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Preclinical investigations addressing contributions of phospholipases to α1-adrenergic contractions, and antagonism of α1-adrenoceptors and growth inhibition by antihypertensive carvedilol in the human prostate

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Place, Date

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List of abbreviations

5-ARIs	5α-reductase inhibitors
AC	adenylate cyclase
ARF6	ADP-ribosylation factor 6
ARs	adrenergic receptors
AUR	acute urinary retention
BPE	benign prostatic enlargement
BPH	benign prostatic hyperplasia
BPO	benign prostatic obstruction
BOO	bladder outlet obstruction
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
DAG	diacylglycerol
DHT	dihydrotestosterone
EAU	European Association of Urology
EFS	electric field stimulation
FAK	focal adhesion kinase
GHRH	growth hormone-releasing hormone
GRK 2/3	G protein-coupled receptor kinase 2/3
GWAS	genome-wide association studies
HESR	hexane-extracted saw palmetto
IFIS	intraoperative floppy iris syndrome
ILK	integrin-linked kinase
IP3	inositol-1,4,5-trisphosphate
IPSS	international prostate symptom score
JNK	c-Jun N-terminal kinase
LHRH	luteinizing hormone-releasing hormone
LIMK	LIM domain kinase
LUTS	lower urinary tract symptoms

MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MLCs	myosin light chains
MYPT1	myosin phosphatase target subunit 1
NO	nitric oxide
NSAIDs	nonsteroidal anti-inflammatory drugs
OR	odds ratio
PAK	p21-activated kinase
PDE5I	phosphodiesterase 5 inhibitor
PDEs	phosphodiesterases
PGs	prostaglandins
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phospholipase A_2
PLC	phospholipase C
PLK	polo-like kinase
PSA	prostate-specific antigen
PVR	post-voiding residual urine volume
Qmax	maximum urinary flow rate
QOL	quality of life
RhoA	Ras homolog family member A
Src	non-receptor Proto-oncogene tyrosine-protein kinase Src
sGC	soluble guanylate cyclase
SR	sarcoplasmic reticulum
STK	serine/threonine kinase 16
TXA_2	thromboxane A_2
TXS	thromboxane synthase

List of publications

Publications included in the thesis:

Sheng Hu, Ayhanim Elif Muderrisoglu, Anna Ciotkowska, Oluwafemi Kale, Patrick Keller, Melanie Schott, Alexander Tamalunas, Raphaela Waidelich, Christian G. Stief, Martin Hennenberg. Effects of carvedilol on human prostate tissue contractility and stromal cell growth pointing to potential clinical implications. Pharmacological Reports. Pharmacol Rep, 2024 Aug;76(4):807-822

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Other publications not included in the thesis:

Ayhanim Elif Muderrisoglu, Anna Ciotkowska, Beata Rutz, **Sheng Hu**, Siwei Qian, Alexander Tamalunas, Christian G. Stief and Martin Hennenberg. Dynamic Phenotypic Shifts and M2 Receptor Downregulation in Bladder Smooth Muscle Cells Induced by Mirabegron. Frontiers in Pharmacology, 2024 Jul 24:15:1446831

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Sheng Hu, Moritz Trieb, Ru Huang, Alexander Tamalunas, Patrick Keller, Melanie Götz, Raphaela Waidelich, Christian G Stief, Martin Hennenberg. Organ-specific off-target effects of Pim/ZIP kinase inhibitors suggest lack of contractile Pim kinase activity in prostate, bladder, and vascular smooth muscle. Naunyn Schmiedebergs Arch Pharmacol, 2024 Feb;397(2):1219-1231

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Ru Huang, Yuhan Liu, **Sheng Hu**, Alexander Tamalunas, Raphaela Waidelich, Frank Strittmatter, Christian G Stief, Martin Hennenberg. Inhibition of α_1-Adrenergic, Non-Adrenergic and Neurogenic Human Prostate Smooth Muscle Contraction and of Stromal Cell Growth by the Isoflavones Genistein and Daidzein. 2022 Nov 22;14(23):4943

Contribution to the publications

1.1 Contribution to paper I

The first author, Sheng Hu, collected and conducted all the organ bath experiments, and additionally was involved in analyzing and interpreting data and findings, preparing figures and drafts as well as the revision of the manuscript.

1.2 Contribution to paper II

The first author, Sheng Hu contributed to the acquisition, analysis and interpretation of data for the work as well as drafting the manuscript.

The co-authors agreed to the MD student Sheng Hu to include the above two publications in his thesis for the doctoral degree in Human Medicine. All authors confirm that the publications will not be used as part of another dissertation.

2. Introduction

2.1 Definition and pathological mechanism

Benign prostatic hyperplasia (BPH), emphasizing hyperplasia but not hypertrophy, is characterized as increased numbers but not related to the sizes of glandular and smooth muscle cells in the periurethral zone and transitional zone of prostates [1, 2]. As a result of hyperplasia, prostates may show macroscopically enlarged volumes, known as benign prostatic enlargement (BPE) [1]. Strictly speaking, however, BPH is defined histologically and does not necessarily indicate an increase in prostate volume. In contrast, BPE typically refers to an increase in prostate volume that occurs on the basis of BPH [3]. Besides, prostatic inflammation, which results in tissue damage and continuous repair, also contributes to BPE [4]. Although BPH and BPE can lead to compression of the prostatic urethra and increased intraurethral pressure, resulting in benign prostatic obstruction (BPO) or bladder outlet obstruction (BOO), this is not inevitable [1]. For example, canines, like human beings, naturally experience BPH and BPE but do not develop BPO or BOO, possibly because dogs lack a prostatic capsule [3]. Additionally, clinical studies have indicated that only 3.4% of the variability in symptom scores and 5.7% of the variability in peak flow rates in BPH can be attributed to prostate volume [5]. In fact, the progression of BPH and BPE to BPO or BOO primarily occurs through two mechanisms: 1) the static obstruction (passive strength) by enlarging prostate; 2) the dynamic obstruction (active strength) by enhanced smooth muscle contraction [6]. Notably, BPO is not synonymous with BOO, nor is it the sole causative factor of BOO. This is evidenced by the occurrence of BOO in females who inherently lack a prostate, indicating that factors beyond prostatic enlargement can precipitate BOO and that its occurrence is not rare in this demography [7, 8].

The urethral resistance stemming from BPH can manifest as voiding symptoms, encompassing excess efforts to initiate and maintain micturition, a weak and diminished urinary stream, terminal dribbling, and incomplete emptying (the latter two also known as post-micturition symptoms) [3]. To overcome the BPO caused by BPH, the bladder is required to increase its contractile force to facilitate the expulsion of urine. Prolonged exposure to these conditions can consequently lead to hypertrophy and dysfunction of the detrusor [9, 10]. This long-term chronic presence of urethral resistance can result in compensatory adaptions in detrusor function, typically occurring in two distinct phases: 1) Initial phase: characterized by increased frequency and urgency of micturition, as well as nocturia, attributed to diminished stability and compliance of the detrusors; 2) Advanced phase: marked by declining voiding force and heightened susceptibility to urinary retention due to impaired detrusor contractility, indicative of decompensation [11]. Both voiding and storage symptoms, collectively termed lower urinary tract symptoms (LUTS), can compromise the quality of life (QOL) [12]. In addition to the aforementioned distressing symptoms, BPH-associated augmentation of post-voiding residual (PVR) urine formation and urinary retention can heighten

the susceptibility to urinary tract infections, bladder calculi, bilateral upper urinary tract dilation, and renal dysfunction, thereby exerting a substantial influence on the overall health and well-being of affected individuals [1].

It is noteworthy that BPH neither serves as a sufficient nor a necessary condition for LUTS, as male individuals with large prostates may not necessarily experience voiding symptoms and the severity of LUTS does not correlate strictly with the size of the prostates [13, 14]**.** Nevertheless, in clinical practice and real world settings, the diagnosis of BPH relies predominantly on symptomatic presentation and corresponding clinical indices (uroflowmetry, imaging, urodynamics, et al.), as histological evidence can typically only be obtained through autopsy, puncture biopsy (reserved for suspected prostate cancer cases), or postoperative pathological examination [1, 15]. Hence, the term "LUTS suggestive of BPH" appears to be more precise for the clinical diagnosis of BPH, and is widely used to describe the yet unconfirmed association between BPH/BPE and LUTS [3]. Conversely, the term BPO denotes a causal relationship between the BPH/BPE and LUTS, typically confirmed through invasive urodynamics/pressure flow studies [15].

2.2 Epidemiology

BPH, as an age-dependent disease, is not exhibited among men aged 21 to 30 years. However, the histological diagnostic prevalence increases dramatically from only 8% in the fourth decade of age to 50% in the sixth decade, and finally proceeding to 90% by the ninth decade [1, 16]. In accordance with the variance of histological changes, the macroscopical volume of the transitional zone increases with a rate of 1.6% in the fourth decade of age and this rate continues to increase with age [17]. For men with age more than 50 years, the growth rate of the transitional zone is around 3.5%, and the growth rate of whole prostate volume also reaches 2.2% [18]. With the extension of average life expectancy and the intensification of population aging, the global prevalence of BPH increased by approximately 84% from 2000 to 2019, rising from 51.1 million to 94 million cases [19]. The global prevalence of individuals afflicted by any LUTS (i.e., symptoms resulting from BPH, or storage symptoms attributed to the bladder) is also projected to escalate from an estimated 1.9 billion people worldwide in 2008 to approximately 2.3 billion by the year 2018 [20]. BPH typically exhibits histological progression with advancing age. Nevertheless, in clinical settings, the peak absolute burden of BPH is observed in individuals aged 65 to 69 years, while the highest prevalence rates stratified by age are recorded among those aged 75 to 79 years [19]. This trend is in line with other community studies and may be explained by that the diagnosis of BPH in clinical practice mainly relies on LUTS as clinical manifestations and prostate enlargement [21, 22]. The high prevalence rate implies a significant economic burden. In 2018, the U.S. National Institute of Diabetes and Digestive and Kidney Diseases estimated that globally, men aged 65 and above contribute to an economic burden of $73.8 billion annually due to BPH [19].

2.3 Etiology and risk factors

BPH is a hyperplastic condition characterized by an absolute increase in the number of epithelial and stromal cells within the transitional and periurethral zone of the prostate [1]. Although BPH is primarily a proliferative process, increased smooth muscle tone within the prostate also contributes to the development of male LUTS [6]. In some instances, voiding symptoms can manifest without evidence of prostatic hyperplasia, underscoring the significance of smooth muscle dysfunction in the pathogenesis of these symptoms [13, 14].

