Inhibition of smooth muscle contraction and ARF 6 activity by the inhibitor for cytohesin GEFs, secinH3 in the human prostate

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- Design, planning, and implementation of the study: CGS, CG, MH.
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- RT-PCR, Western-blot analyses, and fluorescence stainings: AH, AC, BR, YW, MH.
- Organ bath experiments: AH, PK, MS, AT, MH.
- Pull down assays: AC, BR, MH.
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Abstract

Prostate smooth muscle contraction is critical for etiology and treatment of male lower urinary tract symptoms (LUTS), and is promoted by small monomeric GTPases (RhoA, Rac). GTPases may be activated by guanosine nucleotide exchange factors (GEFs). GEFs of the cytohesin family may indirectly activate Rac, or ADP ribosylation factor (ARF) GTPases directly. Here we investigated the expression of cytohesin family GEFs, and effects of the cytohesin inhibitor secinH3 on smooth muscle contraction and GTPase activities in human prostate tissues. Of all four cytohesin isoforms, cytohesin-1 and -2 showed the highest expression in RT-PCR. Western blot and fluorescence staining suggested that cytohesin-2 may be the predominant isoform in prostate smooth muscle cells. Contractions induced by norepinephrine, the α₁-adrenoceptor agonist phenylephrine, the thromboxane A₂ analogue U46619, endothelins 1 and 3, as well as neurogenic contractions induced by electric field stimulation (EFS) were reduced by secinH3 (30 µM). Inhibition of EFS-induced contractions appeared to have similar efficacy than inhibition by the α₁-adrenoceptor antagonist tamsulosin (300 nM). Combined application of secinH3 plus tamsulosin caused larger inhibition of EFS-induced contractions than tamsulosin alone. Pull down assays demonstrated inhibition of the small monomeric GTPase ARF6 by secinH3, but no inhibition of RhoA or Rac1. In conclusion, we suggest that a cytohesin/ARF6 pathway takes part in smooth muscle contraction. This may open attractive new possibilities in medical treatment of male LUTS.

221 words
Key words

- lower urinary tract symptoms (LUTS)
- benign prostatic hyperplasia (BPH)
In the prostate, smooth muscle contraction is critically promoted by small monomeric GTPases, including RhoA and Rac (26, 63). Increased prostate smooth muscle tone may contribute to urethral obstruction in patients with benign prostatic hyperplasia (BPH), resulting in bladder outlet obstruction (BOO) and lower urinary tract symptoms (LUTS) (26). Consequently, inhibition of prostate smooth muscle contraction by α₁-adrenoceptor antagonists or other medications represents an important strategy for medical treatment of male LUTS suggestive of BPH (45). However, efficacy of available treatment options remains limited, often leading to unsatisfying results, frequent use of combination therapies, and high rates of discontinuation, hospitalization, and surgeries (18, 26, 45). Newer findings proposed that the limitations of α₁-adrenoceptor antagonists may be explained by non-adrenergic mediators, which also contribute to prostate smooth muscle tone and subsequent voiding symptoms in parallel to α₁-adrenoceptors (26). In order to overcome these difficulties, alternative options with improved efficacy are of high demand.

Intracellular signalling mechanisms mediating smooth muscle contraction in the prostate and in other organs involve the small monomeric GTPases RhoA and Rac (14, 26, 63). While the role of RhoA for mediating receptor-induced contraction has been demonstrated by various studies, Rac-dependent control of prostate smooth muscle contraction has recently become evident (14, 26, 63). Monomeric GTPases including RhoA, Rac, and others are activated by replacement of guanosine diphosphate (GDP) being bound in the inactive state, by guanosine triphosphate.
(GTP) being bound in the inactive state (11). This GDP-GTP exchange results in subsequent activation, and is accomplished by guanosine exchange factors (GEFs) (11).

Therefore, it appears very likely that GEFs are involved in the regulation of prostate smooth muscle tone. While the roles of RhoA and Rac, as well as their effector kinases have been well established for contraction of prostate smooth muscle, no data on the involvement of GEFs or about the activating mechanisms of these GTPases in the prostate are available to date (14, 26, 55, 63). Cytohesins are a group of GEFs, which exist in four different isoforms, referred to as cytohesin-1 to -4 (33, 37, 50). Apart from indirect, downstream Rac activation by cytohesin-dependent mechanisms, direct activation of ADP ribosylation factor 6 (ARF6) by cytohesin-2 has been repeatedly reported (30, 33, 50, 57). ARF6 is another small monomeric GTPase, which has been associated with actin organization and with functions depending on correct actin assembly in different studies (17). However, a possible role of cytohesins or ARF6 in smooth muscle contraction of any organ has not yet been considered. With secinH3, a cytohesin-specific small molecule inhibitor has now become available (19, 22, 23).

