

NAME treatment decreased MI compared to that at baseline (Figure 3-5), which is consistent with a previous finding that L-NAME suppresses the inhibitory effect of NO on bladder excitability and induces bladder hyperactivity(Aizawa et al., 2011; Caremel et al., 2010; Masuda et al., 2007; Persson et al., 1992). ASP6432 suppressed the L-NAME-induced decrease in micturition frequency (Figure 3-6), suggesting that inhibition of LPA_1 activity can ameliorate bladder overactivity caused by impaired NO production. However, it should be noted that L-NAME can also affect bladder functions as a result of inhibiting urethral relaxation during voiding (Persson et al., 1992) or suppressing the sensory signaling from the urethra that facilitates bladder contraction (Danziger and Grill, 2017; Yokoyama et al., 2007), although it is unknown whether L-NAME decreases MI by affecting the afferent signaling from the urethra.. That ASP6432 improved the L-NAME-induced decrease in MI suggests that LPA_1 may be involved in bladder dysfunction, and that ASP6432 has the potential to improve bladder overactivity on top of its potent urethral relaxation effects during voiding and potential suppression of prostate stromal hyperplasia. The exact site/mechanism of action of LPA_1 in the regulation of bladder functions, however, needs further investigation.

In summary, the present study demonstrated a number of novel findings. *In vitro* experiments confirmed that ASP6432 is a potent and selective LPA_1 antagonist (Table 1-1). Studies using LPA and ASP6432 indicate that LPA_1 may be responsible for LPA-induced prostate and urethral contraction (Figure 1-3) and prostate stromal cell proliferation (Figure 1-6). *In vivo* studies using LPA and ASP6432 further confirmed that LPA_1 plays an important role in mediating the LPA-induced increase in urethral pressure (Figure 1-4) and urinary frequency

(Figure 3-3). In addition, ASP6432 potently decreased the urethral pressure, including during urine voiding, to a greater extent than that induced by tamsulosin (Figure 1-5, 2-2). Further, ASP6432 prevented voiding dysfunction (Figure 2-4) and reversed the decreased micturition frequency induced by L-NAME (Figure 3-6). These results suggest extensive roles for LPA_1 in the regulation of the lower urinary tract, ranging from prostate cell proliferation to bladder micturition interval, and the potential of ASP6432 in improving BPH and associated LUTS. The effects of ASP6432 clearly differentiate it from existing pharmacotherapies, such as its potent urethral relaxation effect during urine voiding, improvement of bladder function, and the potential suppression of prostate stromal cell hyperplasia.

While the present findings provide various insights for further investigations into the role of LPA_1, detailed mechanisms underlying the LPA_1-mediated modulation of the lower urinary tract has not been fully unraveled, including the intracellular signaling pathway responsible for controlling smooth muscle contraction, interaction between LPA_1 and other signaling pathways, the mechanism governing the modulation of bladder functions, and the *in vivo* prostate growth inhibition by suppressing LPA_1. Future studies on these aspects will allow for a more extensive characterization of the therapeutic potential of LPA_1 antagonists in the treatment of BPH and associated LUTS. In addition to urological diseases, ASP6432 may have other indications, given that a number of previous studies have demonstrated that LPA_1 antagonists are effective in a variety of disease models. Findings in other research areas may help researchers in urology to better understand the function of LPA_1 in the lower urinary tract.

In conclusion, the present study results suggest a role for LPA_1 in regulating the function of the lower urinary tract, and the potential of ASP6432 as a new therapeutic option for LUTS associated with BPH and other lower urinary tract diseases.

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List of Published Articles

Sakamoto K, Noguchi Y, Ueshima K, Yamakuni H, Ohtake A, Sato S, Ishizu K, Hosogai N, Kawaminami E, Takeda M, Masuda N. Effect of ASP6432, a Novel Type 1 Lysophosphatidic Acid Receptor Antagonist, on Urethral Function and Prostate Cell Proliferation. Journal of Pharmacology and Experimental Therapeutics. 2018;366:390-6.

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Sakamoto K, Noguchi Y, Ueshima K, Ohtake A, Sato S, Imazumi K, Takeda M, Masuda N. Modulation of urinary frequency via type 1 lysophosphatidic acid receptors: effect of the novel antagonist ASP6432 in conscious rats. European Journal of Pharmacology. *in press.* doi: 10.1016/j.ejphar.2019.03.011

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