DOI:10.14753/SE.2024.3019

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3019.

MOLNÁR PÉTER JÓZSEF

Kórélettan és transzlációs medicina
című program

Program- és témavezető: Dr. Benyó Zoltán, egyetemi tanár

DOI:10.14753/SE.2024.3019

The role and signaling of isoprostanes in the regulation of detrusor muscle tone in murine and human urinary bladder

PhD Thesis

Péter József Molnár, MD

Doctoral School of Theoretical and Translational Medicine

Semmelweis University

Supervisor: Zoltán Benyó, MD, PhD, DSc

Official reviewers: Ákos Pytel, MD, PhD
Márton Keszthelyi, MD, PhD

Head of the Complex Examination Committee:
Gábor Varga, MD, PhD, DSc

Members of the Complex Examination Committee:
Barna Vásárhelyi, MD, PhD, DSc
Csaba Ambrus, MD, PhD

Budapest
2024

DOI:10.14753/SE.2024.3019

Table of Contents

DOI:10.14753/SE.2024.3019

List of Abbreviations

αβ-methylene ATP: α,β-Methyleneadenosine 5’-triphosphate

CTRL: control

COX: cyclooxygenase enzyme

$[Ca^{2+}]_{ic}$: intracellular Ca^{2+} concentration

CaM: calmodulin

DO: detrusor overactivity

IP_3: inositol trisphosphate

isoP: isoprostane

KO: knockout

MLCK: myosin light chain kinase

MLCP: myosin light chain phosphatase

OAB: overactive bladder

PPADS: pyridoxalphosphate-6-azophenyl-2',4'-disulfonate

PLC-β: phospholipase Cβ

PG: prostaglandin

PGE_2: prostaglandin E_2

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$

RhoA: small GTPase RhoA

ROCK: Rho kinase

SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase

SMMHC: smooth muscle myosin heavy chain

TXA_2: thromboxane A_2

TP: thromboxane prostanoid receptor

UBSM: urinary bladder smooth muscle

VDCC: voltage dependent Ca^{2+} channel

DOI:10.14753/SE.2024.3019

1. Introduction

Regulation of urinary bladder function under physiological and pathophysiological conditions has not been understood completely to date despite numerous animal studies and human investigations. Disorders of the urinary bladder affect a large number of patients and are often associated with systemic inflammation. Release of inflammatory mediators may have a substantial impact on the impairment of micturition and therefore the elucidation of these pathways may help to understand the pathogenesis of frequent urinary bladder disorders like overactive bladder syndrome (OAB) [1, 2]. The prostaglandin-like isoprostanes are released in large amounts during systemic inflammation and previous studies indicated their potential role in the pathogenesis of urinary bladder dysfunctions [3].

1.1. Regulation of urinary bladder function

The urinary bladder is a hollow muscle organ and has two main functions: accommodating the urine during the storage phase and emptying the bladder in a controlled manner at the right time through the urethra in the voiding phase. Both of these functions of the bladder are under complex neuronal and humoral regulation. Most importantly, the urinary bladder is under the control of the parasympathetic and the sympathetic nervous system [4, 5].

1.2. Mediators and receptors of voiding mechanism

Under physiological circumstances, during the storage phase, noradrenaline (NA) released from sympathetic nerve endings plays the pivotal role in regulating bladder functions. First, via activation of $ß_2/ß_3$-adrenoreceptors in the detrusor muscle it promotes smooth muscle relaxation allowing filling of the bladder without a significant rise of the intraluminal pressure. At the same time, the released NA also binds to α_1-adrenoreceptors of smooth muscle cells in the bladder neck (trigonum) and the internal urethra sphincter resulting in bladder neck and sphincter contraction and consequent outflow obstruction therefore promoting urine storage [6, 7]. Finally, NA inhibits synaptic transmission in parasympathetic ganglia via activation of prejunctional α_2-adrenoreceptors.

In contrary, during the voiding phase, acetyl-choline (ACh) is released from the parasympatic postganglionary nerves binds to M_2/M_3 muscarinic receptors in the detrusor muscle evoking voiding smooth muscle contractions [8]. Although M_2-receptors are more abundant in the urinary bladder, the M_3-receptor subtype has a greater role in mediating the contractile effect [9]. Simultaneously, beside ACh, ATP is released from the nerve terminals as co-transmitter. ATP binds mainly to P2X1-purinergic receptor in the bladder and can elicit detrusor muscle contraction [6]. Furthermore, the urethra also has parasympatic innervation. NO released from these parasympatic nerves promotes relaxation of the internal sphincter [10]. In summary, during voiding, ACh evokes detrusor contraction which elevate the intravesical pressure and NO relaxes the internal sphincter muscle to allow emptying the bladder.

1.3. Role of inflammatory mediators in the urinary bladder

Prostanoids play an essential role in platelet aggregation, renal function, regulation of smooth muscle tone and inflammation. Within the eicosanoid family, prostanoids share roughly the same saturated fatty acid moiety, and minor alterations of the ring structure divide them into prostaglandins (PG) (PGD, PGE, PGF), prostacyclin (PGI) and thromboxanes (Tx) (TxA, TxB) [11]. Prostanoid production requires the activity of cyclooxygenase enzymes (COX) that catalyze the metabolism of arachidonic acid to form PG and Tx compounds. The various effects of prostanoids are mediated by G protein-coupled receptors (GPCR) named by their primary ligands (DP, EP, FP, IP, TP). The thromboxane A_2 prostanoid receptor (TP) is expressed in several tissues (e.g. thymus, spleen, lung, uterus, brain, ileum and the urinary bladder) and cell types (e.g. platelets, cardiac myocytes, endothelial and smooth muscle cells) [12].

It has been demonstrated that prostanoids are synthetized as a result of urinary bladder distension, and they are potent mediators of bladder contraction either through exciting bladder afferent nerve fibers or through activating receptors directly in the detrusor muscle [13, 14]. In the human detrusor, among the PGs, PGE_2 appears to be the most potent constrictor. EP_1, EP_3 as well as TP and neurokinin receptors have been proposed to mediate this effect [13, 15-18], but the exact mechanism remains to be elucidated.

DOI:10.14753/SE.2024.3019

1.4. Isoprostanes

1.4.1. Formation of isoprostanes

Isoprostanes are members of the isoeicosanoid family, they are PG-like compounds, however there are marked differences between the two lipid groups. Unlike PGs that are produced from arachidonic acid via COX enzymes, isoprostanes are formed primarily non-enzymatically through lipid peroxidation, as a result of reactive oxygen species (ROS) interaction with polyunsaturated fatty acids (e.g. arachidonic acid) [11]. Beside the difference in formation, PGs and isoprostanes also differ in the orientation of the side-chains in their structure: isoprostanes' side-chains are usually positioned *cis* to the cyclopentane ring, whereas PG side-chains are *trans* isomers. Isoprostane families designated by the letters D, E, F, A and J are distinguished by the type of the cyclopentane ring in their structure [19, 20]. This nomenclature is similar to that of PGs', for instance, F2-isoprostanes are structural isomers of $PGF_{2\alpha}$ [21].

1.4.2. Effects of isoprostanes on smooth muscle

Systemic isoprostane formation is remarkably increased under pathological conditions associated with oxidative stress and systemic chronic inflammation, such as diabetes, atherosclerosis, Alzheimer's disease, asthma and OAB [22-24]. Isoprostane levels are elevated in these disorders, and they are suitable compounds for serving as biomarkers of diseases associated with oxidative stress, as they are formed *in vivo* in the human body as a result of elevated ROS concentration. Moreover, they are chemically stable compounds and are present in detectable amounts in both human urine and plasma [22]. In addition, isoprostanes have been identified recently as biological mediators in the progression of the above-mentioned conditions linked to oxidative stress, including their possible role in inducing smooth muscle contraction in the airways and the vasculature [25-28]. Interestingly, isoprostanes have also been implicated in human neurogenic bladder dysfunction, as their urinary concentration is elevated in patients with hyperreflexic bladder whereas decreased in patients affected by areflexic bladder [3].

DOI:10.14753/SE.2024.3019

1.5. Regulation of the smooth muscle contraction

The general concept of smooth muscle regulation through Ca^{2+}-dependent and Ca^{2+}-sensitizing pathways apply to the detrusor muscle as well. Ca^{2+}-dependent contraction requires elevated intracellular Ca^{2+} concentration and formation of Ca^{2+}-calmodulin complex which activates the myosin light chain kinase (MLCK) leading to the phosphorylation of myosin light chain and consequently to contraction. The cross-bridge cycle is also regulated by myosin light chain phosphatase (MLCP), which cleaves the phosphate from MLC reducing its activity. The Rho – Rho-kinase (ROCK) pathway inhibits MLCP resulting in a sustained smooth muscle contraction [29, 30].

1.5.1. $G_{q/11}$ protein-coupled signaling pathway

Many contractile stimuli, acting through G protein–coupled receptors, induce MLC phosphorylation via activation of the G_q and G_{11} proteins and subsequent stimulation of phospholipase C-β, resulting in inositol 1,4,5-trisphosphate (IP3)-mediated Ca^{2+} release from the sarcoplasmic reticulum and the Ca^{2+} and calmodulin–dependent activation of MLCK [31].

1.5.2. $G_{12/13}$ protein-coupled signaling pathway

The receptors of many contractile mediators also couple to the G_{12} and G_{13} proteins to activate the Rho/ROCK pathway, resulting in the inhibition of MLCP [31-33]. Activation of RhoA through G_{12} and G_{13} is mediated by a subgroup of Rho guanine nucleotide exchange factors (RhoGEFs), which are activated through the direct interaction with $G\alpha_{12}$ and $G\alpha_{13}$ [34]. Thus, the dual regulation of MLC phosphorylation through Ca^{2+}-dependent MLCK activation and Rho/ROCK–mediated myosin phosphatase inhibition is initiated by the coupling of receptors to G_q-G_{11} and G_{12}-G_{13}, respectively[31, 32].

TP receptors have been reported to mediate vascular, bronchial, and prostate smooth muscle contractions [35-38]. The downstream signaling pathways mediating TP receptor activation have been examined extensively in platelets and smooth muscle as well. It has been reported that both $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins can couple to TP receptors and both

DOI:10.14753/SE.2024.3019

the PLC-ß – IP_3 – CaM-$(Ca^{2+})_4$ – MLCK and the RhoGEF – RhoA – ROCK – MLCP pathways can be involved in the signal transduction of TP-mediated responses (e.g. platelet aggregation, vasoconstriction, bronchoconstriction) [12, 39].

DOI:10.14753/SE.2024.3019

2. Objectives

The working hypothesis of our study is that isoprostanes that are present in the urine are not only useful biomarkes of systemic oxidative stress, but may be possible mediators of detrusor contractions leading to detrusor overactivity under pathological conditions.

The aim of this study was to investigate the potential effects of isoprostanes and identify the receptor(s) mediating these effects in the murine detrusor muscle. Furthermore, we aimed to examine the intracellular signaling of isoprostane-evoked contractions with the help of transgenic mouse models and pharmacological tools in order to provide novel, more specific therapeutic targets for the treatment of the bladder overactivity. To gain further clinical significance, we have performed experiments investigating the effects of isoprostanes and the signaling of the responses in human urinary bladders, as well.

3. Materials and methods

Animals

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (Permission number: PEI/001/2709-13/2014).