Many etiological factors directly contribute to the occurrence and development of BPH, accompanied by various risk factors increasing the probability of developing BPH without being directly causative. Given that BPO is caused by both dynamic and static forces, it follows that factors associated with these two forces may collectively contribute to LUTS suggestive of BPH. Hyperplasia serves as the foundational basis for static obstructions and has undergone extensive investigation, yielding the identification of numerous contributing factors, including androgens, estrogens, various growth factors, neurotransmitters, et al. [11]. These factors may act independently or synergistically to promote hyperplasia. Conversely, dynamic obstruction primarily arises from contractions of stromal smooth muscle, necessitating further inquiry to elucidate associated factors. Such factors may encompass non-adrenergic agonists, elements influencing actin polymerization, and the regulation of intracellular calcium concentration and sensitivity [23, 24].

2.3.1 Genetic predisposition

Genes are always thought to be involved in causing monogenic disorders by a single gene, increasing the susceptibility of polygenic disorders, or serving as genetic predispositions that elevate the probability of developing the condition but not guaranteeing it. Though up to 72% of patients suffering voiding symptoms or requiring surgery in BPH could be accounted for genetic predisposition according to a twins' study [25], evidence from both candidate gene approaches and genome-wide association studies (GWAS) appears to be insufficient.

A meta-analysis of candidate gene approaches in LUTS found that while the vitamin D receptor variant (variant rs731236) exhibits the highest potential as a protective factor for LUTS with an odds ratio (OR) of 0.64, its epidemiological credibility remains only moderate [26]. Further studies utilizing candidate gene approaches have identified three polymorphisms in estrogen receptor 2 and one variant in estrogen receptor as risk factors for the voiding symptoms or histological BPH, with OR ranging from 1.94 to 6.3 [27, 28]. Interestingly, phytoestrogens demonstrated a protective effect on BPH-related voiding symptoms by inhibiting both contraction and growth, as evidenced by *in vitro* experiments [29]. Four research studies employing GWAS have respectively delineated associations between various specific genetic variants and BPH/voiding symptoms. The initial investigation revealed an association between a variant (rs17144046, located near the *GATA3* gene, which encodes a transcription factor) and the occurrence of voiding symptoms [30].

Subsequently, the second study identified 23 variants from 14 genomic loci exhibiting a significant and robust correlation between prostate-specific antigen (PSA) levels and BPH, confirmed with a genetic correlation coefficient of 0.77 [31]. The third study elucidated a protective effect against BPH conferred by a genetic variant at rs2710383, situated proximate to the *SYN3* gene, with an OR of 0.69 [32]. Lastly, the fourth investigation identified an intronic variant at rs1237696 within the progesterone receptor gene as a risk factor for BPH, characterized by an OR of 1.36 [33]. Two studies employing Mendelian Randomization based on GWAS have additionally documented that elevation in bioavailable testosterone possesses the potential to instigate BPH [34], while subclinical hypothyroidism and clinically diagnosed hypothyroidism have been identified as protective factors against BPH [35].

Quantifying the individual or cumulative contributions of risk variants to the overall genetic risk remains currently unattainable and requires additional data and the application of genetic metrics [36].

2.3.2 Growth

Androgens have long been considered a prerequisite for the occurrence of BPH. The contributions of androgens and androgen receptors in the initiation and advancement of BPH has been extensively and deeply investigated [1]. Nevertheless, at least two phenomena persist awaiting elucidation: 1) while all prostates from mammalian animal models contain testosterone, dihydrotestosterone (DHT) and androgen receptors along with the growth signaling pathways, spontaneous BPH solely manifests in humans, nonhuman primates like chimpanzees, and canines [11]; 2) the seminal vesicle, another glandular organ responsive to androgen throughout the life, does not undergo hyperplasia [11]. So far, we can only conclude that androgens play a permissive role in the initiation of BPH [11]. Therefore, further research is required to elucidate the "etiological" effects mediated by androgens.

There is no doubt that hyperplasia is caused by imbalanced cell proliferation and death including apoptosis [37]. Interestingly, though androgens are deployed to establish BPH in experimental animal models, conclusive evidence regarding their promotion of proliferation effects remains elusive. In parallel to this, studies have shown that androgens may not only sustain normal proliferation of prostate cells but also promote BPH by inhibiting apoptosis [38, 39], as depletion of androgens can activate apoptosis-related molecules in the prostate [40, 41]. Although the occurrence of BPH requires the presence of testosterone, testosterone is not the primary form in the prostate but rather DHT converted by 5α-reductase [42]. Even as testosterone decreases with age in the elderly, DHT and androgen receptors in the prostate can still maintain unchanged levels [43, 44]. The lifelong high expression levels of androgen receptors in the prostate, along with their high affinity and stability for DHT, may form the basis for the progression of BPH with increasing age. The exploration of androgen receptors remains intricate, with shortened CAG repeats serving as one of the best examples. Short CAG repeats in the androgen receptor gene were once reported to parallel increased prostate sizes and risk of disease progression [45]. However,

a later study pointed out that this relationship does not actually exist among the Dutch population [46]. Moreover, a further study showed that short CAG repeats were less common in Finnish BPH patients compared to control subjects [47]. Finally, a study concluded that there is no causal or parallel relationship between shortened CAG repeats and BPH [48].

Though the estrogen has been proven to be associated with the pathogenesis of animal BPH models, its precise role in the initiation of human BPH remains elusive [49]. Other factors or mechanisms like regulation of apoptosis, stromal-epithelial interactions, growth factors, inflammatory signaling pathways and neurotransmitters have not established clear and firm cause-and-effect relationships [11].

2.3.3 Contractility

Prostatic smooth muscle contractility, the most important if not the only source of active urethral resistance, serves as an indispensable part of the etiologies underlying LUTS suggestive of BPH [50]. In BPH, the prostatic stroma, which primarily consists of smooth muscle cells, constitutes 2-3 times the amount of epithelial components and the stroma-to-epithelium ratio is also higher in symptomatic BPH patients compared to those without symptoms [2]. Without regard to the precise epithelial-to-stromal cell ratio in BPH, it is unequivocal that smooth muscles constitute a substantial volume of the whole prostate [51].

The stromal hyperplasia can happen alone or together with epithelial hyperplasia [52]. The early nodules, which exhibit stromal characteristics, initially manifest either within or in close proximity to the preprostatic sphincter [53]. There are two different dominant cell types of hyperplasia indicated in BPH: 1) primarily composed of fibromuscular stroma from a relatively small-volume prostate (with hyperplasia, but no significant volume increase, for instance, less than 40 ml) [2]; 2) primarily composed of epithelial nodules from large-volume prostate [54, 55]. Parallelled by clinical practice, the relatively small-volume prostates exhibit better responsiveness to α_1-blockers [56, 57], while larger prostates exhibit better responsiveness to 5α-reductase inhibitors (5-ARIs) [58]. Considering that the severity of LUTS suggestive of BPH does not correlate strictly with the size of the prostates [13], it is assumed that contraction from stromal smooth muscle affects the prostatic urethral resistance.

In fact, smooth muscle cells are not only involved in the composition of the prostatic stroma but are also a crucial component of the prostatic capsule. As previously mentioned, canines, like humans and other primates such as chimpanzees, also experience naturally occurring BPH and develop BPE [59]. However, unlike in humans or chimpanzees [59], canines do not develop BPO. Even in severe cases of BPE in canines, where the enlarged prostate may compress nearby rectums leading to constipation, BPO does not occur [3]. This is primarily because the canine prostate lacks the capsule that encases the human prostate. This theory is validated in the other experiment: when the canine prostate was encased with a material mimicking the muscular

capsule of the human prostate, the dogs similarly developed symptoms of urethral obstruction [60].

The prostatic capsule and the prostatic stroma are not two independent components. The fibromuscular elements of the prostatic capsule can extend into the prostatic stroma and become part of the stroma [61]. A subsequent study also indicated that the outer fibromuscular tissue (prostatic capsule) infiltrated into the prostatic stroma [62], however this study doubted about the existence of the prostatic capsule due to its absence in the apex and anterior portions of the prostate [62]. This doubt was refuted by later research, which demonstrated the widespread presence of smooth muscle fibers enclosing the prostate, including the apex region [63]. This research further revealed that the prostatic capsule is not only composed of smooth muscle and collagen but also includes fast-twitch and slow-twitch striated muscle fibers [63]. The smooth muscle component accounts for 25-75% of the total volume of the prostatic capsule, with a tendency to increase from the apex to the base and from the anterior to the posterior sections. Conversely, the distribution of striated muscle shows the opposite trend [63]. Under normal physiological conditions, the contraction of the striated muscle in the prostatic capsule may act as a supplementary component of the urethral sphincter, contributing to urethral closure and preventing urinary incontinence [63]. However, in the presence of BPE, this function, due to the integration of the muscular fibers of the capsule with the prostatic stroma, will exacerbate BPO in turn. For patients with symptomatic BPH, the contractile force of the prostatic capsules' smooth muscle in response to phenylephrine stimulation is four times greater than that of asymptomatic patients, suggesting that the smooth muscle fibers of the prostatic capsule in BPH do not merely facilitate the passive strength by enclosing the enlarged hyperplastic prostate but also enhance active strength due to their increased contractile capacity [64].

Considering the following points: 1) the smooth muscle fibers within the prostatic stroma facilitate the expulsion of secretions from the prostatic acini during ejaculation [65]; 2) the contraction of stromal smooth muscle closes the proximal urethra assisting to prevent retrograde ejaculation [66]; 3) the smooth muscle fibers of the prostatic capsule extend into the stroma, potentially interweaving with the stromal smooth muscle fibers to form a smooth muscle network; and 4) the smooth muscle of the prostatic capsule, along with its striated muscle, participates in urinary continence [63]. It is not difficult to infer that excessive contraction of the prostatic smooth muscle will lead to significant BPO/BOO and LUTS.

2.3.4 Other factors

The previous paragraph has discussed the etiological factors that may directly contribute to LUTS suggestive of BPH. Among the factors that do not directly impact the prostate, age is a definite and significant factor in the occurrence and progression of BPH [1]. Specifically, age may promote LUTS suggestive of BPH through a combination of hormonal changes, cellular and molecular mechanisms, genetic predisposition, and others [67].