In this study, we hypothesized that secinH3 may interfere with smooth muscle contraction and GTPase activities in the human prostate. Therefore, we examined the expression of cytohesins, and the effects of secinH3 on smooth muscle contraction and GTPase activities in human prostate tissues.
Methods

Human prostate tissues

Human prostate tissues were obtained from patients undergoing radical prostatectomy for prostate cancer (n=95). Patients who underwent previous transurethral resection of the prostate (TURP) were excluded. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and has been approved by the ethics committee of the Ludwig-Maximilians University, Munich, Germany. Informed consent was obtained from all patients. Samples and data were collected and analyzed anonymously. Samples were taken immediately after prostatectomy, following macroscopical examination by an uropathologist. All tissues were taken from the periurethral zone, considering that most prostate cancers arise in the peripheral zone (49, 54). Upon pathologic evaluation, only tissue samples which did not exhibit histological signs of neoplasia, cancer, or inflammation were collected. BPH is present in 80-83% of patients with prostate cancer (2, 48). For macroscopic examination and sampling, the prostate was opened by a single longitudinal cut from the capsule to the urethra. Subsequently, both intersections were checked macroscopically for any obviously malignant infiltration. Because prostate cancer is usually located in the peripheral zone, tumor infiltration in the periurethral zone (where sampling was performed) was very rare (found in less than 1% of all prostates included). Prostates with signs of cancer in the periurethral zone upon macroscopic inspection were not subjected to sampling and were not included in this study. Organ bath studies were performed immediately after...
sampling, while samples for molecular analyses were shock frozen in liquid nitrogen and stored at -80 °C.

**Real time polymerase chain reaction (RT-PCR)**

RNA from frozen prostate tissues was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). For isolation, 30 mg of tissue was homogenized using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). RNA concentrations were measured spectrophotometrically. Reverse transcription to cDNA was performed with 1 µg of isolated RNA using the Reverse Transcription System (Promega, Madison, WI, USA). Real time- (RT-) PCR for cytohesin isoforms, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with a Roche Light Cycler (Roche, Basel, Switzerland) using primers provided by Qiagen (Hilden, Germany) as ready-to-use mixes, based on the RefSeq Accession numbers NM_004762 for cytohesin-1 (CYTH1), NM_004228 for cytohesin-2 (CYTH2), NM_004227 for cytohesin-3 (CYTH3), NM_013385 for cythohesin-4 (CYTH4), and NM_002046 for GAPDH. PCR reactions were performed in a volume of 25 µl containing 5 µl LightCycler® FastStart DNA MasterPlus SYBR Green I (Roche, Basel, Switzerland), 1 µl template, 1 µl primer, and 18 µl water. Denaturation was performed for 10 min at 95 °C, and amplification with 45 cycles of 15 sec at 95 °C followed by 60 sec at 60 °C. The specificity of primers and amplification was demonstrated by subsequent analysis of melting points, which revealed single peaks for each target. Results were expressed using the ΔΔCP method, where number of cycles (Ct) at which the fluorescence signal exceeded a defined threshold for
GAPDH was subtracted from Ct values for cytohesins (\(C_{\text{cytohesin}} - C_{\text{GAPDH}} = \Delta CP\)), and values were calculated as \(2^{\Delta CP}\) and normalized to each other.

**Western blot analysis**

Frozen prostate tissues were homogenized in a buffer containing 25 mM Tris/HCl, 10 \(\mu M\) phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 10 \(\mu g/ml\) leupeptine hemisulfate, using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). After centrifugation (20,000 g, 4 min), supernatants were assayed for protein concentration using the Dc-Assay kit (Biorad, Munich, Germany) and boiled for 10 min with sodium dodecyl sulfate (SDS) sample buffer (Roth, Karlsruhe, Germany). Separate from experiments with prostate tissues, electrophoresis and Western blot analyses were performed with recombinant cytohesin-1 and cytohesin-2 (Cloude-Crone Corp., Houston, TX, USA). Samples (20 \(\mu g\)/lane for prostate tissue, 0.1 \(\mu g\)/lane of recombinant cytohesins) and Precision Plus Protein™ molecular weight size marker (Biorad, Munich, Germany) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were blotted on Protran® nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For detection of ARF6, Rac1, RhoA, and PSA, electrophoresis was performed using 10 % gels, while 8 % gels were used for all others. Membranes were blocked with phosphate-buffered saline (PBS) containing 5 % milk powder (Roth, Karlsruhe, Germany) over night, and incubated with rabbit anti cytohesin-1 (USC-PAB580-HU01) (Cloude-Cone Corp., Houston, TX, USA) (1:500), goat anti cytohesin-1 (sc-9733) (1:500), rabbit anti cytohesin-2 (USC-PAB583-HU01) (Cloude-Cone Corp., Houston, TX, USA) (1:500), goat anti cytohesin-2 (sc-9727) (1:500), mouse monoclonal anti ARF6 (sc-7971) (1:500),
mouse monoclonal anti pan-cytokeratin (sc-8018) (1:1000), mouse monoclonal anti calponin 1/2/3 (sc-136987) (1:500), mouse monoclonal anti prostate-specific antigen (PSA) (sc-7316) (1:800), mouse monoclonal anti β-actin antibody (sc-47778) (1:1000) (if not other stated, from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or Rac1 and RhoA antibodies (1:500) provided with pull-down assay kits (BK035 or BK036, Cytoskeleton, Denver, CO, USA). Primary antibodies were diluted in PBS containing 0.1 % Tween 20 (PBS-T) and 5 % milk powder. Subsequently, detection was continued using secondary biotinylated horse anti mouse or horse anti goat IgG (BA-1000, BA-2000, BA-9500) (Vector Laboratories, Burlingame, CA, USA), followed by incubation with avidin and biotinylated HRP from the “Vectastain ABC kit” (Vector Laboratories, Burlingame, CA, USA) both diluted 1:200 in PBS. Membranes were washed with PBS-T after any incubation with primary or secondary antibodies, or biotin-HRP. Finally, blots were developed with enhanced chemiluminescence (ECL) using ECL Hyperfilm (GE Healthcare, Freiburg, Germany).