Urinary bladders were obtained from adult male (90-120 day-old, 30-35 g) wild-type mice (C57BL/6 strain from Charles River Laboratories, Isaszeg, Hungary, referred to as WT) and from animals deficient for the TP receptor (TP-KO), or from mice in which either the $G\alpha_{q/11}$- or the $G\alpha_{12/13}$-protein encoding genes were conditionally inactivated in a smooth muscle specific manner ($G\alpha_{q/11}$-KO and $G\alpha_{12/13}$-KO). The mouse lines with smooth muscle specific inducible deletion of the $G_{q/11}$ or $G_{12/13}$ signaling pathway were generated on $G\alpha_{11}$-deficient ($G\alpha_{11}^{-/-}$) or $G\alpha_{12}$-deficient ($G\alpha_{12}^{-/-}$) background [40, 41] with floxed alleles of the genes coding Gαq ($Gnaq^{flox/flox}$) or Gα13 ($Gn\alpha13^{flox/flox}$), and expressing a fusion protein of the Cre recombinase with a modified estrogen receptor binding domain (Cre-ERT2) [42] under the control of the smooth muscle myosin heavy chain (SMMHC) promoter. Deletion of Gnaq or Gna13 was induced by intraperitoneal tamoxifen treatment (1 mg/day for five consecutive days) in SMMHC-$CreERT2^{+/-}$;$Gnaq^{flox/flox}$;$Gna11^{-/-}$ and SMMHC-$CreERT2^{+/-}$;$Gna12^{-/-}$;$Gna13^{flox/flox}$ mice, respectively as described [30, 43]. Tamoxifen-treated SMMHC-$CreERT2^{-/-}$;$Gnaq^{flox/flox}$;$Gna11^{+/+}$ and SMMHC-$CreERT2^{-/-}$;$Gna12^{+/+}$;$Gna13^{flox/flox}$ mice served as controls and are referred to as $G\alpha_{q/11}$-CTRL and $G\alpha_{12/13}$-CTRL.

Human tissues

All procedures involving human urinary bladder tissues have been approved by the Scientific and Research Committee of the Medical Research Council of Hungary (License No.: 21545-2/2019/EKU). Urinary bladder tissue samples were obtained from 19 patients (15 males, 4 females; age 65.5±9.3 years, range between 44-78 years) undergoing open radical cystectomy due to muscle-invasive bladder cancer after having obtained informed patient consent. None of the patients has had any symptom of overactive bladder syndrome or any voiding dysfunction before surgery, nor was taking drugs for OAB. The urinary bladders were placed into physiological saline solution immediately after the

surgical removal and examined by an expert uro-pathologist who has cut out 3x2 cm tumor-free parts of the urinary bladders containing full width of bladder wall. Samples were collected preferentially from either the dome or the cranial part of the lateral bladder wall. These specimens were placed into Hank's Balanced Salt Solution (HBSS) containing 10^{-6} M of indomethacin and were transported to the place of the experiments. The addition of indomethacin was necessary in order to avoid any potential prostanoid receptor desensitization, since there is evidence for *in vitro* prostanoid synthesis in the urinary bladder, which can be increased by mechanical stretch [16, 44]. However, in order to avoid any potential side effects of COX inhibition, indomethacin was not present in the Krebs solution during the experiments [45].

Preparation of UBSM strips

After the mice were sacrificed with cervical dislocation the urinary bladders were removed from a lower midline incision and placed into Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2 \cdot H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 20 mM $NaHCO_3$, 0.03 mM EDTA, and 10 mM glucose, pH 7.4) at 37 °C. Under dissection microscope (M3Z; Wild Heerbrugg AG, Gais, Switzerland), adipose and connective tissues were removed from the serosal surface. The whole mucosal layer (urothelium + submucosa) was also gently and completely removed in order to prevent the potential release of paracrine factors from the mucosal epithelium or submucosal tissue and to avoid potential tension changes induced by myofibroblasts. The cleaned detrusor muscle was cut into four strips of equal length for myography. In some specific experiments, the mucosal layer was not removed to evaluate whether its presence alters the contractile responses.

Human urinary bladder specimens were also placed into Krebs solution (same as described above, T = 37 °C) containing 10^{-6} M of indomethacin during the preparation. Under dissection microscope, the serosal tissue and the mucosal layer were removed. The isolated, denuded detrusor specimens were cut into equal, approximately 10x3x3 mm strips for myography.

Myography

Both murine and human detrusor muscle strips were mounted perpendicularly on two parallel tissue-holding needles of a myograph evenly spaced from the end of the strips (200 μm needles, 6 ml chambers, 610 M Multi Wire Myograph System, Danish Myo Technology A/S, Aarhus, Denmark). Chambers were filled with 6 ml of 37 °C Krebs solution aerated with carbogen (mixture of 5% CO_2 and 95% O_2). Detrusor muscle contractions were registered under isometric conditions. The direction of emerging contraction force was measured along the longitudinal axis of the samples parallel with the axis of the force transducer.

Every experiment started with a 60-minute resting period while the UBSMs were stretched to and stabilized at a resting tension of 5 mN (murine) or 3 mN (human). After the resting period, UBSMs were challenged twice with 124 mM K^+-containing Krebs solution to examine the viability of the tissues. After several washes with normal Krebs solution, the contractile effect of U-46619 [selective TP agonist (10^{-5}M)], PGE_2 (10^{-5} M), $PGF_{2\alpha}$ (10^{-5} M), 8-iso-PGE_2 (10^{-5} M), 8-iso$PGF_{2\alpha}$ (10^{-5} M), carbamoylcholine chloride [carbachol (10^{-6} M)] or α,β-methyleneadenosine 5'-triphosphate [α,β-methylene ATP, ATP-analogue (10^{-5} M)] was measured. In some of the strips one of the following inhibitors was applied without washing out: SQ-29548 (selective TP antagonist, 10^{-5} M, 20 min), L-798106 (EP_3-receptor antagonist, 10^{-5} M, 20 min), atropine (muscarinic-ACh-receptor antagonist, 10^{-6} M, 20 min), pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS; P2 purinergic receptor antagonist, 10^{-5} M, 20 min), Y-27632 (ROCK inhibitor, 10^{-5} M, 20 min), tetrodotoxin (TTX, voltage-gated sodium channel blocker to inhibit potential neurotransmitter release from nerve endings, 10^{-6} and 10^{-5} M, 30 min). When DMSO was the solvent of the inhibitor, it was applied in matched concentration as vehicle control. Furthermore, in some experiments, non-cumulative (single-dose) dose-response relationship assessments of U-46619, 8-iso-PGE_2 and 8-iso$PGF_{2\alpha}$ were performed at the following concentrations: 10^{-8} M, 10^{-7} M, 10^{-6} M, $3x10^{-6}$ M, 10^{-5} M and $3x10^{-5}$ M. Finally, bladder strips were exposed to 124 mM K^+-containing Krebs solution to retest the viability of the detrusor strips. Agonist-induced tension changes were normalized to the reference contraction induced by 124 mM K^+-containing Krebs solution (second administration).

MP100 system and AcqKnowledge 3.72 software from Biopac System (Goleta, CA) were used for the acquisition and analysis of myographic measurements. The moving average smoothening function of the software was applied on recordings in order to eliminate the noises arising from the bubbling of the medium and to reduce the high frequency - low amplitude spontaneous tension oscillations. The parameters of the smoothening filter were carefully chosen in order to eliminate only the tension oscillations but not to alter the amplitude of the isoprostane-induced responses. The sample rate of the recordings was 10 samples/sec (10 Hz), the smoothing factor was between 10 and 40 samples.

Reagents

U-46619, PGE_2, $PGF_{2\alpha}$, 8-iso-PGE_2, 8-iso-$PGF_{2\alpha}$, SQ-29548 and L-798106 were purchased from Cayman Chemical (Ann Arbor, MI), and were dissolved in DMSO to stock solutions of 10^{-2} M. Carbachol (carbamoylcholine chloride) was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in saline to a stock solution of 10^{-3} M. Atropine was purchased from Egis Pharmaceutical PLC (Budapest, Hungary) and was diluted in water to a stock solution of 10^{-3} M. α,β-methylene ATP, PPADS and Y-27632 were purchased from Cayman Chemical (Ann Arbor, MI) and were dissolved in saline (α,β-methylene ATP: 10^{-2} M, PPADS: 10^{-2} M and Y-27632: 10^{-3} M). Indomethacin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO (10^{-2} M stock concentration), as its aqueous solutions are unstable [46]. Tetrodotoxin citrate (TTX) was purchased from Tocris Bioscience (Bristol, UK) and was dissolved in DMSO to stock solutions of 10^{-2} M.

Data analysis

Maximum contraction was defined as the peak value of tension developed after the addition of agonists. Average curves of individual contraction responses were also determined and presented on the left side of the figures, where they were plotted as mean ± SD. All data are presented as mean ± SD except dose-response curves, where mean ± SEM is shown. For statistical analysis, all data sets were subjected first to the D'Agostino-Pearson normality test. If the normal distribution of the data was verified, the *p* values

were determined by Student's unpaired *t* test or one-way ANOVA, depending on the number of experimental groups, whereas Mann-Whitney test or Kruskal-Wallis test was used if the normality test failed or if the case numbers were too small for the normality test. Statistical analysis and graph plotting were performed with GraphPad Prism software (v.6.07; GraphPad Software Inc., La Jolla, CA, USA), and $p < 0.05$ was considered as statistically significant difference.

DOI:10.14753/SE.2024.3019

4. Results

4.1. Contractile effects of isoprostanes in murine urinary bladder

4.1.1. Isoprostanes evoke detrusor muscle contraction independent of the mucosal layer

First, we wanted to examine if the isoprostane analogues of PGE_2 and $PGF_{2\alpha}$ do have a contractile effect in murine UBSM *ex vivo*. Similarly, to PGs, 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ evoked smooth muscle contractions in isolated murine detrusor muscle strips (Fig. 1/ A-B). Next, we aimed to evaluate the potential contribution of cells of the mucosal layer to the mediation of the contractile responses. Therefore, the effects of isoprostanes were compared in the presence [M(+)] and absence [M(-)] of the mucosal layer. There was no significant difference in the responses evoked by the isoprostanes, indicating that mucosa-derived mediators do not contribute to the contractile effect (Fig. 1/A-B).

DOI:10.14753/SE.2024.3019

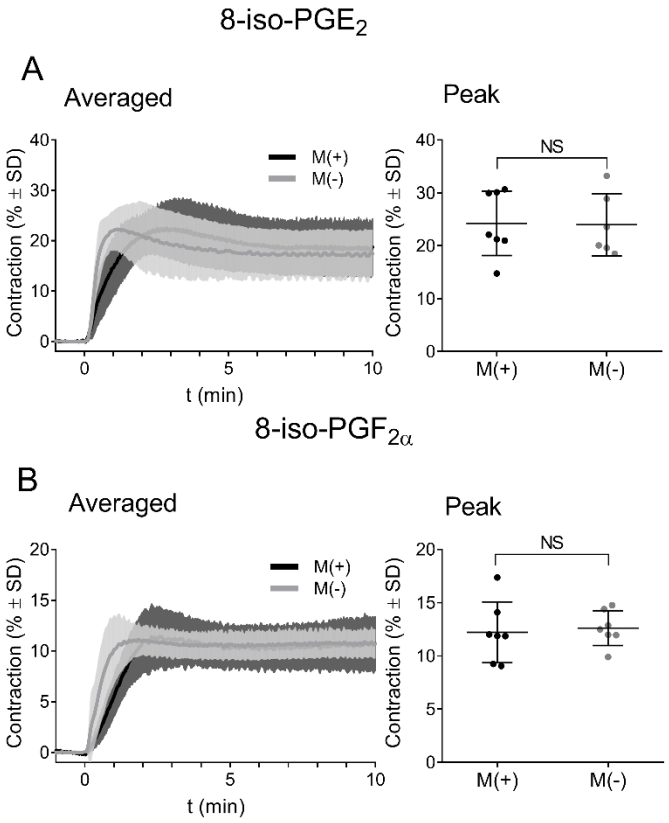
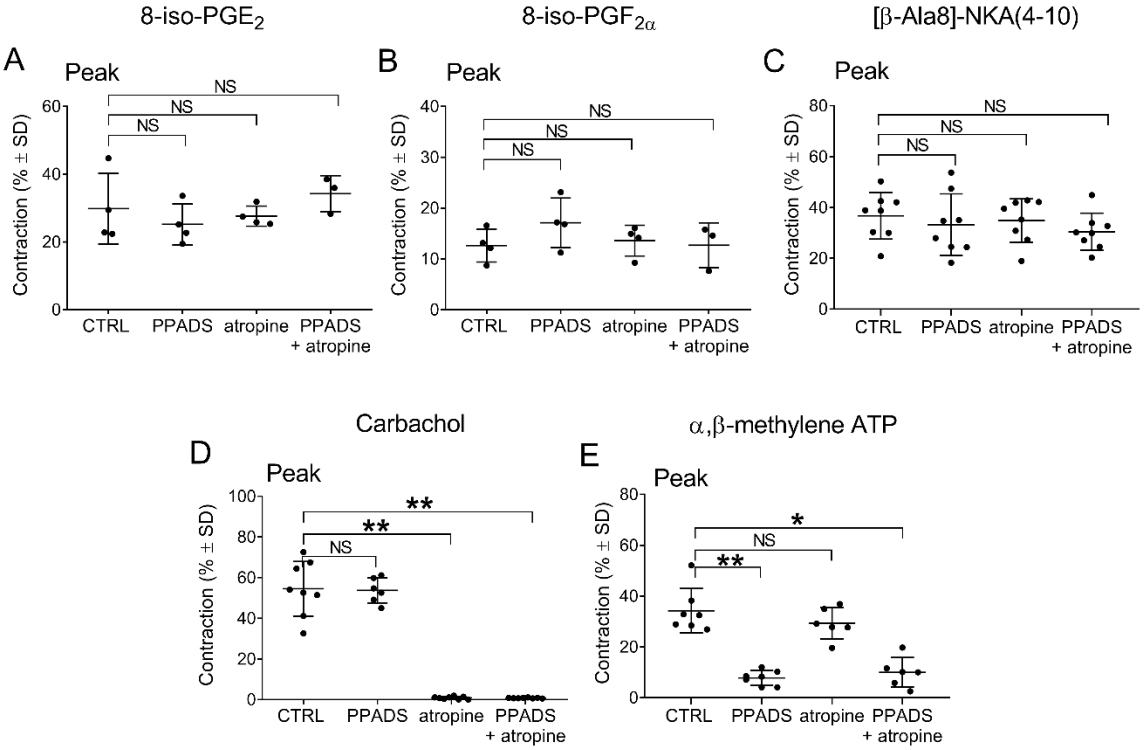