In the analysis of common risk factors for BPH, a previous study indicated that obesity and diabetes are potential risk factors for BPH, while physical exercise and moderate avoiding alcohol consumption appears to be protective factors [68]. The influence of factors such as blood lipids, blood pressure, smoking, and diet on BPH appears to be less significant [68]. Subsequent meta-analyses supported that moderate alcohol consumption may be associated with a lower likelihood of developing BPH [69, 70]. The earlier meta-analysis indicated no significant association between alcohol intake and the occurrence of LUTS, despite the association with BPH [69]. However, the more recent systematic review suggested that moderate alcohol consumption is linked to a reduced risk of developing LUTS, while high alcohol consumption (e.g., >40 g/day) may be involved in an increased risk of mixed LUTS [70]. Other recently identified risk factors include insomnia [71], bioavailable testosterone [34], glucose levels and insulin use [72], diabetes mellitus [73], heavy metal toxicity by lead and cadmium [74], obesity and sedentary behavior [75, 76]. Conversely, the emerging protecting factors include dietary phytochemical index [77], uric acid [78], and adequate sleep duration [79].

2.4 Contraction and relaxation of prostatic smooth muscles

The tension of prostatic smooth muscle varies depending on its state of contraction or relaxation. Contraction is mediated by neurohumoral and paracrine sources, including adrenergic and non-adrenergic mediators, which transduce and induce intracellular signaling pathways to initiate contraction [3]. Relaxation, on the other hand, is primarily mediated by the generation of cyclic guanosine monophosphate (cGMP) induced by inducible nitric oxide (NO), or by the generation of cyclic adenosine monophosphate (cAMP) induced by β-ARs and prostaglandin receptors [3]. Notably, prostaglandin F_{2a}, also named thromboxane A_2 (TXA_2), which is supposed to induce relaxation, induces contractions of prostate smooth muscle instead [3].

2.4.1 Contraction

The regulation of prostatic smooth muscle contraction, like that in other smooth muscle-rich organs, involves the activation of G protein-coupled receptors and is facilitated through three main intracellular signaling pathways (as illustrated in Figure 1): 1) inositol-1,4,5-trisphosphate (IP3)-mediated increase in cytosolic calcium ions, 2) protein kinase C (PKC) along with 3) Rho kinase-mediated increase in “calcium sensitization” [80-82]. The ultimate achievement of contraction requires three preconditions: 1) phosphorylation of myosin light chains (MLCs) by myosin light chain kinase (MLCK), 2) polymerization of monomeric actin into filaments, and 3) attachment of these filaments to the cell membrane and anchorage of cells to the extracellular matrix [3].

The following describes the detailed process concerning the classic contraction model mediated by G protein-coupled receptors. When activated by agonists or other stimuli, the receptor triggers G protein-associated intracellular signaling pathways, specifically by activating phospholipase C (PLC) and the monomeric GTPase, Ras homolog family member A (RhoA) via the Gα subunit

[24, 83]. Firstly, activated PLC hydrolyzes phosphatidylinositol diphosphate, producing the second messengers IP3 and diacylglycerol (DAG) [24]. IP3 primarily participates in the elevation of calcium ions: it opens calcium channels on the sarcoplasmic reticulum (SR), causing an increase in cytosolic calcium concentration, which leads to membrane depolarization. Subsequently, voltage-gated calcium channels on the cell membrane open, allowing a large influx of extracellular calcium ions into the cell, rapidly increasing cytosolic calcium concentration. The elevated calcium then binds to and activates calmodulin (CaM), which further activates MLCK [84]. MLCK phosphorylates MLCs, fulfilling the first prerequisite for smooth muscle contraction. Simultaneously, inhibiting myosin light chain phosphatase (MLCP) to maintain the phosphorylated state of MLCs (calcium sensitization) is also important and includes two molecular mechanisms [83, 85]: On the one hand, DAG activates PKC, which inhibits MLCP, either by directly phosphorylating the myosin phosphatase target subunit 1 (MYPT1) of MLCP or indirectly through the phosphorylation of MLCP inhibitory protein, protein phosphatase 1 regulatory subunit 14A. On the other hand, RhoA, activated by the Gα subunit, further activates Rho kinase, which can inactivate MLCP by phosphorylating MYPT1.

Though the α_{1A}-AR is recognized as the predominant receptor responsible for inducing smooth muscle contraction in the prostate of humans and underlines the clinical application of α_1-blockers for BPH treatment [86], non-adrenergic mediators including TXA_2 and endothelin also promote the human prostate smooth muscle contraction concurrently. In fact, the effectiveness of α_1-blockers in inhibiting prostatic contraction is limited. A supposed reason for this limitation is that non-adrenergic meditators also participate in the contraction of the prostate, and these mediators are resistant to α_1-blockers [23]. Other non-adrenergic mediators such as acetylcholine, dopamine, histamine, serotonin and adenosine triphosphate can only induce slight contraction or even no contraction in human prostate tissues [23, 87-89].

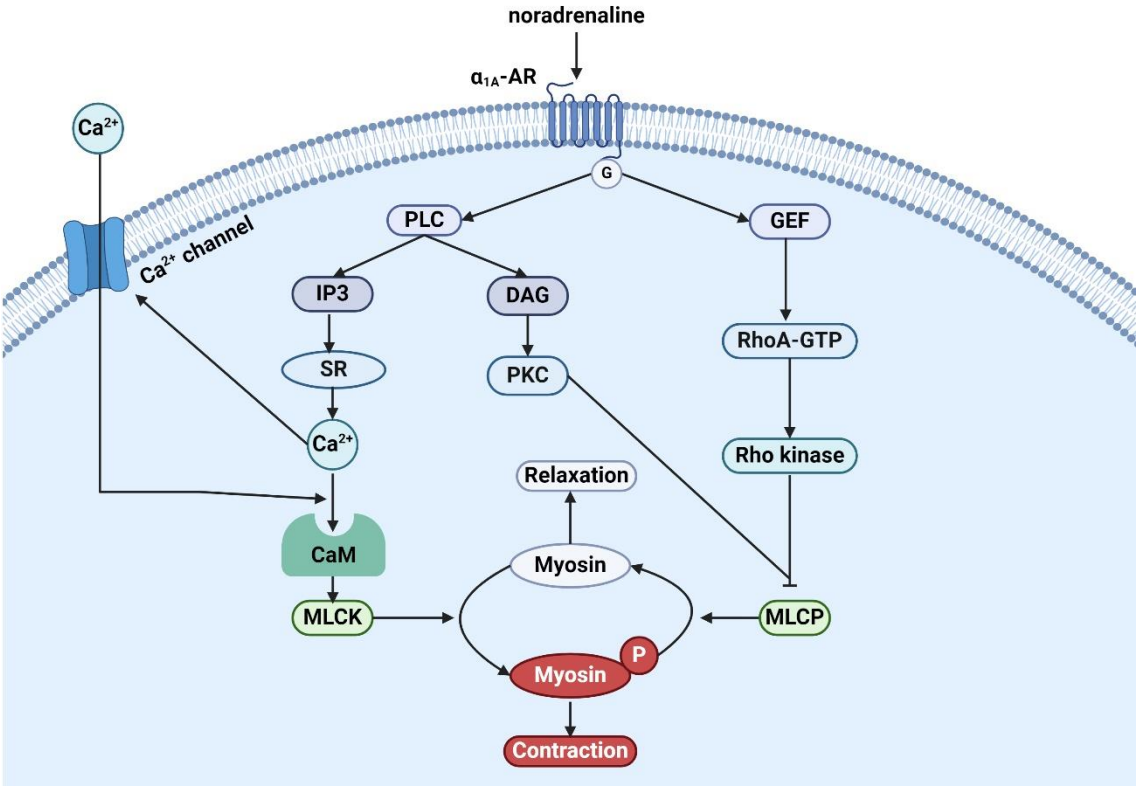

Figure 1. Process of adrenergic prostate smooth muscle contraction. Upon activation, the α_{1A}-AR transduces signals through calcium-dependent pathways to promote the phosphorylation of MLC and through calcium-independent pathways to inhibit the dephosphorylation of MLC, thereby facilitating muscle contraction. (This figure is adapted from the main structure of a corresponding figure in a previous thesis of Li, B. (2021), *Effects of the integrin inhibitors BTT3033 and BOP, and the integrin-linked kinase inhibitor Cpd22 on human prostate smooth muscle contraction*, Ludwig-Maximilians-Universität München.)

2.4.2 Relaxation

The relaxation of smooth muscle primarily relies on the activation of MLCP and decrease intracellular calcium concentrations, which is dependent on protein kinase A (PKA) and protein kinase G (PKG), regulated by cAMP and cGMP, respectively (as illustrated in Figure 2) [3, 82, 90]. cAMP is produced by the catalytic action of adenylate cyclase (AC), which can be activated by prostaglandins (PGs) and β-adrenergic agonists. cGMP, on the other hand, is produced by the catalytic action of soluble guanylate cyclase (sGC), which is activated byNO [3]. Both cAMP and cGMP can be hydrolyzed by phosphodiesterases (PDEs).

Though both mechanisms mediated by cAMP and cGMP are better established for other smooth muscles compared to prostatic smooth muscles [3], the effects of these mechanisms in relaxing prostatic smooth muscles are well-evidenced. PDEs degrade cAMP, thereby reducing cAMP-mediated smooth muscle relaxation. Multiple organ bath experiments based on human prostatic smooth muscle have demonstrated the relaxation-promoting effects of inhibitors for PDE4 and

PDE5, which hydrolyze cAMP and cGMP, respectively [91-93]. Furthermore, the approval of the PDE5 inhibitor (PDE5I) tadalafil for the clinical treatment of BPH supports the involvement of cGMP in the relaxation of human prostatic smooth muscle [91-93]. Regarding cAMP-related mechanisms, forskolin, an AC activator, can induce relaxation of human prostatic smooth muscle contractions to varying degrees, including those induced by varied adrenergic and non-adrenergic agonists [91, 94, 95].