Immunofluorescence

Human prostate specimens, embedded in optimal cutting temperature (OCT) compound, were snap-frozen in liquid nitrogen and kept at -80 °C. Sections (8 µm) were cut in a cryostat and collected on Superfrost® microscope slides. Sections were post-fixed in methanol at -20 °C and blocked in 1 % bovine serum albumin before incubation with primary antibody over night at room temperature. For double labelling, following primary antibodies were used: goat anti cytohesin-1 (sc-9733), rabbit anti cytohesin-2 (USC-PAB583-HU01) (Cloude-Cone Corp., Houston, TX, USA), goat anti cytohesin-2 (sc-9727), mouse anti pan-cytokeratin (sc-8018), or
mouse anti calponin 1/2/3 (sc-136987) (if not other stated, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Binding sites were visualized using Cy3-conjugated goat anti mouse IgG (AP124C), fluorescein isothiocyanate- (FITC-)conjugated rabbit anti goat IgG (AP106F) (both from Millipore, Billerica, MA, US, and Cy5-conjugated goat anti rabbit IgG (ab6564) (Abcam, Cambridge, UK). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole-dihydrochloride (DAPI) (Invitrogen, Camarillo, CA, USA). Immunolabeled sections were analyzed using a laser scanning microscope (Leica SP2, Wetzlar, Germany). Fluorescence was recorded with separate detectors. Control stainings without primary antibodies did not yield any signals.

Tension measurements

Prostate strips (6 x 3 x 3 mm) were mounted in 10 ml aerated (95% O₂ and 5% CO₂) tissue baths (Föhr Medical Instruments, Seeheim, Germany), containing Krebs-Henseleit solution (37 °C, pH 7.4) with following composition: 118 mM NaCl, 4.7 mM KCl, 2.55 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 7.5 mM glucose. Preparations were stretched to 4.9 mN and left to equilibrate for 45 min. In the initial phase of the equilibration period, spontaneous decreases in tone are usually observed. Therefore, tension was adjusted three times during the equilibration period, until a stable resting tone of 4.9 mN was attained. After the equilibration period, maximum contraction induced by 80 mM KCl was assessed. Subsequently, chambers were washed three times with Krebs-Henseleit solution for a total of 30 min. Cumulative concentration response curves for norepinephrine, phenylephrine, endothelins -1, -2, and -3, and for U46619, or frequency response
curves induced by electric field stimulation (EFS) were created 30 min after addition of secinH3 or dimethylsulfoxide (DMSO) for controls. Application of EFS simulates action potentials, resulting in the release of endogenous neurotransmitters, including norepinephrine. For EFS, tissue strips were placed between two parallel platinum electrodes connected to a Grass S48 stimulator (Grass Instruments, West Warwick, RI, USA). Square pulses with durations of 1 ms were applied with a voltage of 50 V, for a train duration of 10 s and using a delay of 1 ms between single pulses. EFS-induced contractile responses were studied at frequencies of 2, 4, 8, 16, and 32 Hz, with train intervals of 30 s between stimulations. For calculation of agonist- or EFS-induced contractions, tensions (peak height in EFS-induced contractions and maximum contractions following agonist-exposure) were expressed as percentage (%) of KCl-induced contractions, as this may correct differences in stromal/epithelial ratios and smooth muscle content, varying degree of BPH, or any other heterogeneity between prostate samples and patients. Only one curve was recorded for each sample (agonist or EFS, either with or without secinH3/tamsulosin).

**Pull down assays**

Tissues from each prostate included were cut into several small strips (6 x 1 x 1 mm), which were allocated to two samples using 6-well plates filled with custodiol solution, in which tissues were incubated with secinH3 (30 µM) or DMSO for 90 min at 37 °C under continuous shaking. Following incubations, tissues were shock frozen with liquid nitrogen, and homogenization, centrifugation, and protein determination were subsequently performed as described for Western blot analysis. Aliquots containing 300 µg of protein were subjected to pull-down assays for Rac1, RhoA, and Arf6.
Assays were performed using pull-down activation assay kits BK033, BK035, and BK036 obtained from Cytoskeleton (Denver, CO, USA), according to the manufacturer's instructions, and included Western blot analysis as described above. Series for each pull-down were repeated in in at least five independent experiments using five different prostates.

Intensities of resulting bands were quantified densitometrically using “Image J” (NIH, Bethesda, Maryland, USA). For semiquantitative calculation, control samples without secinH3 were set to 100 %, and samples with agonists were expressed as percentage (%) of controls. This normalization is inevitable due to high variation of control values obtained by densitometric quantification (providing “arbitrary units”). These variations mostly result from detection conditions (slight differences of incubation periods, exposure times), and from different adjustments during digitization of blots (required due to varying background or GTPase content). These variations of control values may not be avoided in practice; therefore, normalization of controls was required.