Figure 1. Isoprostane 8-iso-PGE$_2$ and 8-iso-PGF$_{2\alpha}$ *(A, B)* evoked smooth muscle contractions in the wild-type (WT) murine detrusor muscle. The contractile responses evoked by the isoprostanes showed no difference between urinary bladder strips with intact [M(+)] or denuded [M(-)] mucosal layer. (*A-B:* Mann-Whitney test; NS indicates non-significant, *A:* n = 6-7, *B:* n = 7-7)

4.1.2. Isoprostanes act directly on the detrusor muscle independently of neuronal transmitter release

Similarly, we wanted to examine whether isoprostanes' contractile actions are mediated by neurotransmitter release from nerve terminals innervating the bladder. To examine this, UBSM strips were pretreated with either the muscarinic-ACh-receptor antagonist atropine (10^{-6} M, 20 min incubation) or the purinergic P2-receptor antagonist PPADS (10^{-5} M, 20 min incubation) or with the combination of the two antagonists. Neither atropine, nor PPADS, nor even the combination of the two decreased the contractile responses evoked by 8-iso-PGE$_2$ and 8-iso-PGF$_{2\alpha}$ (10^{-5} M) (*Fig. 2/A-B*). As tachykinins acting on the NK2 receptor are also thought to be involved in the neuronal control of micturition [47], we also tested whether UBSM contractions induced by the

NK2 agonist [β-Ala^8]-NKA(4–10) are mediated by ACh or ATP release. However, similarly to isoprostanes, [β-Ala^8]-NKA(4–10)-induced contractions also remained unaltered in the presence of atropine or PPADS (*Fig. 2/C*). The effectiveness of the inhibitory effect of atropine and that of PPADS was verified by the loss of UBSM contraction induced by the ACh analogue carbachol (10^{-6} M) and the ATP analogue α,β-methylene ATP (10^{-5} M), respectively (*Fig. 2/D-E*). Therefore, we concluded that the detrusor muscle contractions induced by isoprostanes and tachykinins acting on NK2



receptors are not mediated by ACh or ATP release from nerve terminals.

Figure 2. Isoprostane- and [β-Ala^8]-NKA(4–10)-induced detrusor contractions remain unaltered after incubation with atropine or PPADS in murine urinary bladder smooth muscle (UBSM).

Neither the muscarinic-acetylcholine-receptor antagonist atropine (10^{-6} M, 20 min incubation), nor the purinergic P2-receptor inhibitor pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS; 10^{-5} M, 20 min incubation) decreased the responses evoked by 10^{-5} M 8-iso-PGE_2 (*A*), 8-iso-$PGF_{2\alpha}$ (*B*) or [β-Ala^8]-NKA(4–10) (*C*). At the same time the contractions evoked by 10^{-6} M carbachol (*A*) were diminished by atropine and those elicited by the stable ATP-analogue α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP, 10^{-5} M, *B* panel) were abolished by PPADS. (*A-E:* Kruskal-Wallis test; NS indicates non-significant, * $p < 0.05$, ** $p < 0.01$; *A:* n = 3-4, *B:* n = 3-4, *C:* n = 8-8, *D:* n = 6-8, *E:* n = 6-7)

DOI:10.14753/SE.2024.3019

4.1.3. The isoprostane-evoked detrusor contractions are mediated by the thromboxane prostanoid TP receptor

The TP receptor has been suggested to mediate smooth muscle contractions in various organs and species in response to prostanoids [16, 35-38] and isoprostanes [25]. Therefore, we examined the role of TP receptors in mediating the effect of prostanoids and isoprostanes in the mouse detrusor muscle. The contractile responses evoked by PGs were decreased significantly, and the contractions evoked by the isoprostanes were almost entirely abolished in mice deficient for the TP receptor (TP-KO) (*Fig. 3-4*). Pretreatment with the TP-specific antagonist SQ-29548 (10^{-5} M, 20 min incubation) induced similar changes (data not shown). The remaining responses to PGs in the TP-KO mice decreased further in the presence of the EP_3 receptor antagonist L-798106 (10^{-5} M, 20 min incubation, *Fig. 3/A and 4/A*). These results indicate that both PGs and isoprostanes evoke contractile responses in the UBSM, and the responses of the PGs are mediated partly, whereas those of the isoprostanes mainly by TP receptors.

DOI:10.14753/SE.2024.3019

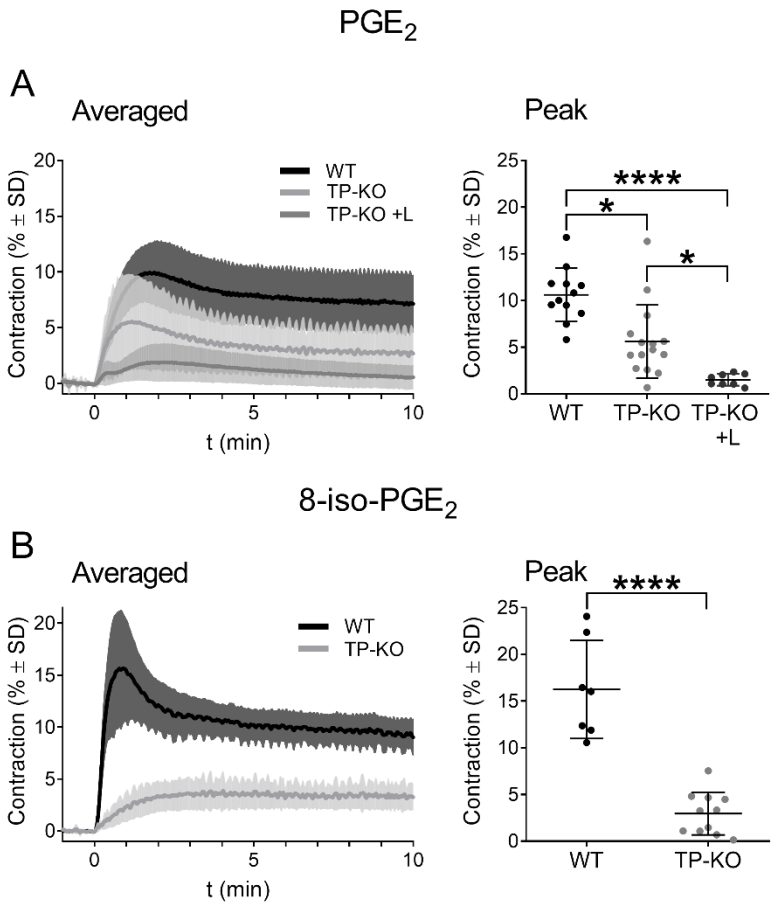


Figure 3. The role of thromboxane prostanoid (TP) receptors in mediating the E_2-prostanoid- and E_2-isoprostane-evoked detrusor muscle contraction in murine urinary bladder smooth muscle (UBSM). *A-B:* Prostaglandin (PG) E_2 (*A*), as well as isoprostane 8-iso-PGE_2 *(B)* evoked contractions in the wild-type (WT) and thromboxane prostanoid receptor knock-out (TP-KO) murine detrusor muscle. The contractile responses evoked by the PGE_2 were reduced (*A*), whereas those evoked by the E_2-isoprostane-analogue were abolished *(B)* in the bladder strips of TP-KO mice. The remaining contractions evoked by the PGE_2 in the TP-KO strips were abolished in the presence of the EP-3 receptor antagonist L-798106 (TP KO+L). (*A-B:* Mann-Whitney test; * $p < 0.05$, **** $p < 0.0001$; *A:* n = 12-15, *B:* n = 8-11)

DOI:10.14753/SE.2024.3019

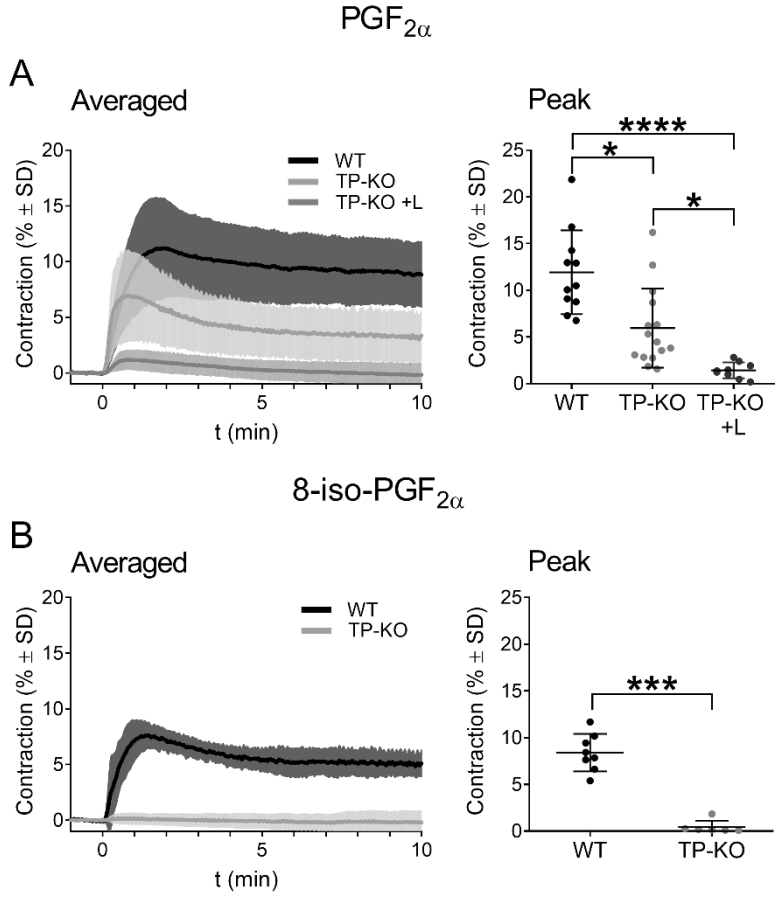
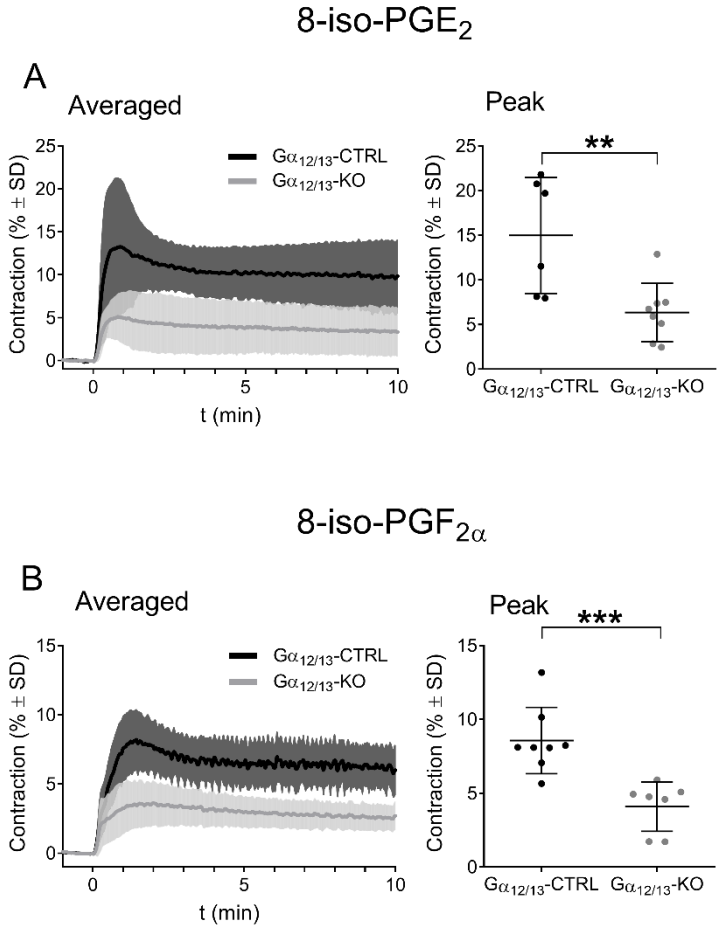