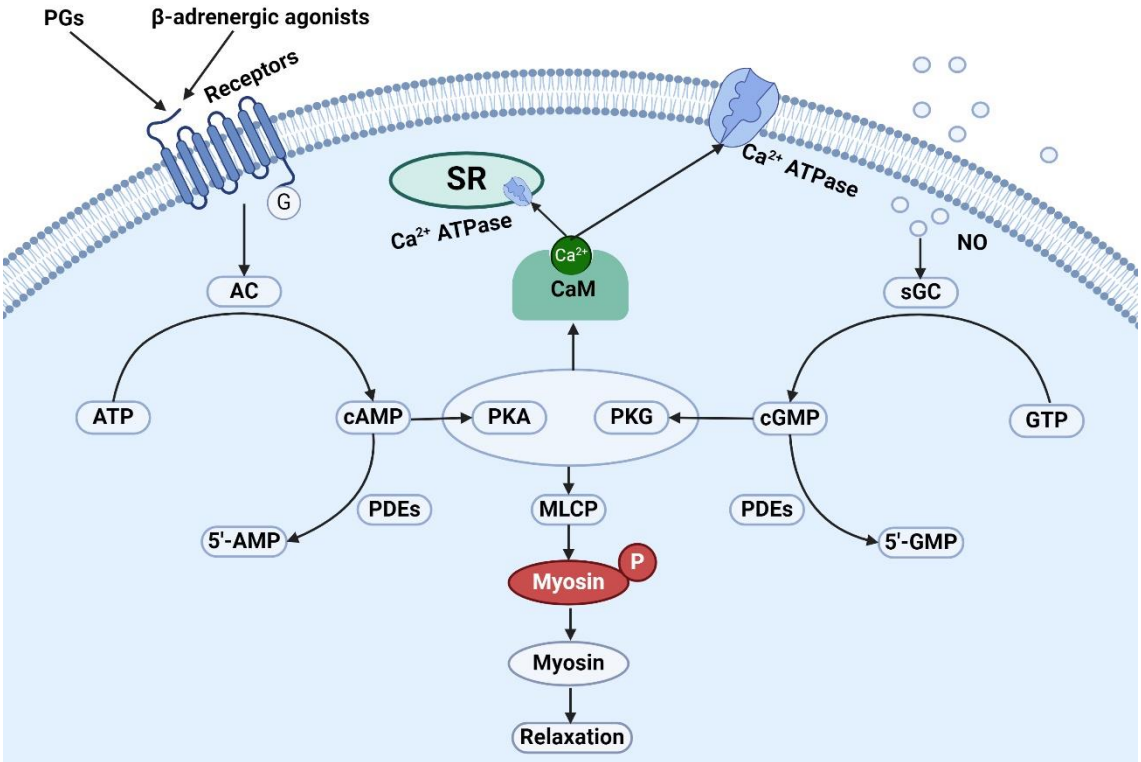

Figure 2. The process of prostate smooth muscle relaxation. The increase in cytoplasmic cAMP and cGMP mediated by β-ARs or NO promotes the dephosphorylation of MLC, thereby facilitating relaxation. (This figure is adapted from the main structure of a corresponding figure in a previous thesis of Li, B. (2021), *Effects of the integrin inhibitors BTT3033 and BOP, and the integrin-linked kinase inhibitor Cpd22 on human prostate smooth muscle contraction*, Ludwig-Maximilians-Universität München.)

2.5 Pharmacotherapy

As previously mentioned, static forces (growth) and dynamic forces (contraction) are the two fundamental mechanisms underlying BPH. Pharmaceutical inhibition of static forces requires a prolonged treatment period before clinical effects become evident, and is primarily employed to control the progression of BPH and mitigate the occurrence of complications. Conversely, inhibition of dynamic forces exhibits a rapid onset of action, facilitating the prompt alleviation of voiding symptoms. The corresponding representative drugs for these two mechanisms are α-blockers, which inhibit contraction, and 5-ARIs, which suppress growth. In addition, the PDE5I

tadalafil has been licensed as an option in BPH through the relaxation of prostate smooth muscle. Long-standing BPO can lead to secondary spontaneous detrusor contractions, which may manifest as storage symptoms. Intriguingly, despite the fact that these spontaneous detrusor contractions are neither cholinergic nor neurogenic in origin [96], both anticholinergic agents and β_3-AR agonists have demonstrated efficacy in alleviating the storage symptoms. There are also some phytotherapies that are reported to manifest effects on both voiding symptoms and the progression of BPH. The combinations of these drugs are more conducive to coping with the mixture of voiding and storage symptoms as well as the high risk of disease progression.

2.5.1 α_1-Blockers

α_1-Blockers are theoretically thought to inhibit prostatic contraction by antagonizing α_{1A}-ARs, which induce contraction in the prostate, thereby reducing resistance in the prostatic urethra. However, other mechanisms might also be involved, as their effects on BOO assessed urodynamically are evidentially very limited, and improvement in symptoms is not closely related to obstruction [97]. Despite these observations, their practical efficacy makes them a first-line treatment for BPH [15]. Currently, the main α_1-blockers include five agents (Silodosin, Tamsulosin, Terazosin, Doxazosin and Alfuzosin), with silodosin exhibits the highest degree of selectivity for the α_{1A}-AR. In clinical trials, these agents have shown similar efficacy levels based on International Prostate Symptom Score (IPSS, reduction of 30-50%) and maximum urinary flow rate (Qmax, increase of 20-40%) [98, 99]. They share the following characteristics: 1) rapid onset of action: effective within hours to days, with full effects achieved within a few weeks [100]; 2) initial symptom severity and age-independent efficacy: the patients' age does not affect the drugs' efficacy and improvements do not depend on severity of various initial symptoms [100]; 3) prostate size effects on efficacy: efficacy is not influenced by prostate size during the first year of use, but in long-term treatment, smaller prostates (less than 40 ml) respond better than larger prostates [101-104]; 4) sustained effect duration: the effect in symptoms improvement lasts for at least four years or more [99]; 5) no reduction in prostate size or risk of acute urinary retention (AUR): long-term use does not shrink the prostate or decrease the risk of AUR [101-103, 105].

The administration of α-blockers is frequently associated with a range of adverse effects, including asthenia, dizziness, and hypotension. Notably, silodosin exhibits a lower incidence of hypotension compared to other α-blockers, owing to its high selectivity for α_{1A}-ARs [106]. Conversely, silodosin exhibits the highest incidence of abnormal ejaculation [107]. It was previously hypothesized that the ejaculatory dysfunction associated with α-blocker use was attributed to retrograde ejaculation, resulting from decreased contraction of the bladder neck and proximal urethra of the prostate. However, studies have demonstrated that tamsulosin administration significantly increases the incidence of reduced ejaculated volume during orgasm, when compared to control groups, yet sperm were rarely detected in midstream urine post-ejaculation [108]. These findings suggest that α-blocker-induced ejaculatory abnormalities are more likely attributable to a reduction in semen volume per se, rather than retrograde ejaculation [3]. Furthermore, α-blockers have been shown to increase the risk of intraoperative floppy iris syndrome (IFIS), with tamsulosin being involved

in a higher incidence of this complication [109]. Therefore, it is recommended that patients scheduled for ophthalmic surgery discontinue α-blocker therapy and inform their ophthalmologist of their medication history to minimize the risk of IFIS.

2.5.2 5-ARIs

5-ARIs function by inhibiting the activity of the enzyme 5α-reductase in the prostate, leading to diminished synthesis of DHT. As BPH is androgen-dependent, this reduction in DHT ultimately induces apoptosis in glandular epithelium, resulting in a decrease in prostate volume. The principal agents in this pharmacological class are dutasteride and finasteride. Dutasteride exerts an inhibitory effect on both isoforms of 5α-reductase, with type 1 (predominantly in the glandular epithelium of the prostate and skin, with lesser amounts in the prostatic stroma) and type 2 5α-reductase (mainly in the prostatic stroma) [110], and finasteride primarily inhibits type 2 5α-reductase [111]. Interestingly, despite dutasteride's ability to inhibit both isoenzymes, it does not show superior efficacy to finasteride in the treatment of BPH [99].

5-ARIs can reduce IPSS by 10-30%, diminish prostate volume by 18-28%, and increase Qmax by 1.5-2.0 ml/s [15]. For patients treated for up to four years, they can reduce the risk of AUR by 57-68% and lower the necessity for surgery by 55-64% [15]. The diminution in prostate volume affected by luminal epithelial-focused 5α-RIs is reversible upon cessation of the treatment [112]. The therapeutic profile of 5-ARIs encompasses: 1) delayed onset of action: the clinical effects of 5-ARIs are typically observed after 6-12 months of treatment [99]; 2) prostate volume dependence: 5-ARIs may be less effective in individuals with smaller prostate volumes (less than 40 ml) [58]; 3) broad therapeutic efficacy: 5-ARIs not only improve voiding symptoms but also reduce prostate volume, thereby lowering the risk of AUR and the need for surgical intervention.

The adverse effects of 5-ARIs are mainly related to reduced DHT levels, including decreased libido and erectile dysfunction. Less common are ejaculatory abnormalities (such as retrograde ejaculation, decreased semen volume, or azoospermia) and gynecomastia. It is important to note the increased incidence of high-grade prostate cancer in patients treated with 5-ARIs, although a causal relationship has not been established [113]. Additionally, since 5-ARIs can lower PSA levels, any increase in PSA during treatment warrants screening for prostate cancer risk [15].

2.5.3 PDE5I

PDE5Is are conventionally believed to induce the relaxation of prostate smooth muscle through augmentation of intracellular cGMP levels. However, this hypothesis encounters skepticism as they do not consistently enhance the Qmax when administered as monotherapy [3]. Moreover, research suggests that these inhibitors may exert their effects on urinary function through the modulation of spinal reflex pathways [114]. Among the available PDE5Is, tadalafil is currently the only one approved for the treatment of BPH [15]. A meta-analysis showed that tadalafil monotherapy significantly improved International Index of Erectile Function scores by 5.5 points

and decreased IPSS by 2.8 points, with no significant change in Qmax [115]. Interestingly, when tadalafil was combined with α-blockers, a significant improvement in Qmax (1.5 ml/s) was observed compared to α-blocker monotherapy [115]. Another meta-analysis revealed comparable efficacies between tadalafil and tamsulosin with respect to IPSS, QOL, Qmax, and PVR [116]. Adverse events commonly associated with tadalafil usage encompass headaches, nasal congestion, backache, erythema, gastroesophageal reflux disease, and indigestion. Its prescription is cautiously avoided in patients taking nitrates or GC stimulators, those with unmanaged hypertension or recent cerebrovascular incidents, and those who have encountered vision impairment attributed to PDE5Is [15].