Drugs and nomenclature

N-[4-[5-(1,3-Benzodioxol-5-yl)-3-methoxy-1H-1,2,4-triazol-1-yl]phenyl]-2-(phenylthio)acetamide (secinH3) is a cytohesin family-specific inhibitor, showing IC_{50} values of 2.4, 5.4, 5.4, 5.6, 5.6, 6.5 and > 100 μM for human cytohesin-2, human cytohesin-1, mouse cytohesin-3, human cytohesin-3, drosophila steppke, yGea2-S7 and hEFA6-S7 respectively. Tamsulosin is an α₁-adrenoceptor antagonist, which completely inhibits norepinephrine-induced contractions of human prostate tissues if
applied at a concentration of 300 nM (24). Stock solutions (10 mM) were prepared with DMSO, and kept at -20 °C until use. Phenylephrine (\((R)-3-[-1\text{-hydroxy-2-}\
\text{(methylamino)ethyl]}\text{phenol)}\) is a selective agonist for \(\alpha_1\)-adrenoceptors. U46619 (\((Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3\text{-hydroxyoct-1-}\
\text{enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]}\text{hept-5-enoic acid)}\) is an analogue of thromboxane A\(_2\) (TXA\(_2\)) and frequently used as an agonist for TXA\(_2\) receptors. Aqueous stock solutions of phenylephrine and norepinephrine (10 mM) were freshly prepared before each experiment. Stock solutions of U46619 were prepared in ethanol, and stored at -80 °C until use. Aqueous stock solutions of endothelin-1, -2, and -3 were stored at -20 °C until used. SecinH3 and tamsulosin were obtained from Tocris (Bristol, UK), phenylephrine and norepinephrine were obtained from Sigma (Munich, Germany), U46619 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and endothelins from Enzo Life Sciences (Lörrach, Germany).

**Statistical analysis**

Data are presented as means ± standard error of the mean (SEM) with the indicated number \((n)\) of experiments. Two-tailed student t test, or one-way analysis of variance (ANOVA) and multivariate ANOVA were used for paired or unpaired observations. P values <0.05 were considered statistically significant.
Results

Detection of cytohesin isoforms in human prostate tissues

By RT-PCR, mRNAs of cytohesin isoforms 1-4 were detectable in each prostate sample included in the analysis (fig. 1A). Quantification revealed varying levels in different prostates for each isoform, but suggested higher contents of cytohesin-1 and -2 mRNAs compared to cytohesin-3 and 4 mRNA (fig. 1A).

Subsequent evaluation of possible protein expression was focussed on cytohesin-1 and -2, as mRNA levels of other isoforms were low. Western blot analyses of human prostate tissues revealed bands with sizes expected for cytohesin-1 (47 kDa) and cytohesin-2 (46 kDa), which were confirmed using two different antibodies for each of both isoforms (fig. 1B). Similar to the results of RT-PCR, in which the content of mRNA levels of each isoform varied between samples, at least the intensity of cytohesin-2 bands also varied between samples (fig. 1A,B).

Apart from bands matching the expected sizes for cytohesins, additional bands were observed in Western blot analysis of prostate tissues, as can be seen regarding complete lanes (fig. 1C). As no validation data for these antibodies were available, we performed Western blot analyses using recombinant cytohesin-1 and -2 (fig. 1D). Antibody sc-9733 was raised against cytohesin-1 and showed strong binding to recombinant cytohesin-1 and almost no binding to cytohesin-2 (fig. 1D). Antibody USC-PAB580HU01 was also raised against cytohesin-1 but showed limited
specificity, as binding to cytohesin-1 was only slightly higher than to cytohesin-2 (fig. 1D). Sc-7971 was raised against cytohesin-2 and showed some cross reactivity for cytohesin-1, but binding to cytohesin-2 was considerably higher than binding to cytohesin-1 (fig. 1D). USC-PAB583-HU01 was also raised against cytohesin-2 and also showed some cross reactivity; again binding to cytohesin-2 was clearly higher than binding to cytohesin-1, but appeared be stronger than that of sc-7971 (fig. 1D).

Histology of prostate sections showed typical architecture, being composed of stroma and glands (fig. 2). Staining for the smooth muscle marker calponin was restricted to the stroma, while staining for the epithelial marker pan-cytokeratin was exclusively observed in the glandular epithelium (fig. 2). Immunoreactivity after staining with cytohesin-2 antibodies showed colocalization with calponin in the stroma, which was observed for both included cytohesin-2 antibodies (fig. 2). In addition, immunoreactivity in the glandular epithelium was observed for both cytohesin-2 antibodies (fig. 2). Staining for putative cytohesin-1 was only performed with sc-9733, as USC-PAB580HU01 did not distinguish sufficiently between cytohesin-1 and -2 in Western blot analyses with recombinant cytohesins (fig. 1D). Immunoreactivity after staining with this cytohesin-1 antibody in the stroma was rather local, and did not clearly colocalize with the smooth muscle marker calponin (fig. 2).

Western blot analysis demonstrated varying contents of PSA, calponin, and pan-cytokeratin (fig. 1B), possibly reflecting different degree of BPH (PSA), and individual heterogeneity and variation (calponin, pan-cytokeratin) in our samples. Despite this heterogeneity, detection of calponin indicated the presence of smooth muscle in each sample (fig. 1B) and tissues showed typical architecture of prostate tissues (fig. 2).
For cytohesin-2, but not for cytohesin-1, bands with the correct size were observed in each prostate sample included to this analysis (fig. 1B). For cytohesin-1, strong bands with the correct size were observed only in two from eight analyzed samples (fig. 1B).