Figure 4. The role of thromboxane prostanoid (TP) receptors in mediating the $F_{2\alpha}$-prostanoid- and $F_{2\alpha}$-isoprostane-evoked detrusor muscle contraction in murine urinary bladder smooth muscle (UBSM). *A-B:* Prostaglandin (PG) $F_{2\alpha}$ (*A*), as well as isoprostane 8-iso-$PGF_{2\alpha}$ *(B)* evoked contractions in the wild-type (WT) and thromboxane prostanoid receptor knock-out (TP-KO) murine detrusor muscle. The contractile responses elicited by the $PGF_{2\alpha}$ were reduced (*A*), whereas those evoked by the isoprostanes 8-iso-$PGF_{2\alpha}$ were abolished *(B)* in the bladder strips of TP-KO mice. The remaining contractions evoked by the PG in the TP-KO strips were abolished in the presence of the EP-3 receptor antagonist L-798106 (TP KO+L). (*A-B:* Mann-Whitney test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; *A:* n = 11-15, *B:* n = 6-8)

4.1.4. Intracellular signaling of the isoprostane-evoked contractions in murine detrusor muscle

The general concept of smooth muscle tone regulation through Ca^{2+}-dependent and Ca^{2+}-sensitizing pathways apply to the detrusor muscle as well. Thus, we intended to

investigate the roles of the Ca^{2+}-dependent, $G\alpha_{q/11}$- and phospholipaseCβ (PLCβ)-mediated as well as the Ca^{2+}-sensitizing, $G\alpha_{12/13}$- and Rho-kinase (ROCK)-mediated signaling in isoprostane-evoked detrusor contractions. Therefore, we first examined whether the Ca^{2+}-independent pathway plays a role in the downstream signaling of the TP receptor. The contractile responses evoked by isoprostanes (10^{-5} M) were decreased in mice with smooth-muscle specific $G\alpha_{12/13}$ deficiency compared to WT animals (*Fig 5/A, B*) Similarly, the responses decreased significantly in the presence of the ROCK inhibitor Y-27632 (10^{-5} M, 20 min incubation) compared to the vehicle-treated control



group (*Fig 6/A, B*).

Figure 5. The $G\alpha_{12/13}$-coupled signaling pathway plays a significant role in mediating the contractile effect of isoprostanes in murine urinary bladder smooth muscle.
A, B: The detrusor muscle contractions elicited by 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ were reduced in the urinary bladders from mice deficient for the $G\alpha_{12/13}$-protein ($G\alpha_{12/13}$-KO) compared to the strips from control animals ($G\alpha_{12/13}$-CTRL). (*A-B:* Mann-Whitney test; ** $p < 0.01$, *** $p < 0.001$; *A:* n = 6-8, *B:* n = 7-8)

DOI:10.14753/SE.2024.3019

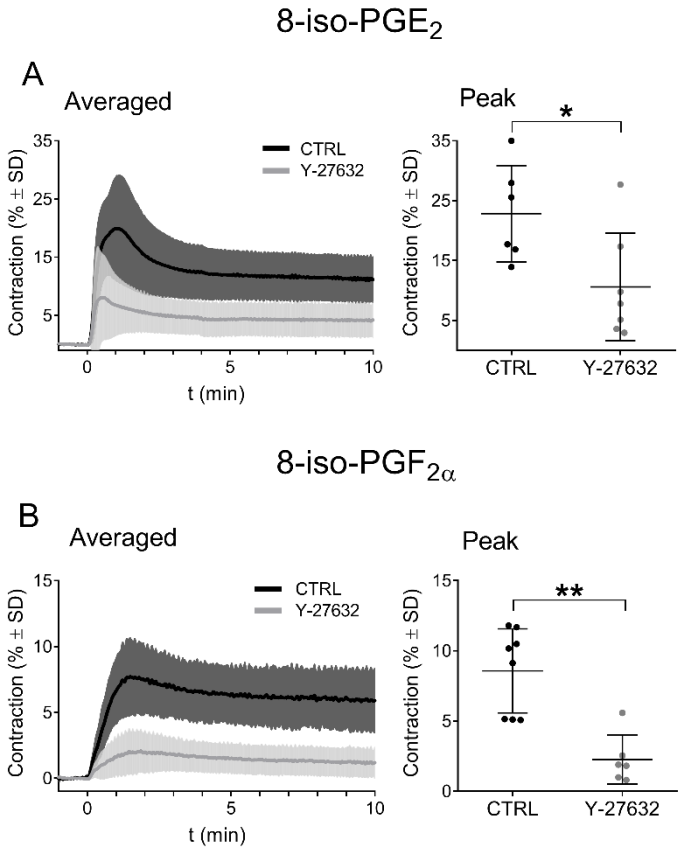


Figure 6. The Rho-ROCK pathway plays a significant role in mediating the contractile effect of isoprostanes in murine urinary bladder smooth muscle.

A, B: Contractile responses evoked by the 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ were decreased in the presence of the Rho-kinase inhibitor Y-27632 (10^{-5} M, 20 min incubation) as compared to vehicle-treated controls (CTRL), similarly to the changes observed in the $G\alpha_{12/13}$-KO strips. (*A-B:* Mann-Whitney test; * $p < 0.05$, ** $p < 0.01$; *A:* n = 6-7, *B:* n = 6-8)

DOI:10.14753/SE.2024.3019

We also examined the role of the $G\alpha_{q/11}$-mediated, classical Ca^{2+}-dependent signaling pathway in mediating isoprostane-induced contractions. In UB strips of mice deficient for $G\alpha_{q/11}$-proteins in smooth muscle cells, the contractions evoked by isoprostanes (10^{-5} M) decreased significantly. Furthermore, in $G\alpha_{q/11}$-KO strips the contractile responses to isoprostanes were abolished completely when the ROCK-inhibitor Y-27632 (10^{-5} M, 20 min incubation) was co-administered (*Fig. 7/A-B*).

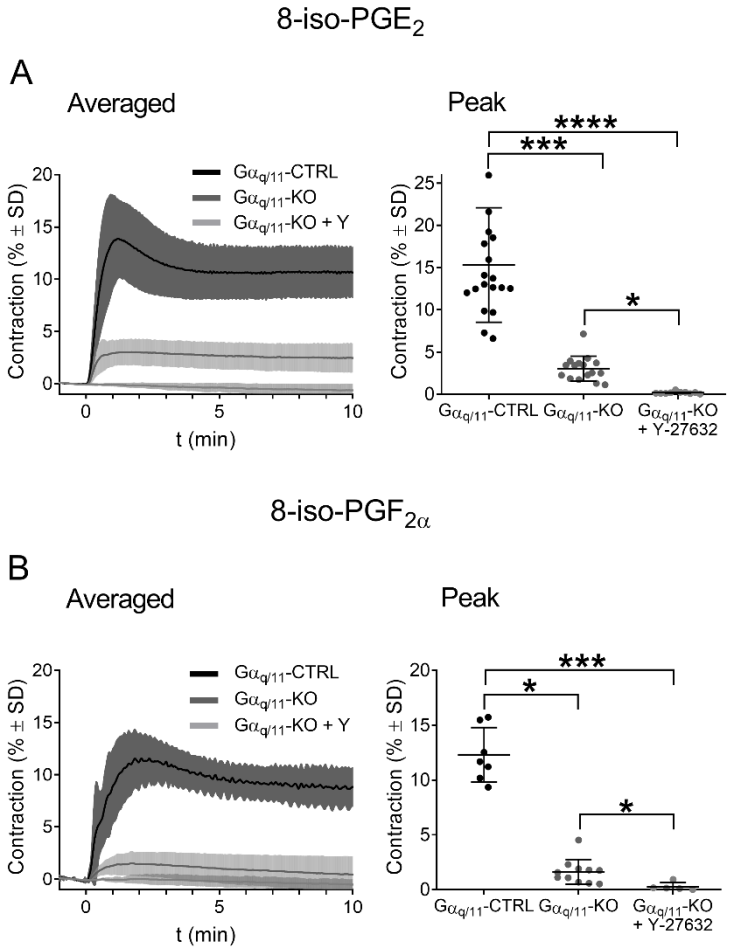


Figure 7. The $G\alpha_{q/11}$ and the $G_{12/13}$-Rho-ROCK signaling pathways mediate the effects of isoprostanes in murine urinary bladder smooth muscle.

A-B: Contractile responses evoked by the isoprostane 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ were reduced in bladder strips from mice deficient for the $G\alpha_{q/11}$-protein ($G\alpha_{q/11}$-KO) compared to the strips from control ($G\alpha_{q/11}$-CTRL) animals. Furthermore, in the urinary bladders from $G\alpha_{q/11}$-KO mice, the contractile effects elicited by isoprostanes were diminished completely in the presence of the Rho-kinase inhibitor Y-27632 (10^{-5} M, 20 min incubation). (*A-B:* Kruskal-Wallis test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; *A:* n = 10-19, *B:* n = 5-11)

DOI:10.14753/SE.2024.3019

4.2. Isoprostanes evoke contraction in the human detrusor muscle

4.2.1. TP receptor activation leads to detrusor contraction in human UBSM strips

In the next part of the study, we first investigated whether functional TP receptors are also present in the human UBSM. We found that the synthetic TP receptor agonist, U-46619 (10^{-5} M) evoked contractions in the human UBSM strips, which were comparable to the effect induced by the muscarinic-ACh-R agonist carbachol (10^{-6} M) (*Fig. 8/A*). The responses to U-46619, but not to carbachol were abolished in the presence of the TP-receptor antagonist SQ-29548 (10^{-5} M, 20 min incubation) (*Fig. 8/B and 8/C*).

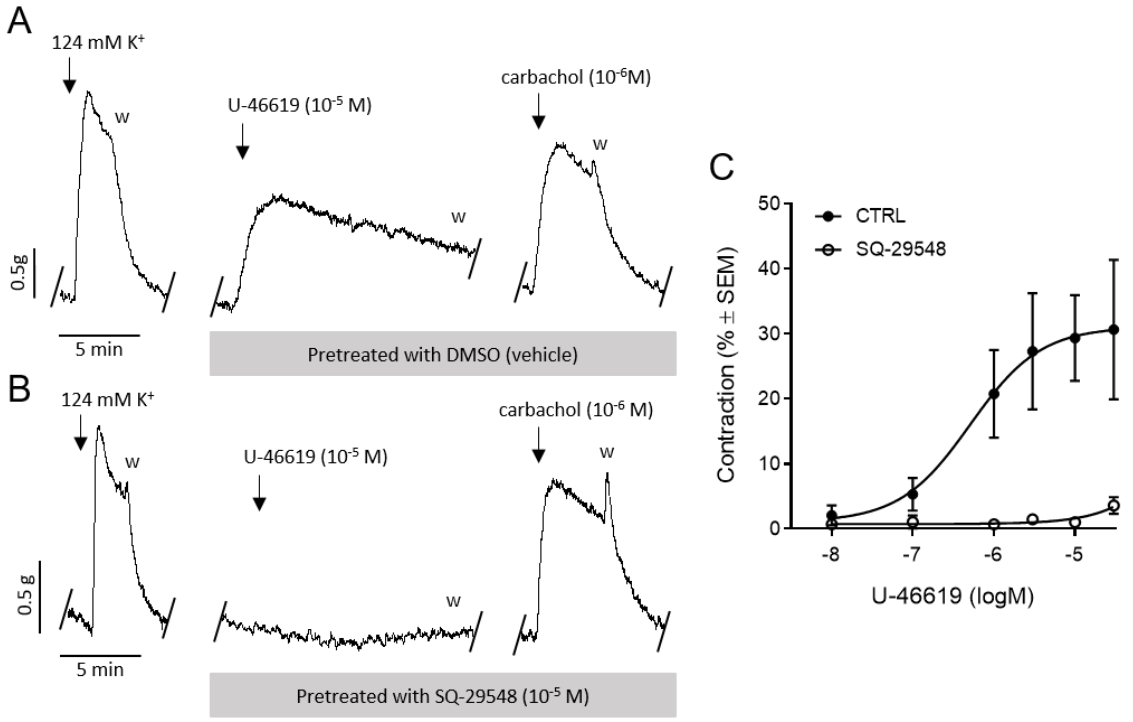


Figure 8. Functional thromboxane prostanoid (TP) receptors are present in the human urinary bladder smooth muscle and their activation leads to significant contraction.