2.5.4 Plant extracts and phytotherapeutics

The multifaceted nature of plant extracts, characterized by their diverse chemical constituents, presents a significant challenge in identifying specific molecular targets for their therapeutic effects. While precise mechanisms of action remain under investigation, existing evidence suggests that plant extracts primarily exert their effects through pathways associated with inflammation, sex hormone modulation, and regulation of smooth muscle contraction [3].

The therapeutic potential of plant extracts for the management of LUTS suggestive of BPH has garnered significant attention in contemporary research, with numerous studies exploring various phytotherapies. However, a critical challenge arises from the substantial variability in the active ingredients and their concentrations within extracts derived from the same plant species. This variability stems from the diverse extraction methodologies employed by different manufacturers, resulting in inconsistent efficacy levels across products from distinct sources [117]. Notably, even batches produced by the same manufacturer may exhibit variations in the concentration of active components, thereby hindering the precise analysis of the pharmacokinetic properties of specific plant extracts [118]. This inherent variability further complicates the conduct of large-scale clinical trials to assess safety and efficacy.

Currently, the only plant extract recommended by the European Association of Urology (EAU) for the clinical treatment of LUTS suggestive of BPH is hexane-extracted saw palmetto (HESR) [15]. A meta-analysis has demonstrated that HESR effectively reduces nocturia and improves Qmax, when compared to the placebo [119]. Furthermore, it revealed comparable efficacy in alleviating LUTS, when compared to α-blockers and short-term finasteride use [119]. Notably, HESR exhibits favorable tolerability and minimal adverse effects on sexual function [119]. These findings are corroborated by another meta-analysis, further solidifying the therapeutic potential of HESR in the management of BPH [120].

2.5.5 Muscarinic receptor antagonists

Muscarinic receptor antagonists, widely known as antimuscarinic or anticholinergic drugs, offer therapeutic potential in alleviating storage symptoms by antagonizing cholinergic

neurotransmission in the detrusor muscle of the bladder. These agents do not exhibit a significant amelioration of voiding symptoms correlated with BPH, despite the presence of muscarinic receptors in modest concentrations within the prostatic smooth muscle [99]. Their primary action of inhibiting bladder contraction potentially contributes to reduced urinary expulsion force, raising concerns about their potential to exacerbate voiding difficulties. In a theoretical framework, anticholinergic medications are posited to inhibit detrusor muscle contractions, thereby diminishing the expulsive force of micturition, which may have the unintended consequence of exacerbating existing voiding difficulties. While empirical data indicates an increase in PVR [121], the extent to which muscarinic receptor antagonists contribute to the risk of AUR remains an ambiguous aspect of their pharmacological profile [15].

2.5.6 β_3-AR agonists

β_3-AR agonists are hypothesized to ameliorate storage symptoms through the relaxation of detrusors [3]. Currently, mirabegron stands as the sole β_3-AR agonist authorized for the treatment of male LUTS in Europe, demonstrating efficacy in reducing urinary frequency, urgency, and urge incontinence [15]. When administered as a monotherapy, mirabegron does not provide a significant benefit in the relief of voiding symptoms and does not appear to exert a substantial effect on the nonspontaneous detrusor contractions [122].

2.5.7 Combination therapies

Contemporary clinical guidelines recommend a dual pharmacotherapy approach in the management of BPH, with no recommendation for the application of triple or higher-order drug regimens. The most prevalent pharmacological strategy combines α-blocker with 5-ARIs [15]. Additional therapeutic pairings, such as α-blocker with muscarinic receptor antagonists, α-blocker with β_3-AR agonists, PDE5I (tadalafil) with α-blocker, and tadalafil with finasteride, have been conditionally suggested based on limited clinical evidence [123]. In the context of non-neurogenic LUTS, utilization of a combined treatment regimen involving muscarinic receptor antagonists and β_3-AR agonists has been reported as well, but needs further investigation [124].

The combination of α-blockers and 5-ARIs is generally deemed appropriate for individuals manifesting a prostate volume exceeding 40 ml, elevated PSA levels, older age, and a disposition towards adherence with long-term pharmacotherapy (exceeding 12 months). Within the initial year, this combination therapy does not demonstrate superior enhancement in the IPSS and Qmax relative to monotherapy; nevertheless, the benefits become more discernible in the period extending from the second to the sixth year [125]. Although this regimen is capable of ameliorating voiding symptoms and decelerating disease progression at once, it concomitantly augments the cumulative adverse effects associated with both medications [15].

In cases of moderate to severe LUTS, particularly those manifest during the storage phase, the concomitant administration of a muscarinic receptor antagonist or a β_3-AR agonist alongside an

α-blocker may demonstrate more effectiveness compared with α-blocker monotherapy in patients with mixed LUTS, i.e. showing voiding and storage symptoms simultaneously. While both combination therapies exhibit comparable improvements in symptoms, further extensive studies are requisite to ascertain whether such improvement is attributable to the combined therapeutic approach or to the α-blocker alone [123].

Regarding the conjunctive use of tadalafil and an α-blocker, a study has discerned a marginal difference in efficacy when contrasted with silodosin monotherapy; however, this marginal benefit is of constrained clinical import [123, 126]. The dual prescription of tadalafil and 5-ARIs is subject to scrutiny as it may represent an instance of cascading prescriptions and a low-value treatment strategy, which could potentially intensify concerns surrounding polypharmacy [127].

Although the combination of muscarinic receptor antagonists and β_3-AR agonists is regarded as more effective in the amelioration of both subjective and objective parameters of LUTS than monotherapy, this dual-drug approach does not address the foundational cause of urethral resistance. Moreover, the prevailing studies predominantly include female participants, thus diminishing the applicability of these findings to the male demographic afflicted with BPH suggestive of LUTS [127].

2.5.8 Challenges of current medications

A paramount challenge in the pharmacological management of BPH lies in the suboptimal adherence to prescribed medication and high rates of treatment discontinuation. The primary catalyst for this lack of adherence and subsequent self-discontinuation is the disproportion between adverse drug reactions and the limited efficacy of therapeutic outcomes [128, 129]. Furthermore, the escalating concern of polypharmacy constitutes another significant factor influencing medication adherence [130]. The cessation of pharmacological intervention precipitates the progression of the disease, potentially leading to AUR and upper urinary tract complications such as hydronephrosis, thereby increasing the necessity for hospitalization and surgical procedures [15].

Among first-line treatments for BPH, α-blockers exhibit a discontinuation rate as high as 65% within the inaugural year of treatment [128, 129, 131]. The rate of discontinuation for combination therapies involving α-blockers and 5-ARIs is even more pronounced, and may reach up to 90% [128, 129]. The prevalence of BPH in the middle-aged and elderly male population further compounds the issue of polypharmacy, a concern that is accentuated by the fact that over one-quarter of the global population is affected by multiple chronic conditions, a statistic that is rising alongside an ageing demographic [130]. In the broader context of chronic disease management, medication adherence is a pervasive challenge: for example, discontinuation rates for antihypertensive medications among patients with hypertension range from 43% to 65.5% [132]. The cumulative side effects of drugs, enhanced healthcare expenditures, and a diminished QOL

are all factors that negatively impact the adherence of patients who are navigating the complexities of polypharmacy [130].

The therapeutic effectiveness of current α-blockers, inclusive of those with high selectivity, is deemed comparable and constrained. This evaluation is underscored by two principal observations [3]: 1) the magnitude of their effect does not substantially exceed that of placebo; 2) a considerable proportion of patients exhibit a non-responsive disposition to α-blockers. It has been documented that α-blockers can enhance the IPSS and the Qmax by up to 50% and 40%, respectively. Nonetheless, placebos have been shown to reduce the IPSS by a minimum of 30% and to augment the Qmax by at least 15% [97, 99, 133-135]. Approximately 30-35% of patients experience less than a 25% diminution in IPSS in response to α-blockers [125, 131, 134, 136].

The modest efficacy and substantial non-response rate associated with α-blockers may be attributable to non-adrenergic mediators, such as endothelin-1 and TXA_2, which are implicated in mediating contraction. Organ bath studies indicate that the peak contraction of the prostate elicited by endothelin-1 is akin to that induced by adrenergic agonists (e.g., norepinephrine and phenylephrine) as well as neurogenic contractions induced by electric field stimulation (EFS). Furthermore, the maximal contraction prompted by TXA_2 analogs attains at least one-third of the maximal contraction elicited by the previously mentioned stimuli [23]. Notably, the maximal contractile responses mediated by these non-adrenergic mediators seem to occur independently of α_{1A}-AR activation, and their resultant maximal tension is complementary rather than additive with α_1-adrenergic contractions [23]. From these data, it can be deduced that contractile responses mediated by non-ARs may sustain the tension within the prostate, trigone, and urethra, notwithstanding the inhibition of α-ARs [23], which may elucidate the limited therapeutic efficacy of α-blockers. Additionally, the adverse effect of hypotension associated with these blockers could undermine adherence to BPH pharmacotherapy [15], where first-line treatments for hypertension are typically based on vasodilators [137].

To overcome the previously mentioned challenges, it would be prudent to investigate new chemical entities possessing the following dual properties: 1) the ability to inhibit both adrenergic and non-adrenergic prostatic contractions; 2) the capacity to suppress both the contraction and growth of the prostate. Although current treatments for BPH typically address prostatic contraction and growth separately, prostatic smooth muscle contraction and growth often share regulatory factors such as GTPases and other kinases. Therefore, inhibiting these regulatory factors could potentially suppress both contraction and growth simultaneously.

2.6 Novel potential pharmacotherapy

Novel candidate compounds can be categorized based on their functional properties as follows: 1) inhibitors of prostatic contraction, 2) inhibitors of prostatic growth, 3) agents that demonstrate an improvement in LUTS *in vivo*. Depending on their targets, these compounds can be further

classified into: 1) TXA_2 receptor antagonists and TXA_2 synthase inhibitors; 2) inhibitors of monomeric GTPases; 3) kinase inhibitors; and 4) other targets or molecules.