**Effects of secinH3 on adrenergic contractions**

Norepinephrine and the $\alpha_1$-adrenoceptor-selective agonist phenylephrine induced concentration-dependent contractions of prostate strips (fig. 3). Application of secinH3 (30 µM) resulted in significant inhibition of contractions induced by norepinephrine or phenylephrine. Following multivariate analysis, inhibition of norepinephrine-induced contraction was significant at 3 µM ($p=0.011$), 10 µM ($p=0.005$), 30 µM ($p=0.002$), and 100 µM ($p=0.002$) norepinephrine (fig. 3A). Significant inhibition of phenylephrine-induced contractions was observed at 3 µM ($p=0.012$), 10 µM ($p=0.006$), 30 µM ($p=0.004$), and 100 µM ($p=0.017$) phenylephrine (fig. 3B). One-way ANOVA was conducted to compare concentration response curves in control and secinH3 groups, indicating that inhibition of norepinephrine- and phenylephrine-induced contractions by secinH3 was significant ($p<0.001$ for DMSO vs. secinH3 for norepinephrine, $p<0.001$ for DMSO vs. secinH3 for phenylephrine).

**Effects of secinH3 on non-adrenergic contractions**

The thromboxane A$_2$ analog, U46619, and endothelins -1, -2, and -3 induced concentration-dependent contractions of prostate strips (fig. 4). Application of
secinH3 (30 µM) resulted in significant inhibition of contractions induced by U46619, endothelin-1, and endothelin-3. Following multivariate analysis, inhibition of U46619-induced contraction was significant at 0.3 µM (p=0.024) and 1 µM (p=0.031) U46619 (fig. 4A). Significant inhibition of endothelin-1-induced contraction was observed at 0.3 µM (p=0.038), 1 µM (p=0.014), and 3 µM (p=0.017) endothelin-1 (fig. 4B). Significant inhibition of endothelin-3-induced contraction was observed at 1 µM (p=0.006) and 3 µM (p=0.006) endothelin-3 (fig. 4D). In contrast, contractions induced by endothelin-2 were similar after application of secinH3 and DMSO (fig. 4C). One-way ANOVA was conducted to compare concentration response curves in control and secinH3 groups, indicating that inhibition of U46619-, endothelin-1-, and endothelin-3-induced contractions by secinH3 was significant (p<0.001 for DMSO vs. secinH3 for U46619, p<0.001 for DMSO vs. secinH3 for endothelin-1, p<0.001 for endothelin-3).

Effects of secinH3 on EFS-induced contractions

EFS induced frequency-dependent contractions of prostate strips (fig. 5). Application of secinH3 (30 µM) resulted in inhibition of EFS-induced contractions (fig. 5A). Following multivariate analysis, significant inhibition was observed at 32 Hz (p=0.005) (fig. 5A). At each frequency level, EFS-induced tension after application of secinH3 ranged around 50% of EFS-induced tension after application of DMSO (fig. 5A). One-way ANOVA revealed a significant difference between the control and secinH3 group (p=0.004).

Comparison and combination of secinH3 with tamsulosin
Application of tamsulosin (300 nM) resulted in inhibition of EFS-induced contractions (fig. 5B). Following multivariate analysis, significant inhibition was observed at 8 Hz (p=0.028), 16 Hz (p=0.001), and 32 Hz (p<0.001) (fig. 5B). Similar to secinH3, EFS-induced tension after application of tamsulosin ranged around 50% of EFS-induced tension after application of DMSO at each frequency (fig. 5B). One-way ANOVA revealed a significant difference between the control and secinH3 group (p<0.001).

Combined application of secinH3 and tamsulosin caused greater inhibition of EFS-induced contractions than tamsulosin alone (fig. 5C). After combined application of secinH3 (30 µM) and tamsulosin (300 nM), EFS-induced tensions were lower than EFS-induced tensions after application of tamsulosin alone (fig. 5C). Following multivariate analysis, significant inhibition was observed at 4 Hz (p=0.031), 8 Hz (p=0.004), 16 Hz (p=0.001), and 32 Hz (p<0.001) (fig. 5C). One-way ANOVA revealed a significant difference between the tamsulosin and the combination group (p<0.001).

**Effects of secinH3 on GTPase activities**

Due to the involvement of RhoA and Rac in prostate smooth muscle contraction and since Rac has been reported to be activated by indirectly by cytohesin-2, we hypothesized that the inhibition of prostate smooth muscle contraction by secinH3 was attributable to RhoA or Rac activity (26, 33, 37, 63). In fact, active Rac (“GTP-Rac”) and RhoA (“GTP-RhoA”) were detectable in all prostate tissues analysed by
pulldown assays (fig. 6). However, incubation with secinH3 (30 µM) did not reduce the content of GTP-Rac or GTP-RhoA (fig. 6).

ARF6 may act as another effector GTPase of cytohesin-2, prompting us to examine the effects of secinH3 on ARF6 activity (30, 56, 57). Active ARF6 was detectable in all prostate tissues analysed by pulldown assays (fig. 6). On average, incubation with secinH3 (30 µM) reduced the content of GTP-ARF6 in prostate tissues by 63 ± 7 % (fig. 6) (p<0.001 for secinH3 vs. control). Western blot analysis of prostate tissues from seven patients revealed bands matching the expected sizes for ARF6 in all samples included (fig. 1).
Discussion

The crucial role of small monomeric GTPases, including RhoA and Rac, for smooth muscle contraction in the prostate and other organs has been confirmed by numerous studies (26, 63). In contrast, the role of GEFs in prostate smooth muscle contraction, and a role of ARF6 in smooth muscle contraction in general have not been considered to date. GEFs are direct upstream activators of small monomeric GTPases, which may accomplish GTPase activation through receptors and their associated heterotrimeric G proteins (11). In this study, we demonstrate that secinH3, an inhibitor of cytohesin family GEFs, inhibits α₁-adrenergic, endothelin- and thromboxane-induced, and neurogenic contractions of human prostate tissues. Simultaneously, ARF6 activity was inhibited by secinH3. Our results suggest that a shared intracellular cytohesin-ARF6 pathway plays a role in adrenergic and non-adrenergic contractions in the human prostate, hereby potentially providing new targets for medical treatment of male LUTS.