A: Original trace: the TP receptor agonist U-46619 (10^{-5} M) evokes contraction in isolated human detrusor muscle strips, which is comparable in size to the responses evoked by the muscarinic-acetylcholine-receptor agonist carbachol (10^{-6} M). *B:* The U-46619-evoked contraction is abolished in the presence of the TP-antagonist SQ-29548 (10^{-5} M, 20 min incubation), whereas the response to carbachol remains unaltered.

C: Dose-response curves of U-46619 in control and SQ-29548-treated urinary bladder strips (control: E_{max}: 31.1%, EC_{50}: 5.11 x 10^{-7} M, n = 3-19).

DOI:10.14753/SE.2024.3019

After verifying the presence of functional TP receptors in human UBSM, we aimed to examine whether the TP receptors eventually mediate the effect pre-junctionally via activating neurotransmitter release, as we did previously in murine bladders. The responses evoked by U-46619 (10^{-5} M) were unaltered by the muscarinic-ACh-R antagonist atropine (10^{-6} M, 20 min incubation) or the purinergic P2-receptor antagonist PPADS (10^{-5} M, 20 min incubation), but were abolished by SQ-29548 (10^{-5} M, 20 min incubation) (*Fig. 9/A*). The positive control for the inhibitory effect of atropine was the loss of carbachol (10^{-6} M)-induced contractions, whereas the effectiveness of PPADS was verified by inhibition of the contraction induced by the ATP-analogue α,β-methylene ATP (10^{-5} M) *Fig. 9/B-C*. The contractions evoked by either carbachol or α,β-methylene ATP were unaltered by SQ-29548 *(Fig. 9/B-C)*. These data indicate that the contractions evoked by TP activation are independent of either cholinergic or purinergic signaling, and therefore the TP receptors are likely to be localized directly on the detrusor muscle in the human urinary bladder as well.

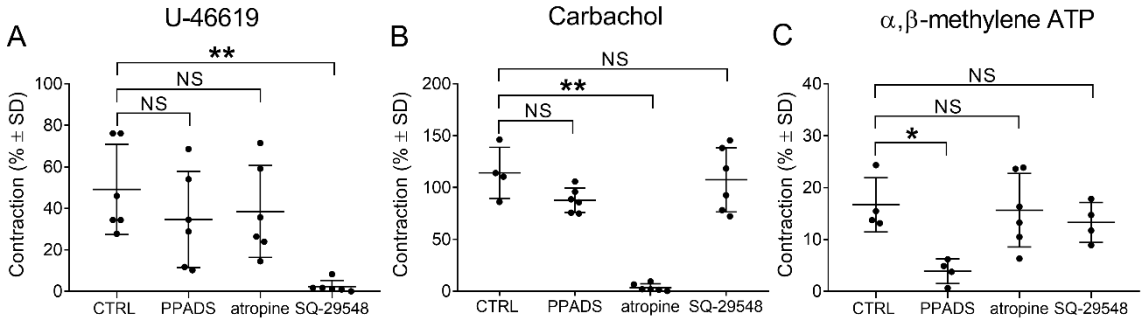


Figure 9. The thromboxane prostanoid TP receptor-mediated smooth muscle contraction in the human urinary bladder is independent of cholinergic or purinergic signaling.

A-C: U-46619-evoked contractions were unaltered in the presence of either the muscarinic-acetylcholine-receptor antagonist atropine (10^{-6} M, 20 min incubation), or the purinergic P2-receptor inhibitor pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS; 10^{-5} M, 20 min incubation), whereas the contractions evoked by carbachol were abolished by atropine and those elicited by the stable ATP-analogue α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP; 10^{-5} M) were diminished completely by the PPADS. (*A-C:* Kruskal-Wallis test; NS indicates non-significant, * $p < 0.05$, ** $p < 0.01$; *A*: n = 6-6, *B*: n = 4-6, *C*: n = 4-6)

DOI:10.14753/SE.2024.3019

4.2.2. Isoprostanes evoke concentration-dependent contractions via TP receptor in human UBSM strips

The isoprostane 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ evoked dose-dependent contractions in the human UBSM strips (*Fig. 10/A-B*) (8-iso-PGE_2: E_{max}: 22.5%, EC_{50}: 1.48 x 10^{-6} M; 8-iso-$PGF_{2\alpha}$: E_{max}: 28.0%, EC_{50}: 1.59 x 10^{-6} M). Importantly, the contractile responses evoked by the two examined isoprostanes were abolished in the presence of the TP receptor antagonist SQ-29548 (10^{-5} M, 20 min incubation) *(Fig. 10/A-B)*, indicating that the effects of isoprostanes are mediated by the TP receptor in the human urinary bladder, similarly to the results obtained in mice.

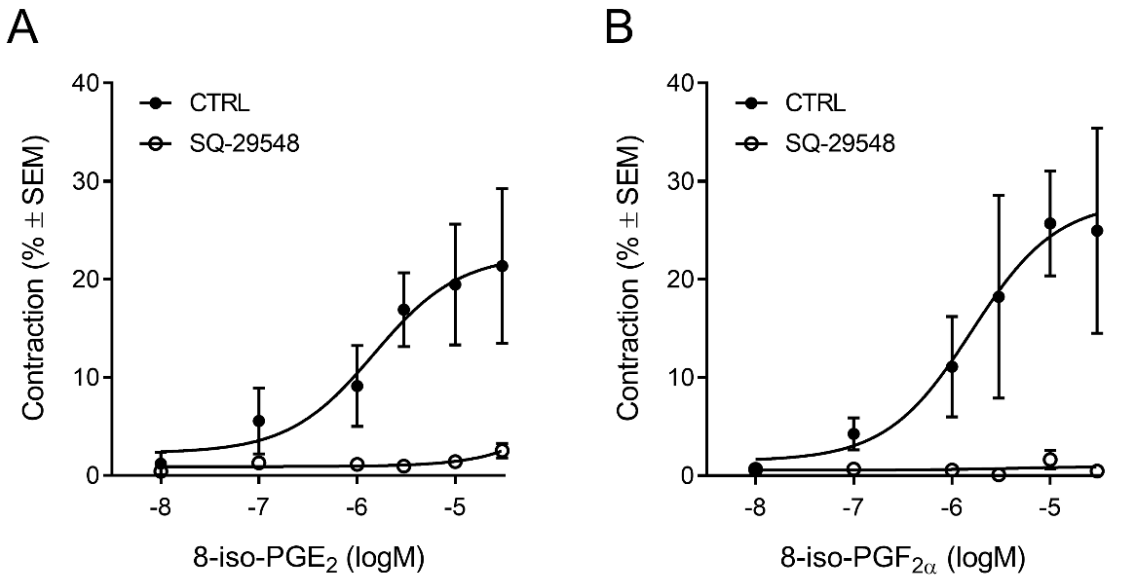


Figure 10. The isoprostane 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ evoke significant, dose-dependent detrusor muscle contraction in human urinary bladder, which is mediated exclusively by the TP receptor.
A-B: 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ evoked dose-dependent contraction in the human urinary bladder detrusor muscle strips, an effect that was abolished in the presence of the thromboxane prostanoid (TP) receptor antagonist SQ-29548 (10^{-5} M, 20 min incubation). (*A:* 8-iso-PGE_2: E_{max}: 22.5%, EC_{50}: 1.48 x 10^{-6} M, n = 2-6; *B*: 8-iso-$PGF_{2\alpha}$: E_{max}: 28.0%, EC_{50}: 1.59 x 10^{-6} M, n = 3-11)

DOI:10.14753/SE.2024.3019

4.2.3. Role of ROCK-enzyme in the signaling of isoprostane-evoked contractions

Finally, we aimed to analyze the intracellular signaling of isoprostanes in the human UBSM. The contractile responses evoked by 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ were markedly reduced in the presence of the ROCK inhibitor Y-27632 (10^{-5} M, 20 min incubation) in human urinary bladder strips (*Fig. 11/A-B*).

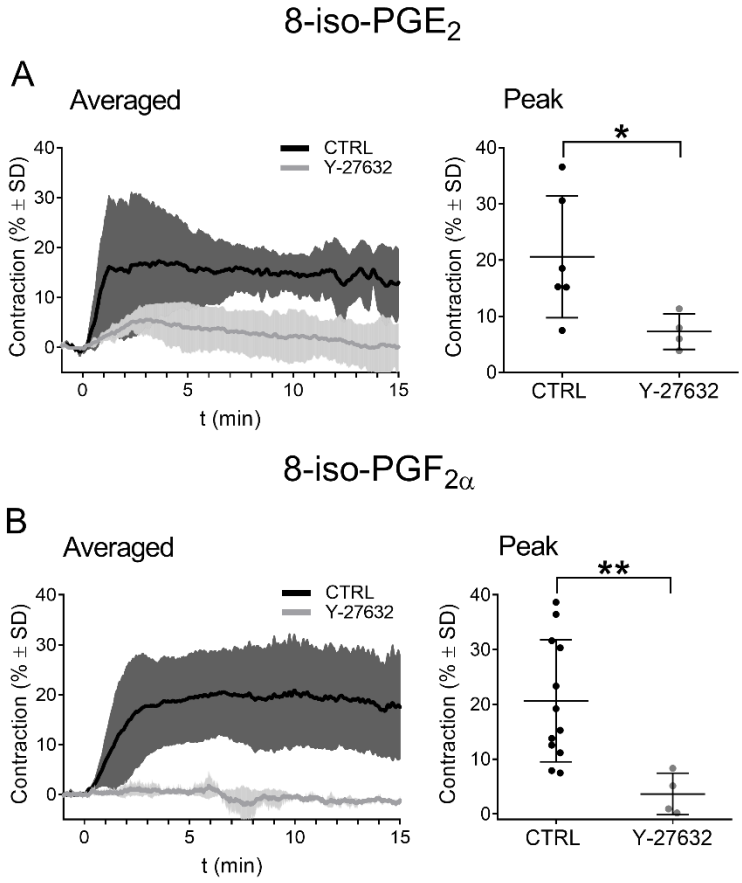


Figure 11. Rho-ROCK signaling plays a major role in mediating the detrusor contraction evoked by isoprostanes in human urinary bladder.
Contractile responses evoked by 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ (10^{-5} M) were reduced in the presence of the Rho-kinase (ROCK) inhibitor Y-27632 (10^{-5} M, 20 min incubation) in human urinary bladder strips. (*A-B:* Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, *A:* n = 4-6; *B:* n = 4-8)

5. Discussion

Overactive bladder syndrome (OAB) is a common urological clinical condition and major health problem worldwide, characterized by frequency, urgency, nocturia with or without urge incontinence. Despite the numerous studies aiming to describe the pathophysiology of OAB, it has not been clarified yet, however four main hypotheses have been proposed [2]. One of them is the myogenic hypothesis, which states that myocytes from bladders with detrusor overactivity show an alteration in their contractile responsiveness [48].