2.6.1 TXA_2 receptor antagonists and TXA_2 synthase inhibitors

Picotamide was initially reported as a competitive antagonist of TXA_2 receptors and an inhibitor of thromboxane synthase [138]. Subsequent studies revealed that picotamide could irreversibly antagonize TXA_2 receptors and inhibit effects induced by other receptors [139, 140]. In fact, picotamide can comprehensively inhibit contractions of human prostatic tissue, including those induced by U46619 (synthetic analog of the endoperoxide prostaglandin PGH_2) and the three isoforms of endothelin, neurogenic contractions, and α_1-adrenergic contractions [141]. It also inhibits contractions of human trigone tissue, including neurogenic contractions and those induced by U46619, phenylephrine, and carbachol [142]. During normal urination, relaxation of the trigone facilitates the opening of the bladder outlet, promoting urine expulsion [143]. Therefore, compared to α_1-blockers, picotamide not only inhibits adrenergic contractions but also non-adrenergic contractions, making it a promising candidate for BPH treatment. Furthermore, since picotamide has already been approved in Italy for the clinical treatment of arterial thromboembolic diseases [144], its established safety and tolerability further support its potential use in BPH therapy.

2.6.2 Monomeric GTPase inhibitors

The role of GTPases, particularly RhoA, in promoting contraction has been extensively studied across nearly all types of smooth muscle tissues. Recently, additional non-RhoA GTPases have been identified as playing roles in prostatic contraction and proliferation, such as Rac GTPase and ADP-ribosylation factor 6 (ARF6) [3]. Inhibitors of Rac GTPase (NSC23766 and EHT1864) and ARF6 (NAV2729) exhibit differential inhibitory sensitivities to neurogenic, adrenergic, and non-adrenergic prostatic contractions [145-147]. SecinH3, a assumed inhibitor of Cytohesin2, a guanine nucleotide exchange factor for ARF6, demonstrate to inhibit all three types of contractions [148]. Beyond inhibiting contraction, NSC23766, EHT1864, and NAV2729 also suppress the proliferation of prostatic stromal cells [145, 146]. Silencing ARF6 produce similar antiproliferative effects as its inhibitor NAV2729 [149]. Thus, targeting GTPases holds the potential for simultaneously addressing dysregulated contraction and growth of the prostate, mitigating the cumulative adverse effects and high discontinuation rates associated with current combination drug therapy regimens.

2.6.3 Kinase inhibitors

Kinases are a popular target in preclinical BPH research due to their diverse roles in signal transduction, metabolism, proliferation, and apoptosis. Common target kinases include polo-like kinase (PLK), c-Jun N-terminal kinase (JNK), serine/threonine kinase 16 (STK), G protein-

coupled receptor kinase 2/3 (GRK2/3), focal adhesion-regulating kinases [focal adhesion kinase (FAK),non-receptor Proto-oncogene tyrosine-protein kinase Src (Src), LIM domain kinase (LIMK)], integrin-linked kinase (ILK) and p21-activated kinase (PAK) [3].

Among the tested supposed PLK inhibitors, onvansertib inhibited neurogenic, adrenergic, endothelin-1 and ATP-induced contractions, with the size of inhibition on neurogenic contractions comparable to that of silodosin and tamsulosin [89]. Additionally, it showed a certain inhibitory effect on the proliferation of cultured prostate stromal cells [89]. In the tested supposed JNK inhibitors, SP600125 demonstrated inhibition of both neurogenic and adrenergic contractions [150]. The STK16 inhibitor STK16-IN-1 appears to have limited translational value, as it only inhibited non-adrenergic contractions [151]. While GRK may phosphorylate GPCRs, leading to their desensitization and inactivation, the G protein-coupled receptor kinase 2/3 inhibitor CMPD101 unexpectedly exhibited inhibitory effects on human prostate smooth muscle contraction [152]. CMPD101 inhibited neurogenic, adrenergic, endothelin, and U46619-induced contractions, although the mechanism remains unclear, with off-target effects on RhoA and MLCK ruled out [152]. Smooth muscle force generation relies on the connection of actin filaments to the cell membrane and adhesion to the extracellular matrix via focal adhesions. These protein complexes, including integrins, are regulated by multiple kinases such as FAK, Src, and LIMK [3]. The assumed LIMK inhibitors SR7826 and LIMKi3 inhibited neurogenic, adrenergic, and U46619-induced contractions [153]. The assumed FAK inhibitors PF-573228 and Y-11 inhibited neurogenic and adrenergic contractions [154]. The supposed Src family kinase inhibitors AZM475271 and PP2 inhibited neurogenic and adrenergic contractions, with only PP2 also inhibiting U46619-induced contraction, and neither affecting endothelin-1-induced contraction [155, 156]. Additionally, the ILK inhibitor Cpd 22 inhibited neurogenic and U46619-induced contractions but had negligible effects on adrenergic and endothelin-induced contractions [157]. The supposed PAK inhibitors FRAX486 and IPA3 inhibited neurogenic and U46619-induced contractions, with FRAX486 also inhibiting endothelin-induced contractions, while neither inhibited adrenergic contractions [156, 158].

2.6.4 Other targets or molecules

Other drug targets or potential therapeutic compounds for BPH include: 1) inhibiting contraction by integrin antagonists, Botulinum toxin A, and oxytocin antagonists; 2) inhibiting growth by vitamin D3 analogs, NX-1207 (fexapotide triflutate), lonidamine, growth hormone-releasing hormone (GHRH), luteinizing hormone-releasing hormone (LHRH) antagonists (degarelix, teverelix, and cetrorelix), PRX302 (topsalysin), GV1001 (tertomotide), afala and progestogen analog (chlormadinone acetate); 3) affecting both contraction and growth by ghrelin; 4) primarily inhibiting inflammatory responses by nonsteroidal anti-inflammatory drugs (NSAIDs) [3, 159, 160].

The integrin $\alpha_2\beta_1$ blocker BTT-3033 inhibited neurogenic and U46619-induced prostate tissue contraction, while another inhibitor, BOP, had no effect on contraction [157]. Botulinum toxin A demonstrated improvements in Qmax and IPSS in three preliminary studies [161-163], but

subsequent placebo-controlled trials found no significant difference compared to placebo [164]. The long-acting oxytocin antagonist cligosiban not only eliminated oxytocin-induced prostate tissue contraction but also exhibited intrinsic relaxation properties in prostate tissue [160].

The vitamin D analog BXL-628 and the receptor agonist CH5036249 both inhibited prostate volume increase in rats and dogs [165, 166]. BXL-628 further demonstrated a significant effect in reducing prostate volume in a placebo-controlled clinical trial [167]. NX-1207, a potential pro-apoptotic peptide compound, has shown improvement in LUTS in clinical trials [168]. Lonidamine, due to its potential role in interfering with energy metabolism and promoting apoptosis, has shown improvement in IPSS, PVR and prostate volume in clinical trials [169, 170]. Based on the role of GHRH in promoting epithelial cell proliferation and inflammatory pathways, GHRH receptor blockers have reduced prostate weight and pro-inflammatory factor expression in BPH mice [171]. LHRH antagonists, thought to inhibit prostate growth by promoting programmed-cell death or inhibiting the plasminogen activation system, include degarelix, teverelix, and cetrorelix, with cetrorelix being the most extensively studied [159]. However, the therapeutic efficacy of cetrorelix is also controversial; while several clinical trials indicated improvements in IPSS and Qmax, two phase III clinical trials showed no significant difference in IPSS compared to placebo [159]. PRX302, a PSA-modified recombinant protein causing membrane pore formation and localized apoptosis [159], demonstrated good tolerance and decreased IPSS by 8-10 points in phase I and II trials [172]. In its phase III clinical trial, PRX302 showed an improvement in IPSS that was 7.6 points higher than the placebo group [159]. In a phase III clinical trial, Afala, an anti-PSA antibody, demonstrated a marginal reduction of 0.8 points in the IPSS in comparison to placebo cohort and incremented the Qmax by 1.1 ml/s beyond the placebo group’s results [173]. GV1001, a telomerase-targeting vaccine, revealed improvements in IPSS and prostate volume compared to placebo in clinical trials [174]. Chlormadinone acetate, a synthetic progestogen analog, exerts its effects through anti-androgenic properties, demonstrating efficacy in improving the IPSS, Qmax, and concurrently reducing prostate volume within clinical practice settings [175, 176]. However, it is important to note that the limited effectiveness of progesterones in the management of BPH was documented in the early 1970s, resulting in the cessation of their application in the treatment of BPH [177].

Ghrelin, a hormone regulating metabolic processes, has been observed to induce an increment in rat prostate volume. Furthermore, the administration of ghrelin agonists has been shown to augment adrenergic prostate tissue contraction in *ex vivo* studies. These findings suggest that compounds designed to block ghrelin signaling may concurrently inhibit prostate hyperplasia and contraction [178]. NSAIDs, while improving IPSS by 2.89 points, decreased Qmax by 0.89 ml/s [179].

2.7 Hypothesis and objective of this thesis

The limited efficacy of α-blockers, currently regarded as the first-line treatment for BPH, is paralleled by the insufficient understanding of ARs function, regulation, and intracellular signaling mediated by them [24]. In the human prostate, α_{1A}-AR, α_{1B}-AR, and three distinct subtypes of β-ARs are expressed, with α-ARs predominantly represented by the α_{1A} subtype and β-ARs predominantly by the β_2 subtype [180]. Historically, α_1-ARs were often considered isolated and static from other ARs, but subsequent experiments have challenged this view. For instance, activation of α_1-ARs can induce phosphorylation of β_2-ARs [181]. Furthermore, α_1-ARs not only couple with G proteins but can also bind to β-arrestin-2 [181], and upon noradrenaline activation, bind to clathrin light chain A [141]. These interactions with various binding partners, partly shared by different subtypes of ARs may mediate different pathways and specific functions [24]. Thus, despite the long-standing and widespread use of α-blockers in the treatment of BPH, further research is required to understand the function and mutual regulation of ARs.