Our data from RT-PCR and Western blot analyses may suggest that all four cytohesin isoforms are expressed in the human prostate, with cytohesin-2 being the best detectable isoform in the human prostate. Interpretation of stainings using cytohesin antibodies may certainly be limited by restricted specificity, as additional bands occurred in addition to bands with correct size in Western blot analysis, and because minor cross reactivity was observed for some (but not all) included antibodies. However, as any immunoreactivity to cytohesin-1 antibodies in the stroma was faint and unclear, and only staining with the cytohesin-2 antibody resulted in
strong immunoreactivity in calponin-positive cells, this may suggest that cytohesin-2
is the predominant isoform in the stroma. Despite limited specificity of antibodies,
these findings may be regarded in context with our other results. Thus, the idea that
cytohesins are present and active in smooth muscle cells of the prostate stroma is
supported if all our molecular and functional results are regarded together.

In our study, we used tissues from prostates of patients undergoing radical
prostatectomy for prostate cancer. Most prostate cancers are located in the
peripheral zone (49, 54). Our samples were exclusively taken from the periurethral
zone, which is usually not affected by prostate cancer. BPH with varying degree may
be present in 80-83 % of patients with prostate cancer, and was reflected by the
varying content of PSA in our Western blot analyses (2, 39, 48). Therefore, our
samples may be regarded as non-malignant, hyperplastic prostate tissue. Varying
content of calponin and pan-cytokeratin reflected variations in stromal-glandular ratio
and different content of smooth muscle. In order to circumvent these variations and
other heterogeneities in our organ bath experiments, agonist- and EFS-induced
contractions were normalized to tension raised by high-molar KCl, as previously
recommended (35). Whether cytohesins or ARF6 are generally upregulated in BPH
tissue may only be determined by comparison of hyperplastic to non-hyperplastic
tissues. However, as surgeries are not performed in healthy prostates, non-
hyperplastic tissues are not available. Most importantly, from a clinical point of view,
only the hyperplastic condition causes LUTS and therefore, is relevant. Our data
suggest that the cytohesin-ARF6 pathway has an important role in BPH.
SecinH3 is an inhibitor with high specificity for cytohesins (19, 22, 23). In biochemical in vitro assays, the IC$_{50}$ for cytohesins ranged around 10 µM (23). The exchange of GDP for GTP at GTPases by cytohesins may be inhibited in the range of 2-5 µM (22). We applied secinH3 in a concentration of 30 µM to prostate tissues, which may be only 3-6-fold higher than IC$_{50}$ values in biochemical assays. In fact, the access of inhibitors to targets, and therefore their efficacy may differ between intact tissue preparations, cell culture or biochemical assays (35, 59, 60). Currently, the next generation of secinH3-based inhibitors with higher affinity to cytohesins are being developed (23). However, these inhibitors are not yet commercially available.

Previous investigations of cytohesins in the prostate were limited to two studies without regarding BPH or smooth muscle contraction (64, 66). Recently, cytohesin expression was detected in the human myometrium, however, no contraction experiments were performed in that study (34). So far, no other attempts have been made to address cytohesins in smooth muscle cells, e.g. vascular smooth muscle cells. Therefore, no conclusions can be made at the current stage about possible cardiovascular side effects, if secinH3 is applied in vivo. In fact, considerations with regard to uroselectivity may be of high relevance for applications in LUTS treatment, as major side effects of LUTS drugs include cardiovascular events such as hypotension or dizziness (3, 51). To date, secinH3 has been rarely applied in vivo. The existing studies did not focus on possible hemodynamic effects and therefore, none have been reported to date (22, 50). SecinH3 was tolerated for a minimum of 2-4 days in mice, although it induced hepatic insulin resistance (22, 50). Thus, in vivo studies to examine urodynamic effects of secinH3 in animal models may be possible,
however, alternative cytohesin inhibitors or appropriate conditions to prevent development of insulin resistance would be required for application in patients.

Based on previous reports together with our current findings, it may be assumed that secinH3 inhibits prostate smooth muscle contraction by inhibition of cytohesin-mediated ARF6 activation. Cytohesin-2 preferentially activates the GTPase ARF6 (30, 56, 57). Pull down assays demonstrated that secinH3 inhibited ARF6, but not RhoA or Rac1 in prostate tissues. ARF6 has been associated with cytoskeletal organization and actin remodelling (17, 30, 31, 36, 40, 53). Consequently, ARF6 promotes these processes depending on correct actin assembly and cytoskeletal organization, including cell adhesion, cell migration, and platelet activation (10, 13, 29, 56, 58). Moreover, ARF6 has been proposed as a possible intracellular effector and regulator of G protein-coupled receptors, which may mediate receptor-induced effects, or may cause posttranslational modification of receptors (6, 20, 25, 32). However, to the best of our knowledge, the present study is the first suggesting that ARF6 takes part in smooth muscle contraction. In fact, this may not be entirely surprising, as smooth muscle contraction in the prostate and other organs requires actin organization (26, 27). On the other hand, our data may not prove whether inhibition of prostate smooth muscle contraction by secinH3 was in fact attributable to ARF6 inhibition, or whether inhibition of contraction and ARF6 activity just paralleled each other. To date, small molecule inhibitors for ARF6 are not yet available; therefore, the role of ARF6 in smooth muscle contraction cannot be confirmed by organ bath studies with human tissues. Finally, ARF6 may even be implicated in the etiology of BPH by producing reactive oxygen species (ROS), which are increased in BPH (42, 62). ROS promote proliferation and smooth muscle contraction, and may
be induced by ARF6 (6). Whether this concept is valid also for prostate cells, may be
subject to future studies.