OAB and detrusor overactivity (DO) are distinct disorders, though there are several similarities between them. Urodynamic studies provide the basis for the diagnosis of DO, which is characterized by involuntary detrusor contractions during the filling phase [49]. Approximately 50% of OAB patients do not experience DO, in addition DO is not necessarily accompanied by urgency [50]. Given these facts, the two conditions are not interchangeable. Thus, it is important to note that our research focused on possible intervention targets in the signaling pathways of detrusor contractions, that might hold therapeutic potential for OAB patients suffering from DO.

Currently available pharmacological therapies (anticholinergic drugs and the β3-receptor agonist mirabegron) have numerous side effects and often fail to reach the desired therapeutic goal [2, 51, 52], thus the management of OAB remains insufficient [51]. Considering the limitations of the available first-line pharmacological treatment of OAB, there is an urgent need for new therapeutic targets as well as for a new approach for managing this pathological condition [50]. Indeed, research of the topic has lately focused on finding novel targets, including P2X3 receptors, the cannabinoid system, TRP channels, serotonergic pathways, tachykinins, neurotrophins and also the prostanoid system [53-56]. OAB often occurs along with other pathological conditions associated with systemic inflammation (e.g. diabetes, metabolic syndrome, atherosclerosis) [1, 57-60]. An increasing body of evidence indicates elevated levels of oxidative stress biomarkers in the urine and blood plasma (e.g. malondialdehyde, 8-hydroxy-20-deoxyguanosine and isoprostanes) of patients with the above-mentioned conditions and OAB [57, 61, 62]. Consequently, attention has been drawn to the role of oxidative stress and reactive oxygen species (ROS) in the etiology of OAB. Therefore, bioactive agents

synthesized during oxidative stress and their signaling pathways are considered as promising novel targets for the treatment of lower urinary tract symptoms.

Earlier observations indicating that diseases associated with systemic inflammation and oxidative stress result in the production of the arachidonic acid derivate prostanoids and isoprostanes prompted us to study the role and signaling of these mediators in detrusor muscle contraction [1, 60]. We found that contractions evoked by PGE_2 and $PGF_{2\alpha}$ were reduced in TP receptor-deficient mouse bladders implying that TP receptors play a significant role in mediating their effects in the murine detrusor muscle. Contractile effects of these two PGs on UBSM have been reported in several species in agreement with our data [14, 63]. With some ambiguity regarding receptor subtypes, it has been proposed previously that these effects are at least partially mediated by EP receptors [16, 38]. Our experimental data provide additional information on the mechanism of PG-evoked contraction of the detrusor via demonstrating that beside EP receptors, TP receptor activation also contributes to the contractile response.

Isoprostanes have been described as potential regulators of bronchial and vascular smooth muscle tone [23, 26]. 8-iso-$PGF_{2\alpha}$-evoked contraction has been described in rabbit detrusor muscle as well [64], however rabbits are considered to have a unique regulation of voiding [65]. As regulation of mouse bladder functions resembles better the situation in humans, data gained from the present study have the advantage of being more relevant to humans [66]. Our observations imply that isoprostanes are also potent constrictors of both murine and human detrusor muscle. To our knowledge, our experiments demonstrate for the first time that isoprostanes evoke contraction in human urinary bladder smooth muscle. An additional novelty of our study is that our results obtained in TP-KO mouse bladders prove that isoprostanes exert their contractile effects in mouse detrusor muscle primarily via TP receptors, similarly to bronchial and vascular smooth muscle [23, 67]. A possible explanation for the remaining weak contractions induced by 8-iso-PGE_2 in TP-KO mouse bladder could be a moderate participation of EP-receptors, as the EP_1 receptor has been implicated in mediating 8-iso-PGE_2-elicited contractions in rat gastric fundus and guinea-pig ileum [68]. In addition, we observed that SQ-29548, the specific antagonist of TP receptors inhibits isoprostane-evoked contractions in human bladder, indicating a similar signal transduction in murine and human UBSM. Data presented here further emphasize that ROS-derived isoprostanes are

important biological mediators and implies that beside asthma and cardiovascular diseases, their role in detrusor overactivity might be significant.

Clinical reports were recently published by Hann-Chorng Kuo's workgroup support our *ex vivo* experimental findings and give further clinical relevance to our results [69-71]. They examined urinary biomarker levels in patients simultaneously with urodynamic studies to investigate *in vivo* urinary bladder function. They found, that urinary 8-isoprostane levels were significantly elevated in patients' with idiopathic detrusor overactivity (IDO) or neurogenic detrusor overactivity (NDO), and also in patients with stress urinary incontinence (SUI) with detrusor overactivity compared to patients without detrusor overactivity component. Therefore, elevated urinary 8-isoprostane levels showed correlation with the prevalence of detrusor overactivity in human clinical studies.

Contractile responses evoked by U-46619, the synthetic analogue of the natural ligand of TP receptors (TXA_2), and their inhibition with selective TP receptor antagonist in human have been reported by Palea et al [16]. Our findings on the U-46619-induced contractions and their attenuation by SQ-29548 in human bladder confirms these results. In addition, we demonstrated that these contractions are independent of cholinergic and purinergic signaling, as neither atropine nor PPADS inhibited them. These results together are strong evidence for the presence of functional TP receptors in the human detrusor muscle, supporting our findings on the TP receptor-mediated effects of isoprostanes.

The mucosal layer of the urinary bladder is now acknowledged as not only a passive barrier, but also a source of several biological mediators. Therefore we investigated whether contractile responses evoked by isoprostanes are mediated or modulated by mucosa-derived substances [72]. In our experiments, however, there was no significant difference between smooth muscle contractions induced by isoprostanes in the presence or absence of the mucosal layer, so we can exclude the role of mucosal cells in mediating or modulating the response. Furthermore, the role of presynaptic TP receptors has been proposed in U-46619-evoked contractions in bronchial smooth muscle [73]. Accordingly, we investigated whether TTX pretreatment attenuates U-46619-evoked contractions. As TTX exerted no effect on the U-46619-induced detrusor contractions, we concluded that TP activation occurs most likely in the detrusor muscle.

The solid proof of the direct, TP-mediated effect of isoprostanes in the detrusor muscle encouraged our further studies. In equal submaximal concentration (10^{-5} M) in mouse bladder 8-iso-PGE_2 exerted a stronger contractile effect (17%) as compared to 8-iso-$PGF_{2\alpha}$ (8%); on the contrary, in human detrusor the maximum amplitude of 8-iso-$PGF_{2\alpha}$-evoked contraction (25%) was higher than that of 8-iso-PGE_2 (21%). Prostanoid-evoked smooth muscle contractions along with other TP-mediated responses show species-dependent differences, which might be the explanation for the different potencies of isoprostanes in murine and human detrusor muscles [12, 38].

As mentioned above, the general concept of smooth muscle regulation through Ca^{2+}-dependent and Ca^{2+}-sensitizing pathways apply to the detrusor muscle as well. Ca^{2+}-dependent contraction requires elevated intracellular Ca^{2+} concentration and formation of Ca^{2+}-calmodulin complex which activates the myosin light chain kinase (MLCK) leading to the phosphorylation of myosin light chain and consequently to contraction. The cross-bridge cycle is also regulated by myosin light chain phosphatase (MLCP), which cleaves the phosphate from MLC reducing its activity. The Rho – ROCK pathway inhibits MLCP resulting in a sustained smooth muscle contraction [29].

TP receptors have been reported to mediate vascular, bronchial, and prostate smooth muscle contractions [35-38]. The downstream signaling pathways mediating TP receptor activation have been examined extensively in platelets and smooth muscle as well. It has been reported that both $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins can couple to TP receptors and both the PLC-ß – IP_3 – CaM-$(Ca^{2+})_4$ – MLCK and the RhoGEF – RhoA – ROCK – MLCP pathways can be involved in the signal transduction of TP-mediated responses (e.g. platelet aggregation, vasoconstriction, bronchoconstriction) [12, 39].

We demonstrated that in murine smooth muscle-specific $G\alpha_{q/11}$- and $G\alpha_{12/13}$-KO bladders contractions evoked by isoprostanes are diminished similarly, indicating that both $G\alpha_{q/11}$- and $G\alpha_{12/13}$-proteins couple to TP receptors following isoprostane binding and contribute to the contraction simultaneously. Neither of the two isoprostanes exerted significant contractile effects in $G\alpha_{q/11}$-KO mouse detrusor tissues treated with Y-27632, indicating that these effects are mediated exclusively via the simultaneous activation of $G\alpha_{q/11}$- and $G\alpha_{12/13}$ pathways. Results with Y-27632 application in human bladder tissues led us to the conclusion that ROCK contributes markedly to isoprostane-evoked

contractions in human bladder as well. The Rho/ROCK signal cascade following $G\alpha_{12/13}$-protein activation is highlighted in recent literature on detrusor dysfunctions, and here we further emphasize the significance of ROCK, as the inhibition of this enzyme resulted in a marked decrease in the amplitude of isoprostane-evoked contractions [74]. Our findings describing intracellular signaling following TP receptor activation in detrusor muscle are in line with studies on bronchus smooth muscle, however to our best knowledge, our work is the first detailed analysis of these pathways in mouse and human detrusor muscle [39]. Together with studies reporting the contractile effect of isoprostanes acting on TP receptors in airway and vascular smooth muscle, our findings also support that isoprostanes, beside of being biomarkers of oxidative stress, might contribute to the etiology of several urinary bladder disorders associated with systemic inflammation through TP receptor-mediated signaling [27, 67].

The main findings of our studies are summarized on Figure 12. Interestingly, in blood vessels isoprostanes are more potent constrictors under oxidative stress, which suggests that isoprostane-evoked bladder contraction may be enhanced in the case of elevated ROS concentrations as well [25]. Furthermore, the potential role of urothelial $TRPV_4$ receptors in inflammatory diseases associated with oxidative stress has been studied extensively in the recent years. $TRPV_4$ receptor activation reportedly enhances urothelial ROS production (H_2O_2, O_2^-) [75, 76], which led us to the intriguing assumption that both the release and the detrusor constricting potency of isoprostanes might be under the control of the urothelium. Thus, the mechanism of isoprostane actions in the detrusor muscle appears to be a promising field for further research.

DOI:10.14753/SE.2024.3019

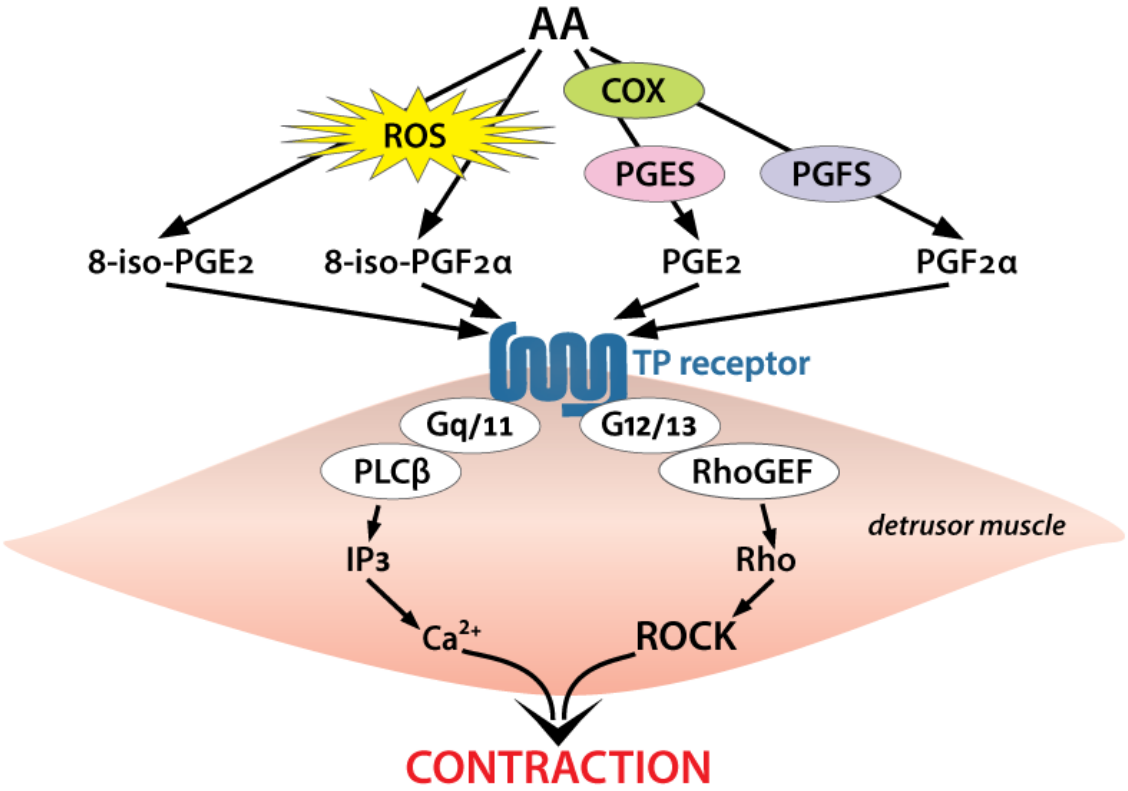


Figure 12. Signaling pathways of the isoprostane- and prostanoid-induced, thromboxane prostanoid TP receptor-mediated urinary bladder smooth muscle contraction