As previously discussed in paragraph 2.5.8, non-AR-mediated prostate contraction compensates for and maintains smooth muscle contraction after α_{1A}-AR blockade, which may account for the limited efficacy of current α_1-blockers [23]. TXA_2, an important component of non-AR-mediated contraction, induces contraction through shared intracellular signaling pathways mediated by α_1-ARs, such as Rho kinase and calcium-dependent mechanisms [182]. The presence of TXA_2 and its receptors in human prostate tissue has also been confirmed [182]. Furthermore, TXA_2 synthase inhibitors and TXA_2 receptor blocker picotamide simultaneously inhibit both adrenergic and TXA_2-induced contraction in human prostate tissue [141]. In vascular smooth muscle, activation of α_1-ARs can further activate PLA_2, leading to the production of TXA_2, which contributed to α_1-AR-mediated contraction [183]. Theoretically, PLA_2 can release arachidonic acid and leukotrienes by catalyzing phospholipids on the cell membrane [184]. Subsequently, arachidonic acid, under the action of cyclooxygenase (COX), generates prostaglandin G2 which is then converted to TXA_2 by thromboxane synthase (TXS) [184]. Therefore, based on picotamides' inhibition of adrenergic contraction, it is speculated that activation of α_1-ARs in human prostate can activate PLA_2-COX-TXS pathway, thereby affecting intracellular TXA_2 generation in prostatic smooth muscle (as illustrated in Figure 3).

Ordinarily, both α_1 and β_2-ARs in the prostate are tethered to G proteins, facilitating signal transduction. Nevertheless, the activation of α_{1A}-AR can instigate a cascade that diverges from this conventional pathway. Specifically, the stimulated α_{1A}-AR can proceed to activate G protein-coupled receptor kinase 2, which then phosphorylates β_2-ARs [181]. This phosphorylation event enables the recruitment of β-arrestin to the β_2-ARs, thereby obstructing the reassociation of G proteins or supplanting them altogether [24, 181, 185]. Furthermore, β-arrestin possesses the capability to activate alternative signaling pathways independently of G proteins. For instance, β-arrestin may engage in the activation of extracellular signal-regulated kinase or p38 mitogen-activated protein kinase pathways thereby contributing to cell growth [24].

Carvedilol, as an unselective β-blocker, can directly bind to β-ARs and activate β-arrestin, potentially participating in the regulation of prostate growth (as illustrated in Figure 3) [186, 187]. Furthermore, as a clinical antihypertensive medication, carvedilol may exert an inhibitory effect on prostate contraction by potentially blocking α_{1A}-AR. This hypothesis may be evidenced by the following (as illustrated in Figure 3): 1) Carvedilol has Ki values for β1-ARs primarily located in the heart, α_{1B} or α_{1D} predominantly distributed in blood vessels, and α_{1A} primarily found in the prostate, which are notably close for all subtypes, and which are lower than the plasma concentration observed at standard doses; 2) β-AR antagonists have been documented to block α1-ARs as an off-target, at least outside the prostate [188, 189]. Consequently, carvedilol may simultaneously interact with these various ARs to confer protective effects on the cardiovascular system and enhance voiding symptoms. In addition, metabolic syndrome components like hypertension, has been demonstrated to significantly contribute to the progression of BPH in several clinical or preclinical studies [190, 191]. Given that BPH and hypertension are prevalent conditions among the elderly, often coexisting and necessitating multiple drug treatments, the ensuing polypharmacy issue may diminish medication adherence for BPH treatment. Carvedilol, as a clinically utilized antihypertensive medication, in conjunction with its potential effects on prostate contraction and growth, may provide a dual-condition management strategy, thereby alleviating the polypharmacy issue to a certain extent and addressing a portion of the challenges posed by current medications.

Considering the limited efficacy of α-blockers, coupled with the incomplete understanding of adrenergic receptor (AR) functionalities and the urgent need to address polypharmacy in the elderly population, this thesis aims to investigate the following:

1) The potential relationship between the activation of α_{1A}-ARs and TXA_2 production by PLA_2, by assessing effects of PLA_2 inhibitors on contractions of human prostate tissues.
2) The feasibility of carvedilol in blocking α_{1A}-AR-induced contractions of human prostate tissues.
3) The impact of carvedilol on growth of human prostate cells.

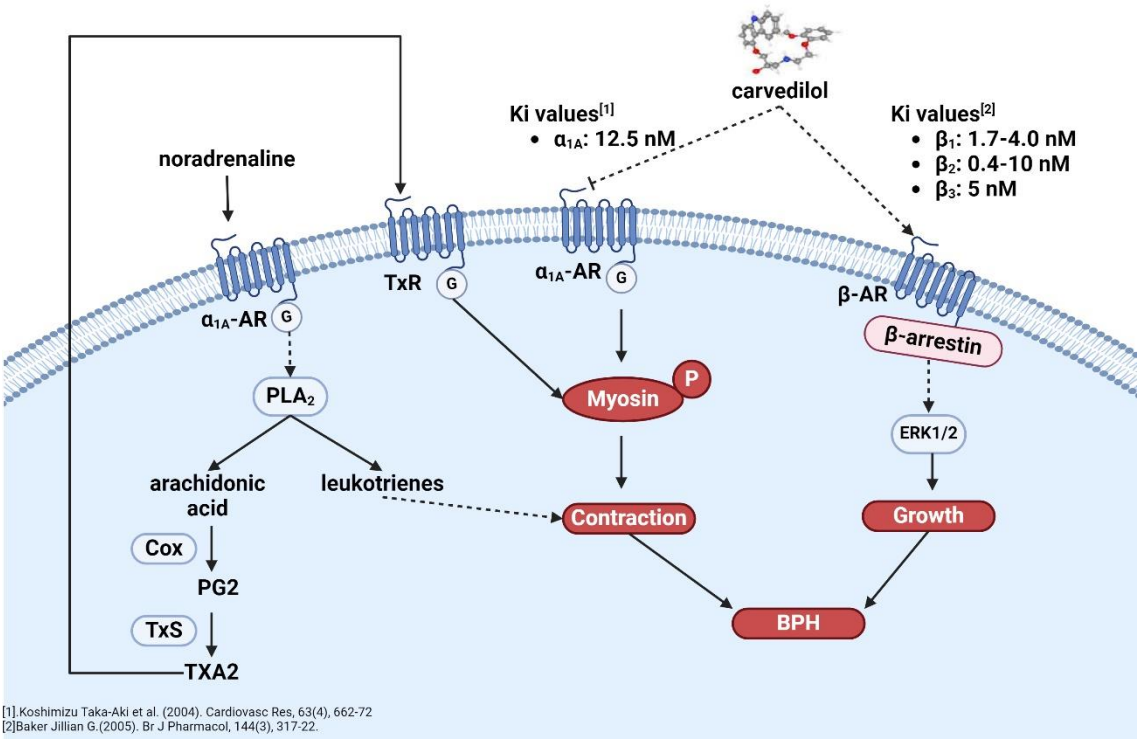

Figure 3. The hypotheses of this dissertation are as follows: 1) activated α_1-ARs may initiate the PLA_2-COX-TXS pathway, thereby promoting the production of TXA_2 within human prostate smooth muscle cells; 2) α and β-ARs in human prostate smooth muscle may be antagonized or activated by carvedilol, leading to the regulation of both contraction and growth.

3. Summary

The contraction induced by non-adrenergic agonists can counteract and impair the inhibitory effect on prostatic contraction and thus, the clinical benefits exerted by α-blockers. TXA_2, as one of these non-adrenergic agonists, can induce approximately one-third of the maximal prostate contraction, thus partially compensating for the contraction inhibited by α-blockers. The source of TXA_2 in the human prostate is not clear. The inhibitor of TXA_2 synthesis and TXA_2 receptor blocker, picotamide, can simultaneously inhibit TXA_2 and α_1-AR-induced contraction of prostate and vascular smooth muscle, suggesting that PLA_2, a key enzyme in TXA_2 synthesis, may be activated by α_1-ARs in the prostate. Therefore, the first study explored the effects of PLA_2 inhibitors on neurogenic and adrenergic contractions of human prostate by applying cytosolic PLA_2 inhibitors (1 μM ASB14780 and 10 μM AACOCF3), secretory PLA_2 inhibitor (3 μM YM26734), and leukotriene receptor antagonists (0.3 and 1 μM Montelukast).

The mechanisms of contraction and relaxation initiated by α_{1A}- and β_2-ARs are considered the primary regulatory components of the human prostate tone. Carvedilol, as a clinically used non-selective β-blocker, may exert blocking effects on α_{1A}-ARs distributed in the prostate with current standard dose. Additionally, carvedilol can also act as a “biased agonist” by binding to β-ARs, thereby activating the β-arrestin pathway, potentially participating in the regulation of prostate growth. BPH and hypertension are frequently co-morbid in the elderly male population requiring multiple medications to manage both conditions. However, the use of a single drug to manage both diseases simultaneously has rarely been studied. Therefore, the second study systematically explored the effects of different concentrations of carvedilol on prostate contraction and growth.

The results of the first study in this thesis indicate that PLA_2 primarily acts on presynaptic transmission in human prostate smooth muscle, thereby modulating contraction. Both cytosolic PLA_2 inhibitors, ASB14780 and AACOCF3, demonstrated inhibitory effects on neurogenic human prostate contraction, while showing no significant impact on contractions induced by different adrenergic agonists. The lack of effects observed with the leukotriene receptor antagonist montelukast suggests the absence of PLA_2-derived leukotrienes involved in the pre-synaptic neural regulation of prostate smooth muscle contraction. In summary, cytosolic PLA_2 may affect prostate contraction through involvement in the transport of presynaptic vesicles and fusion with the cell membrane or synthesis of neurotransmitters, while a mechanism involving the activation of α_1-ARs on the postsynaptic membrane leading to TXA_2 synthesis and contraction seems less likely.

The results of the second study in this thesis demonstrate that carvedilol inhibits prostate smooth muscle contraction from plasma concentrations (115 to 315 nM) onwards, with its maximum inhibitory effect reaching that of current α-blockers used for BPH. Additionally, carvedilol exhibits time-dependent and bidirectional regulatory effects on growth-related functions in the prostate. In this study, carvedilol shifted the concentration-response curve to the right from 100 nM onwards, accompanied by increased EC_{50} values (for agonist) and unchanged E_{max} values (for agonist),

and inhibited neurogenic contraction starting from 10 nM. The inhibitory effects on adrenergic and neurogenic contractions at 10 µM were similar to those achieved with tamsulosin and silodosin under the same conditions, with no inhibitory effect on non-adrenergic contractions. At nanomolar concentrations, carvedilol increased the proliferation of stromal cells, while higher concentrations showed the opposite effect. Prolonged exposure at the same concentration reduced the viability of prostate stromal cells. Although decreased viability of prostate cells may be beneficial in BPH, cytotoxic effects are generally critical. In BPH, and if restricted to prostate cells, both could essentially lead to a beneficial reduction in prostate size. However, concentrations of 10 µM and systemic toxic effects can be excluded in vivo, as maximum plasma concentrations mount to 315 nM. With approved standard dose, carvedilol is safe and clinically applied for treatment of cardiac failure and hypertension, the latter being a common comorbidity of BPH. Overall, carvedilol antagonizes human prostate α_{1A}-ARs within the plasma concentration, with a maximum effect size comparable to that of clinically used α_1-blockers for treating voiding symptoms. The concentration-dependent bidirectional effects of carvedilol on stromal cell growth may be exerted by antagonism or by "biased agonism", which may provisionally, but not necessarily affect prostate size in patients with BPH.