In addition to cytohesin-2-mediated ARF6 activation, inhibition of Rac by secinH3
through indirect mechanisms has been previously reported (33, 50). Thus, Rac itself
may not be activated by cytohesins directly, but downstream of cytohesins by ARF6-
dependent mechanisms. Our present data do not suggest that secinH3-sensitive
cytohesins promote activation of RhoA or Rac in the human prostate. For RhoA or
Rac, several other groups of GEFs have been proposed to mediate their activation in
different cell types (5, 38, 41, 67). PDZ-RhoGEF, LARG, Arhgef1, and p63RhoGEF
have been demonstrated to mediate receptor-induced RhoA activation in smooth
muscle contraction, which was mostly (though not exclusively) investigated in
vascular smooth muscle (1, 4, 9, 12, 28, 43, 44). Kalirin, Vav2, Sos-1, or Trio were
identified as Rac-activating GEFs in cultured smooth muscle cells (16, 21, 52, 65,
68). At present, investigation of these targets is prevented by lack of specific small
molecule inhibitors, so that their function may only be assessed by application of
transgenic models, but not in organ bath studies.

Because secinH3 inhibited prostate smooth muscle contraction, it appears possible
that secinH3 may induce urodynamic effects (e. g., improvements of symptoms and
urinary flow) in vivo. In fact, induction of prostate smooth muscle relaxation (mostly
by α1-blockers) is regarded as an important strategy for medical therapy of male
LUTS (45). Although more than 3.5 billion USD are spent worldwide each year for
LUTS treatment with α1-blockers, their efficacy is surprisingly limited due to
contributions of non-adrenergic mediators (e. g. endothelin, TXA2) (24, 26, 61).
Currently available drugs are only suitable for mild to moderate symptoms, and may show a maximum of improvement by no more than 50% (26, 45). New drugs with increased efficacy could not only improve mild to moderate symptoms, but could also be used to treat more severe symptoms, possibly avoiding surgery in some cases. Several findings support the idea, that urodynamic effects resulting from secinH3 or from combinations of secinH3 with α1-blockers may resemble or exceed those of α1-blockers alone. First, secinH3 inhibited non-adrenergic and α1-adrenergic contractions, while α1-blockers only inhibit α1-adrenergic contractions. Secondly, secinH3 reduced neurogenic contraction with an efficacy similar to that of tamsulosin. Third, inhibition of neurogenic contraction by the combination of secinH3 and tamsulosin was significantly stronger than inhibition by tamsulosin alone. Consequently, secinH3 may be a promising candidate for examination in preclinical animal models of LUTS.

We used tamsulosin in a concentration of 300 nM. This concentration inhibits norepinephrine-induced contractions of human prostate tissues completely, while EFS-induced contractions in our current study were inhibited by 50% (24). A very similar phenomenon has been described recently for another α1-blocker, silodosin (8). Our current data are in line with previous studies, reporting that inhibitions of EFS-induced contractions by α1-blockers remains incomplete even at high antagonist concentrations (8, 15, 46, 47). Obviously, the efficacy of α1-blockers differs between agonist-induced and EFS-induced contractions. The effect of α1-blockers on EFS-induced contractions may stay incomplete due to α1-blocker-insensitive mechanisms, which are involved in neurogenic prostate contraction, including modulation of α1-adrenergic contractions by adenosine 5'-triphosphate (ATP) or other cotransmitters.
(7). These cotransmitters may limit the efficacy of $\alpha_1$-blockers in addition to the contribution of non-adrenergic mediators. Thus, new strategies to enhance the efficacy of $\alpha_1$-blockers may be of interest. Effects of secinH3 on cotransmitters such as ATP or further putative non-adrenergic mediators of prostate smooth muscle contraction (e.g. acetylcholine) may be subject to future studies.

In conclusion, secinH3 inhibits smooth muscle contraction and ARF6 activity in the human prostate. This suggests a new role of ARF6 GTPase in smooth muscle contraction. A possible part of a cytohesin/ARF6 pathway in smooth muscle contraction in the prostate and other organs needs to be considered. SecinH3 may be a promising candidate for further preclinical examinations using animal models of LUTS.


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Disclosures

The authors declare no competing financial interests, and that there are no conflicts of interest.
Figure Captions

Figure 1: Detection of cytohesin isoforms. Detection was performed by RT-PCR (A) or Western blot analyses (B-D) in human prostate tissues (A-C) or with recombinant cytohesins (D). Data in (A) are ΔΔCP values \( 2^{\Delta \Delta CT} \) for each sample (from n=7 patients) and median values (bar). In (B), bands with correct sizes are shown for all investigated samples (from n=8 patients); indicated molecular weights are the expected sizes of proteins. Western blot analysis included calponin as a marker for smooth muscle cells, pan-cytokeratin as a marker of endothelial cells (glands), and prostate-specific antigen (PSA) as a marker for benign prostatic hyperplasia. In (C), complete lanes from Western blot analysis of human prostate tissues are shown for cytohesins and ARF6. Samples (B,C) were run together with a prestained marker to identify molecular weights of bands. In (D), recombinant cytohesin-1 and -2 were subjected to Western blot analyses using cytohesin-1 and -2 antibodies.