DOI:10.14753/SE.2024.3019

6. Conclusion

Since both the storage and voiding phase of the urinary bladder is under a complex regulation, even minor alterations can lead to dysfunctions such as involuntary detrusor contraction resulting in detrusor overactivity and the clinical symptoms of overactive bladder syndrome. The aim of this research work was to examine the potential role of isoprostanes in murine and human urinary bladder functions, based on the hypothesis, that isoprostanes are not only present in the urine as biomarkers of systemic oxidative stress, but may have a significant influence on the tone of the urinary bladder smooth muscle. Furthermore, we aimed to examine the receptor(s) and the intracellular signaling of isoprostane-evoked contractions in the detrusor muscle. Our results gained from the *ex vivo* experiments performed on murine and human urinary bladder detrusor strips are the following:

- We demonstrated, that the isoprostane 8-iso-PGE_2 and 8-iso$PG_{2\alpha}$ evoke significant smooth muscle contraction in both murine and human urinary bladder smooth muscle.
- The isoprostane-evoked contractile effect is independent of the presence of the mucosal layer or the submucosal tissue.
- The contractile responses induced by both isoprostanes are mediated by the thromboxane prostanoid TP receptor in both murine and human urinary bladders.
- The activation of the TP receptor is independent of neurotransmitter release, acetyl-choline or ATP do not play a role in the contractile effect, the isoprostanes evoke the smooth muscle contraction directly acting on the detrusor muscle.
- The isoprostane-induced TP receptor activation is mediated intracellularly by the $G\alpha_{12/13}$-Rho-ROCK and the $G\alpha_{q/11}$-coupled signaling pathways simultaneously.

DOI:10.14753/SE.2024.3019

7. Summary

The urinary bladder has two main functions: storage and voiding. Both functions are under complex regulation. Involuntary detrusor contractions in the storage phase can lead to the symptoms of overactive bladder syndrome (OAB) and can impair the patients' quality of life. The first line pharmacological treatment mainly consists of anti-cholinergic drugs that often have serious adverse effects. Therefore, there is a need for novel, more specific targets in the treatment of OAB. Isoprostanes, which are considered as biomarkers of oxidative stress and are present in the urine, are hypothesized to have a potential role in the development of OAB. The contractile effects of isoprostanes have been examined in other organs such as vascular and airway smooth muscle in other studies. Therefore, we aimed to examine the potential effects of the isoprostanes in the murine and human urinary bladder detrusor muscle with *ex vivo* myography. We found, that the isoprostane 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ elicited smooth muscle contraction in the mouse detrusor muscle, which was unaltered in the presence or absence of the urothelium. These contractions are mediated by the thromboxane prostanoid TP receptor. The isoprostane-evoked contractions were unaltered in the presence of muscarinic acetyl-choline receptor antagonist atropine or the purinergic receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS), suggesting that the TP receptors are located directly on the detrusor muscle and the isoprostane-induced contraction is independent of neurotransmitter release. The contractile effects of the isoprostanes were decreased both in mice deficient for the $G\alpha_{12/13}$ or $G\alpha_{q/11}$-protein, furthermore, the responses were diminished in the $G\alpha_{q/11}$-KO mice pretreated with the ROCK-inhibitor Y-27632. We also examined the effects of the isoprostanes in human detrusor muscle strips. In human urinary bladder, both examined isoptostanes evoked dose-dependent smooth muscle contractions, which were abolished in the presence of the TP-receptor antagonist SQ-29548, suggesting that the TP receptor mediates the contraction, similarly to the results gained in the mouse experiments. Furthermore, in the presence of the ROCK-inhibitor Y-27632, the isoprostane-evoked contractions were decreased.

These results indicate, that 8-iso-PGE_2 and 8-iso-$PGF_{2\alpha}$ can evoke smooth muscle contraction in both murine and human urinary bladders. These contractile effects are mediated mainly by the TP receptors. The downstream signaling involves the $G_{q/11}$-

DOI:10.14753/SE.2024.3019

coupled and the $G_{12/13}$-Rho-ROCK pathways simultaneously. Thus, the mechanism of isoprostane actions in the detrusor muscle may have important clinical implications and appears to be a promising field for further research.

8. References

1. Andersson, K.E., *Oxidative stress and its possible relation to lower urinary tract functional pathology.* BJU Int, 2018. **121**(4): p. 527-533.
2. Leron, E., et al., *Overactive Bladder Syndrome: Evaluation and Management.* Curr Urol, 2018. **11**(3): p. 117-125.
3. Oner-Iyidogan, Y., et al., *Urine 8-isoprostane F2alpha concentrations in patients with neurogenic bladder due to spinal cord injury.* Clin Chim Acta, 2004. **339**(1-2): p. 43-7.
4. Yoshimura, N. and M.B. Chancellor, *Neurophysiology of Lower Urinary Tract Function and Dysfunction.* Reviews in Urology, 2003. **5**(Suppl 8): p. S3-S10.
5. Yoshimura, N., et al., *Therapeutic receptor targets for lower urinary tract dysfunction.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **377**(4-6): p. 437-48.
6. de Groat, W.C. and N. Yoshimura, *Pharmacology of the lower urinary tract.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 691-721.
7. Nomiya, M. and O. Yamaguchi, *A quantitative analysis of mRNA expression of alpha 1 and beta-adrenoceptor subtypes and their functional roles in human normal and obstructed bladders.* J Urol, 2003. **170**(2 Pt 1): p. 649-53.
8. Andersson, K.E. and A.J. Wein, *Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence.* Pharmacol Rev, 2004. **56**(4): p. 581-631.
9. Chapple, C.R., T. Yamanishi, and R. Chess-Williams, *Muscarinic receptor subtypes and management of the overactive bladder.* Urology, 2002. **60**(5 Suppl 1): p. 82-8; discussion 88-9.
10. Bennett, B.C., et al., *Neural control of urethral outlet activity in vivo: role of nitric oxide.* J Urol, 1995. **153**(6): p. 2004-9.
11. Zurier, R.B., *Chapter 24 - Prostaglandins, Leukotrienes, and Related Compounds*, in *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*, G.S. Firestein, et al., Editors. 2017, Elsevier. p. 366-383.e3.
12. Nakahata, N., *Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology.* Pharmacol Ther, 2008. **118**(1): p. 18-35.
13. Rahnama'i, M.S., et al., *The role of prostanoids in urinary bladder physiology.* Nat Rev Urol, 2012. **9**(5): p. 283-90.

14. Stromberga, Z., R. Chess-Williams, and C. Moro, *The five primary prostaglandins stimulate contractions and phasic activity of the urinary bladder urothelium, lamina propria and detrusor.* BMC Urol, 2020. **20**(1): p. 48.
15. Rahnama'i, M.S., G.A. Van Koeveringe, and P.E. Van Kerrebroeck, *Overactive bladder syndrome and the potential role of prostaglandins and phosphodiesterases: an introduction.* Nephro-urology monthly, 2013. **5**(4): p. 934-945.
16. Palea, S., et al., *Pharmacological characterization of thromboxane and prostanoid receptors in human isolated urinary bladder.* Br J Pharmacol, 1998. **124**(5): p. 865-72.
17. Lee, T., et al., *Urodynamic effects of a novel EP(1) receptor antagonist in normal rats and rats with bladder outlet obstruction.* The Journal of Urology, 2007. **177**(4): p. 1562-1567.
18. Ishizuka, O., A. Mattiasson, and K.E. Andersson, *Prostaglandin E2-induced bladder hyperactivity in normal, conscious rats: involvement of tachykinins?* J Urol, 1995. **153**(6): p. 2034-8.
19. Taber, D.F., J.D. Morrow, and L. Jackson Roberts, *A nomenclature system for the isoprostanes.* 1997. **53**(2): p. 63-67.
20. Milne, G.L., H. Yin, and J.D. Morrow, *Human biochemistry of the isoprostane pathway.* The Journal of biological chemistry, 2008. **283**(23): p. 15533-15537.
21. Basu, S., *Isoprostanes: novel bioactive products of lipid peroxidation.* Free Radic Res, 2004. **38**(2): p. 105-22.
22. Czerska, M., M. Zielinski, and J. Gromadzinska, *Isoprostanes - A novel major group of oxidative stress markers.* Int J Occup Med Environ Health, 2016. **29**(2): p. 179-90.
23. Voynow, J.A. and A. Kummarapurugu, *Isoprostanes and asthma.* Biochimica et biophysica acta, 2011. **1810**(11): p. 1091-1095.
24. Hartmann, S. and T.B. Ledur Kist, *A review of biomarkers of Alzheimer's disease in noninvasive samples.* Biomark Med, 2018. **12**(6): p. 677-690.
25. Kromer, B.M. and J.R. Tippins, *The vasoconstrictor effect of 8-epi prostaglandin F2αin the hypoxic rat heart.* British Journal of Pharmacology, 1999. **126**(5): p. 1171-1174.

26. Bauer, J., et al., *Pathophysiology of isoprostanes in the cardiovascular system: implications of isoprostane-mediated thromboxane A2receptor activation.* British Journal of Pharmacology, 2014. **171**(13): p. 3115-3131.
27. Kawikova, I., et al., *8-Epi-PGF2 alpha, a novel noncyclooxygenase-derived prostaglandin, constricts airways in vitro.* Am J Respir Crit Care Med, 1996. **153**(2): p. 590-6.
28. González-Luis, G., et al., *Age-Related Differences in Vasoconstrictor Responses to Isoprostanes in Piglet Pulmonary and Mesenteric Vascular Smooth Muscle.* Pediatric Research, 2005. **57**(6): p. 845-852.
29. Webb, R.C., *Smooth Muscle Contraction and Relaxation.* 2003. **27**(4): p. 201-206.
30. Wirth, A., et al., *G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension.* Nat Med, 2008. **14**(1): p. 64-8.
31. Somlyo, A.P. and A.V. Somlyo, *Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase.* Physiol Rev, 2003. **83**(4): p. 1325-58.
32. Gohla, A., G. Schultz, and S. Offermanns, *Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction.* Circ Res, 2000. **87**(3): p. 221-7.
33. Maguire, J.J. and A.P. Davenport, *Regulation of vascular reactivity by established and emerging GPCRs.* Trends Pharmacol Sci, 2005. **26**(9): p. 448-54.
34. Fukuhara, S., H. Chikumi, and J.S. Gutkind, *RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho?* Oncogene, 2001. **20**(13): p. 1661-8.
35. Strittmatter, F., et al., *Thromboxane A2 induces contraction of human prostate smooth muscle by Rho kinase- and calmodulin-dependent mechanisms.* European Journal of Pharmacology, 2011. **650**(2-3): p. 650-655.
36. Wilson, D.P., et al., *Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca2+ entry and Ca2+ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697.* Biochem J, 2005. **389**(Pt 3): p. 763-74.