Together, α-AR-mediated prostatic smooth muscle contraction does not depend on PLA_2 or PLA_2-derived leukotrienes as PLA_2 regulates prostatic smooth muscle contraction by acting at the presynaptic level. Carvedilol, as a clinical antihypertensive medication, can antagonize α_1 adrenergic prostatic contraction at plasma concentrations, although achieving the effect size of α_1-blockers requires concentrations beyond those in plasma. When considered in conjunction with the concentration-dependent bidirectional growth-regulatory effects of carvedilol, these findings suggest that striking a balance between the therapeutic benefits and adverse effects of carvedilol on both cardiovascular and urological systems may provide a means to address the challenge of polypharmacy. Whether this balance can be implemented by dose titration by prescribers and in real world settings or not needs clinical trials.

4. Zusammenfassung

Die Kontraktion, die durch nicht-adrenerge Agonisten hervorgerufen wird, kann den hemmenden Effekt auf die Kontraktion der Prostata, und damit die klinischen Effekte von α-Blockern ausgleichen und beeinträchtigen. TXA_2, als einer dieser nicht-adrenergen Agonisten, kann etwa ein Drittel der maximalen Kontraktion der Prostata induzieren und kompensiert somit Kontraktions-Hemmungen durch α-Blocker. Die Quelle von TXA_2 in der menschlichen Prostata ist unklar. Der gleichzeitige TXA_2-Synthase-Hemmer und TXA_2-Rezeptorblocker Picotamid hemmt gleichzeitig die TXA_2-induzierte und die α1-adrenerge Kontraktion der Prostata- und der glatten Gefäßmuskulatur, was darauf hindeutet, dass PLA_2, ein Schlüsselenzym in der TXA_2-Synthese, durch α1-ARs in der Prostata aktiviert werden könnte. Daher untersuchte die erste hier vorgestellte Studie die Beteiligung von PLA_2 an neurogenen und adrenergen Kontraktionen der menschlichen Prostata unter Verwendung von cytosolischen PLA_2-Inhibitoren (1 µM ASB14780 und 10 µM AACOCF3), eines sekretorischen PLA_2-Inhibitors (3 µM YM26734) und eines Leukotrienrezeptorantagonisten (0,3 und 1 µM Montelukast).

Die Mechanismen der Kontraktion und Relaxation, die durch α_{1A}- und β_2-ARs initiiert werden, gelten als die primären regulatorischen Komponenten des Tonus der menschlichen Prostata. Carvedilol, ein klinisch verwendeter nicht-selektiver β-Blocker, kann möglicherweise blockierende Effekte auf α_{1A}-ARs ausüben, die in der Prostata mit der aktuellen Standarddosis auftreten könnten. Zudem kann Carvedilol auch als "biased agonist" wirken, indem es an β-ARs bindet und damit den β-Arrestin-Signalweg aktiviert, der potenziell an der Regulation des Prostatawachstums beteiligt ist. BPH und Hypertonie treten häufig gemeinsam bei älteren Menschen auf, was die gleichzeitige Einnahme mehrerer Medikamente zur Behandlung beider Erkrankungen erfordert. Allerdings wurde der Einsatz eines einzigen Medikaments zur gleichzeitigen Behandlung beider Krankheiten selten in Betracht gezogen. Daher untersuchte die zweite hier vorgestellte Studie systematisch die Auswirkungen unterschiedlicher Konzentrationen von Carvedilol auf die Kontraktion humaner Prostatagewebe und das Wachstum von Stromazellen der Prostata.

Die Ergebnisse der ersten Studie in dieser Dissertation zeigen, dass PLA_2 primär auf die präsynaptische Transmission in der glatten Muskulatur der menschlichen Prostata wirkt und dadurch die Kontraktion moduliert. Beide cytosolischen PLA_2-Inhibitoren, ASB14780 und AACOCF3, zeigten hemmende Effekte auf die neurogene Kontraktion der menschlichen Prostata, während sie keinen signifikanten Einfluss auf Kontraktionen hatten, die durch verschiedene adrenerge Agonisten induziert wurden. Das Fehlen von antikontraktilen Effekten in Experimenten mit dem Leukotrien-Rezeptorantagonisten Montelukast legt nahe, dass PLA_2-abgeleitete Leukotriene nicht an der präsynaptischen neuralen Regulation der Kontraktion der Prostata glatten Muskulatur beteiligt sind. Zusammenfassend lässt sich sagen, dass die cytosolische PLA_2 die Kontraktion der Prostata durch Beteiligung am Transport präsynaptischer Vesikel und Fusion mit der Zellmembran oder durch Synthese von Neurotransmittern beeinflussen kann, während ein Mechanismus, der die Aktivierung von α_1-ARs an der postsynaptischen Membran und die nachfolgende TXA_2-Synthese und Kontraktion involviert, weniger wahrscheinlich erscheint.

Die Ergebnisse der zweiten Studie in dieser Dissertation zeigen, dass Carvedilol die Kontraktion der glatten Muskulatur der Prostata von den bekannten Plasmakonzentrationen (115 bis 315 nM) und aufwärts hemmt, wobei seine maximale hemmende Wirkung der von den aktuell verwendeten α-Blocker für BPH entspricht. Darüber hinaus zeigt Carvedilol zeitabhängige und bidirektionale regulatorische Effekte auf wachstumsbezogene Funktionen in der Prostata. In

dieser Studie verlagerte Carvedilol die Konzentrations-Wirkungskurve ab 100 nM nach rechts, begleitet von erhöhten EC_{50}-Werten (für α_1-adrenerge Agonisten) und unveränderten E_{max}-Werten (für α_1-adrenerge Agonist), und hemmte neurogene Kontraktionen ab 10 nM. Die hemmenden Effekte auf adrenerge und neurogene Kontraktionen bei 10 µM waren ähnlich denen, die unter denselben Bedingungen mit Tamsulosin und Silodosin erreicht wurden, ohne jedoch einen hemmenden Effekt auf nicht-adrenerge Kontraktionen zu zeigen. Bei nanomolaren Konzentrationen steigerte Carvedilol die Proliferation stromaler Zellen, während höhere Konzentrationen den gegenteiligen Effekt zeigten. Langzeitexposition bei gleichen Konzentrationen reduzierte die Lebensfähigkeit der stromalen Zellen der Prostata. Obwohl die verringerte Viabilität von Prostatazellen bei BPH vorteilhaft sein kann, sind zytotoxische Effekte generell kritisch zu betrachten. Bei BPH und sofern diese Effekte auf Prostatazellen beschränkt bleiben, könnten beide im Wesentlichen zu einer wünschenswerten Verringerung der Prostatagröße führen. Konzentrationen von 10 µM und systemische toxische Effekte können jedoch in vivo ausgeschlossen werden, da die maximalen Plasmakonzentrationen 315 nM nicht übersteigen. Mit der zugelassenen Standarddosierung ist Carvedilol sicher und wird klinisch zur Behandlung von Herzinsuffizienz und Hypertonie angewendet, wobei letztere eine häufige Komorbidität von BPH darstellt. Insgesamt antagonisiert Carvedilol humane Prostata-α_{1A}-ARs innerhalb der Plasmakonzentrationen, mit einer maximalen Wirkungsstärke die vergleichbar mit der der klinisch verwendeten α_1-Blocker zur Behandlung von Miktionsbeschwerden ist. Die konzentrationsabhängigen bidirektionalen Effekte von Carvedilol auf das Wachstum der Stromazellen können entweder durch Antagonismus oder durch "biased agonism" versursacht sein, was möglicherweise, aber nicht zwingend die Prostatagröße bei Patienten mit BPH beeinflussen könnte.

Zusammenfassend hängt die durch α-AR vermittelte Kontraktion der glatten Muskulatur der Prostata nicht von PLA_2 oder von PLA_2-abgeleiteten Leukotrienen ab, da PLA_2 die Kontraktion der glatten Muskulatur der Prostata auf präsynaptischer Ebene reguliert. Carvedilol, als klinisch verwendetes Antihypertensivum, kann die α_1-adrenerge Kontraktion der Prostata bereits bei nomalen Plasmakonzentrationen antagonisieren, wobei ein maximaler Antagonismus Konzentrationen erfordert, die über denen im Plasma liegen. Zusammen mit den konzentrationsabhängigen bidirektionalen wachstumsregulierenden Effekten von Carvedilol legen diese Befunde nahe, dass ein titriertes Verhältnis zwischen therapeutischem Nutzen und den Nebenwirkungen von Carvedilol auf das kardiovaskuläre und urologische System durch Dosisanpassung eine Möglichkeit bietet, den Herausforderungen der Polypharmazie zu begegnen. Ob dieses Gleichgewicht durch Dosistitration durch verschreibende Ärzte und in realen Settings umgesetzt werden kann, muss durch klinische Studien untersucht werden.

5. Paper I

Title:	Selective inhibition of neurogenic, but not agonist-induced contractions by phospholipase A2 inhibitors points to presynaptic phospholipase A2 functions in contractile neurotransmission to human prostate smooth muscle.
Journal:	Neurourology and Urodynamics
Authors:	Sheng Hu, Ru Huang, Patrick Keller, Melanie Götz, Alexander Tamalunas, Philipp Weinhold, Raphaela Waidelich, Christian G Stief, Martin, Hennenberg.
Accepted:	29th June 2023
Doi:	10.1002/nau.25242
PMID:	37583250

6. Paper II

Title:	Effects of carvedilol on human prostate tissue contractility and stromal cell growth pointing to potential clinical implications.
Journal:	Pharmacological Reports
Authors:	Sheng Hu, A. Elif Müderrisoglu, Anna Ciotkowska, Oluwafemi Kale, Patrick Keller, Melanie Schott, Alexander Tamalunas, Raphaela Waidelich, Christian G. Stief, Martin Hennenberg.
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PMID:	38858312

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