Figure 2: Immunofluorescence staining of human prostate tissues. Sections were double labelled with antibodies raised against cytohesin-1 or -2, together with antibodies for calponin (marker for smooth muscle cells) or pan-cytokeratin (marker for epithelial cells of glands). Yellow color in merged pictures may indicate colocalization of immunoreactivities. Shown are representative stainings from series with tissues from n=5 patients for each combination. Control stainings without primary antibodies (lower panel) did not yield any signals.
Figure 3: Effects of secinH3 on adrenergic contractions of human prostate strips. Contractions of isolated human prostate strips were induced by norepinephrine (A) or phenylephrine (B), after addition of secinH3 (30 µM), or DMSO (control). Tensions have been expressed as % of contraction by highmolar KCl, being assessed before application of secinH3 or solvent. This normalization allows comparisons despite different conditions of tissues or patients, e.g. due to varying degree of BPH, different content of smooth muscle, or any other heterogeneity. Statistical comparisons were performed by one-way analysis of variance (ANOVA) to compare contractions at each agonist concentration between DMSO and secinH3 groups (as indicated in diagrams), while multivariate ANOVA was used to compare DMSO and inhibitor groups with each other (as indicated in the results section). Shown are means ± SEM from series with tissues from n=10 patients for norepinephrine, and n=11 for phenylephrine, in which samples from each patient were allocated to both the control and inhibitor group (# p<0.05 for control vs. secinH3 after one-way ANOVA).

Figure 4: Effects of secinH3 on non-adrenergic contraction of human prostate strips. Contractions of isolated human prostate strips were induced by the thromboxane analog U46619 (A), or by endothelins (B-D), after addition of secinH3 (30 µM), or DMSO (control). Tensions have been expressed as % of contraction by highmolar KCl, being assessed before application of secinH3 or solvent. This normalization allows comparisons despite different conditions of tissues or patients, e.g. due to varying degree of BPH, different content of smooth muscle, or any other heterogeneity. Statistical comparisons were performed by one-way analysis of variance (ANOVA) to compare contractions at each agonist concentration between
DMSO and secinH3 groups (as indicated in diagrams), while multivariate ANOVA was used to compare DMSO and inhibitor groups with each other (as indicated in the results section). Shown are means ± SEM from series with tissues from n=7 patients for U46619, n=10 for endothelin-1, n=4 for endothelin-2, and n=6 for endothelin-3, in which samples from each patient were allocated to both the control and inhibitor group (# p<0.05 for control vs. secinH3 after one-way ANOVA).

Figure 5: Effects of secinH3, tamsulosin, and combination of secinH3 with tamsulosin on EFS-induced contraction of human prostate strips. Contractions of isolated human prostate strips were induced by EFS, after addition of secinH3 (30 µM) (A), tamsulosin (300 nM) (B), secinH3 plus tamsulosin (C), or DMSO (control). Tensions have been expressed as % of contraction by highmolar KCl, being assessed before application of inhibitors or solvent. This normalization allows comparisons despite different conditions of tissues or patients, e. g. due to varying degree of BPH, different content of smooth muscle, or any other heterogeneity. Statistical comparisons were performed by one-way analysis of variance (ANOVA) to compare contractions at each frequency between control and inhibitor/tamsulosin groups (as indicated in diagrams), while multivariate ANOVA was used to compare control and inhibitor/tamsulosin groups with each other (as indicated in the results section). Shown are means ± SEM from series with tissues from n=12 patients for secinH3, n=5 for tamsulosin, and n=5 for tamsulosin vs. tamsulosin plus secinH3, in which samples from each patient were allocated to both the control and inhibitor group (# p<0.05 between indicated groups after one-way ANOVA).
Figure 6: Effects of secinH3 on GTPase activities in human prostate tissues.

Prostate tissues were incubated with secinH3 (30 µM) or DMSO (control). Subsequently, activities for RhoA, Rac1, and ARF6 were semiquantitatively compared between both groups by pull down assays. Statistical comparison was performed by two-tailed student t test. Shown are representative experiments, and means ± SEM from series with tissues from n=5 (RhoA, ARF6) or n=6 patients (Rac1) (# p<0.05 between indicated groups).
Figure 1

A

B

patient #1 - #8

cytohesin-1, 47 kDa
USC-PAB580HU01

cytohesin-1, 47 kDa
sc-9733

cytohesin-2, 46 kDa
USC-PAB583-HU01

cytohesin-2, 46 kDa
sc-9727

ARF6, 20 kDa

calponin, 34 kDa

PSA, 29 kDa

pan-cytokeratin, 37-55 kDa

β-actin, 42 kDa

C

cytohesin-1 (47 kDa), USC-PAB580HU01
cytohesin-1 (47 kDa), sc-9733

cytohesin-2 (46 kDa), USC-PAB583-HU01

cytohesin-2 (46 kDa), sc-9727

ARF6 (20 kDa), sc-7971

D

cytohesin-1 antibody:
USC-PAB580HU01
cytohesin-1 antibody:
sc-9733
cytohesin-2 antibody:
USC-PAB583-HU01
cytohesin-2 antibody:
sc-9727
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6