37. Hennenberg, M., et al., *Inhibition of agonist-induced smooth muscle contraction by picotamide in the male human lower urinary tract outflow region.* Eur J Pharmacol, 2017. **803**: p. 39-47.
38. Root, J.A., D.A. Davey, and K.J. Af Forselles, *Prostanoid receptors mediating contraction in rat, macaque and human bladder smooth muscle in vitro.* Eur J Pharmacol, 2015. **769**: p. 274-9.
39. Hernandez, J.M. and L.J. Janssen, *Revisiting the usefulness of thromboxane-A2 modulation in the treatment of bronchoconstriction in asthma.* Canadian Journal of Physiology and Pharmacology, 2014. **93**(2): p. 111-117.
40. Moers, A., et al., *G13 is an essential mediator of platelet activation in hemostasis and thrombosis.* Nat Med, 2003. **9**(11): p. 1418-22.
41. Wettschureck, N., et al., *Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes.* Nat Med, 2001. **7**(11): p. 1236-40.
42. Indra, A.K., et al., *Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases.* Nucleic acids research, 1999. **27**(22): p. 4324-4327.
43. Der, B., et al., *NK2 receptor mediated detrusor muscle contraction involves Gq/11-dependent activation of voltage-dependent calcium channels and the RhoA-Rho kinase pathway.* Am J Physiol Renal Physiol, 2019.
44. Masunaga, K., et al., *Prostaglandin E2 release from isolated bladder strips in rats with spinal cord injury.* Int J Urol, 2006. **13**(3): p. 271-6.
45. Bolla, M., et al., *Cyclooxygenase Involvement in Thromboxane-Dependent Contraction in Rat Mesenteric Resistance Arteries.* 2004. **43**(6): p. 1264-1269.
46. Curry, S.H., et al., *Preparation and stability of indomethacin solutions.* Can J Physiol Pharmacol, 1982. **60**(7): p. 988-92.
47. Jung, H.J. and R. Priefer, *Tachykinin NK2 antagonist for treatments of various disease states.* Autonomic Neuroscience, 2021. **235**: p. 102865.
48. Brading, A.F. and W.H. Turner, *The unstable bladder: towards a common mechanism.* Br J Urol, 1994. **73**(1): p. 3-8.

49. Abrams, P., et al., *The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society.* Urology, 2003. **61**(1): p. 37-49.
50. Peyronnet, B., et al., *A Comprehensive Review of Overactive Bladder Pathophysiology: On the Way to Tailored Treatment.* European Urology, 2019. **75**(6): p. 988-1000.
51. Nambiar, A.K., et al., *EAU Guidelines on Assessment and Nonsurgical Management of Urinary Incontinence.* Eur Urol, 2018. **73**(4): p. 596-609.
52. Hsiao, S.-M., et al., *Autonomic dysfunction and arterial stiffness in female overactive bladder patients and antimuscarinics related effects.* Maturitas, 2014. **79**(1): p. 65-69.
53. Sacco, E., F. Pinto, and P. Bassi, *Emerging pharmacological targets in overactive bladder therapy: experimental and clinical evidences.* International Urogynecology Journal, 2008. **19**(4): p. 583-598.
54. Andersson, K.E., *Potential Future Pharmacological Treatment of Bladder Dysfunction.* Basic Clin Pharmacol Toxicol, 2016. **119 Suppl 3**: p. 75-85.
55. Yoshida, M., et al., *The Forefront for Novel Therapeutic Agents Based on the Pathophysiology of Lower Urinary Tract Dysfunction: Pathophysiology and Pharmacotherapy of Overactive Bladder.* Journal of Pharmacological Sciences, 2010. **112**(2): p. 128-134.
56. Mills, I.W., et al., *Studies of the pathophysiology of idiopathic detrusor instability: the physiological properties of the detrusor smooth muscle and its pattern of innervation.* J Urol, 2000. **163**(2): p. 646-51.
57. Dokumacioglu, E., et al., *Measuring urinary 8-hydroxy-2'-deoxyguanosine and malondialdehyde levels in women with overactive bladder.* Investigative and clinical urology, 2018. **59**(4): p. 252-256.
58. Masuda, H., et al., *Reactive oxygen species mediate detrusor overactivity via sensitization of afferent pathway in the bladder of anaesthetized rats.* BJU Int, 2008. **101**(6): p. 775-80.
59. Yeniel, A.O., et al., *Is overactive bladder microvasculature disease a component of systemic atheroscleorosis?* Neurourol Urodyn, 2018. **37**(4): p. 1372-1379.

60. He, Q., et al., *Metabolic syndrome, inflammation and lower urinary tract symptoms: possible translational links.* Prostate Cancer and Prostatic Diseases, 2016. **19**(1): p. 7-13.
61. Vona, R., et al., *Biomarkers of Oxidative Stress in Metabolic Syndrome and Associated Diseases.* Oxidative Medicine and Cellular Longevity, 2019. **2019**: p. 8267234.
62. Uno, K. and S.J. Nicholls, *Biomarkers of inflammation and oxidative stress in atherosclerosis.* Biomark Med, 2010. **4**(3): p. 361-73.
63. Parajuli, S.P., A. Provence, and G.V. Petkov, *Prostaglandin E2 excitatory effects on guinea pig urinary bladder smooth muscle: A novel regulatory mechanism mediated by large-conductance voltage- and Ca2+-activated K+ channels.* European Journal of Pharmacology, 2014. **738**: p. 179-185.
64. Tarcan, T., et al., *Isoprostane 8-epi PGF2alpha, a product of oxidative stress, is synthesized in the bladder and causes detrusor smooth muscle contraction.* Neurourol Urodyn, 2000. **19**(1): p. 43-51.
65. Zerhau, P., et al., *Limitations in using rabbit bladders in electrophysiological and urodynamic experiments.* Veterinarni Medicina, 2014. **59**: p. 376-381.
66. Parsons, B.A. and M.J. Drake, *Animal models in overactive bladder research.* Handb Exp Pharmacol, 2011(202): p. 15-43.
67. Audoly, L.P., et al., *Cardiovascular Responses to the Isoprostanes iPF 2α -III and iPE 2 -III Are Mediated via the Thromboxane A 2 Receptor In Vivo.* Circulation, 2000. **101**(24): p. 2833-2840.
68. Sametz, W., et al., *Characterization of prostanoid receptors mediating actions of the isoprostanes, 8-iso-PGE(2) and 8-iso-PGF(2alpha), in some isolated smooth muscle preparations.* British journal of pharmacology, 2000. **130**(8): p. 1903-1910.
69. Chen, W.H., Y.H. Jiang, and H.C. Kuo, *Urinary Oxidative Stress Biomarkers in the Diagnosis of Detrusor Overactivity in Female Patients with Stress Urinary Incontinence.* Biomedicines, 2023. **11**(2).
70. Jhang, J.F., Y.H. Jiang, and H.C. Kuo, *Discriminating Different Bladder and Bladder Outlet Dysfunctions by Urinary Biomarkers in Women with Frequency-Urgency Syndrome.* Biomedicines, 2023. **11**(3).

71. Jiang, Y.H., J.F. Jhang, and H.C. Kuo, *Urinary Oxidative Stress Biomarker Levels Might Be Useful in Identifying Functional Bladder Disorders in Women with Frequency and Urgency Syndrome.* J Clin Med, 2023. **12**(6).
72. Lazzeri, M., *The physiological function of the urothelium--more than a simple barrier.* Urol Int, 2006. **76**(4): p. 289-95.
73. Allen, I.C., et al., *Thromboxane A2 induces airway constriction through an M3 muscarinic acetylcholine receptor-dependent mechanism.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2006. **290**(3): p. L526-L533.
74. Anjum, I., *Calcium sensitization mechanisms in detrusor smooth muscles.* J Basic Clin Physiol Pharmacol, 2018. **29**(3): p. 227-235.
75. Roberts, M.W.G., et al., *TRPV4 receptor as a functional sensory molecule in bladder urothelium: Stretch-independent, tissue-specific actions and pathological implications.* The FASEB Journal, 2020. **34**(1): p. 263-286.
76. Donko, A., et al., *Urothelial cells produce hydrogen peroxide through the activation of Duox1.* Free Radic Biol Med, 2010. **49**(12): p. 2040-8.

9. Bibliography of the candidate's publications

Publications related to the dissertation

P. J. Molnár, B. Dér, K. Borsodi, H. Balla, Z. Borbás, K. Molnár, É. Ruisanchez, I. Kenessey, A. Horváth, A. Keszthelyi, A. Majoros, P. Nyirády, S. Offermanns, and Z. Benyó, "Isoprostanes evoke contraction of the murine and human detrusor muscle via activation of the thromboxane prostanoid TP receptor and Rho kinase," *AMERICAN JOURNAL OF PHYSIOLOGY: RENAL PHYSIOLOGY*, vol. 320, no. 4, pp. F537–F547, 2021. **IF: 4.097**

B. Dér, **P. J. Molnár**, É. Ruisanchez, P. Őrsy, M. Kerék, B. Faragó, P. Nyirády, S. Offermanns, and Z. Benyó, "NK2 receptor-mediated detrusor muscle contraction involves $G_{q/11}$-dependent activation of voltage-dependent Ca^{2+} channels and the RhoA-Rho kinase pathway," *AMERICAN JOURNAL OF PHYSIOLOGY: RENAL PHYSIOLOGY*, vol. 317, no. 5, pp. F1154–F1163, 2019. **IF: 3.191**

Publications not related to the dissertation

H. Balla, K. Borsodi, P. Őrsy, B. Horváth, **P. J. Molnár**, Á. Lénárt, M. Kosztelnik, É. Ruisanchez, J. Wess, S. Offermanns, P. Nyirády, and Z. Benyó, "Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions," *AMERICAN JOURNAL OF PHYSIOLOGY: RENAL PHYSIOLOGY*, vol. 325, pp. F618–F68, 2023. **IF: 4.200**

K. Borsodi, H. Balla, **P. J. Molnár**, Á. Lénárt, I. Kenessey, A. Horváth, A. Keszthelyi, M. Romics, A. Majoros, P. Nyirády, S. Offermanns, and Z. Benyó, "Signaling Pathways Mediating Bradykinin-Induced Contraction in Murine and Human Detrusor Muscle," *FRONTIERS IN MEDICINE*, vol. 8, 2022. **IF: 3.900**

A. Majoros, M. Romics, A. Ali, A. Hamvas, **P. J. Molnár**, A. Keszthelyi, and P. Nyirády, "Hiperaktív (túlműködő) hólyag kezelése botulinumtoxin-A-terápiával," *ORVOSI HETILAP*, vol. 162, no. 36, pp. 1459–1465, 2021. **IF: 0.707**

M. Romics, V. Keszthelyi, V. Brodszky, **P. J. Molnár**, A. Keszthelyi, O. Oláh, P. Nyirády, and A. Majoros, "Narrow vagina as a predictor of obstructive voiding dysfunction after transobturator sling surgery," *UROLOGIA INTERNATIONALIS*, vol. 105, no. 11–12, pp. 1092–1098, 2021. **IF: 1.934**

Cumulative impact factor of the candidate's publication: **18.029**

DOI:10.14753/SE.2024.3019

10. Acknowledgements

I am very grateful to my mentor and supervisor, Professor Zoltán Benyó for his guidance and encouragement. He always made time for me even at the expense of his spare time during my PhD studies. I would like to thank to him for his advices and helping me in every way possible during the years, providing not only the infrastructure for the experiments, but consultations in friendly, but also in a professional environment, and opportunities for taking part in scientific conferences in Hungary and abroad. He was truly inspirational for me.

I am also very thankful to Professor Péter Nyirády, my tutor in the urology training, for his support and professional advices. Also, I would like to thank for Attila Keszthelyi, Attila Majoros, András Horváth and Miklós Romics for their help in designing the research protocol and in obtaining urinary bladder samples for the human studies.

I would like to thank to Bálint Dér, who invited me to the research workgroup during my physiology studies and from whom I have learnt a lot during the years, as he was my first mentor.

I would like to thank Kinga Borsodi, Helga Balla and Ádám Lénárt for their efforts, support and help during the experiments and research work.

I am very grateful to László Hricisák and Ágnes Fülöp for their help with the management of transgenic experimental animals.

I am very thankful to Éva Ruisanchez and Margit Kerék for their enormous help during the myograph experiments, for teaching me the methodology. I am also very grateful for their professional advices.

I am very grateful to Ildikó Murányi for her excellent technical help during the laboratory work.

I would like to express my sincere gratitude to all my co-authors for their effort, and all former and current colleagues at the Institute of Translational Medicine and Department of Urology for their support.

Finally, I am very thankful to my wife, to my parents, and my family. Without their encouragement, inspiration and support this thesis could not have been accomplished.