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RESEARCH PAPER

Inhibition of adrenergic human prostate smooth muscle contraction by the inhibitors of c-Jun N-terminal kinase, SP600125 and BI-78D3

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α_1-adrenoceptor; benign prostate hyperplasia; JNK; mitogen-activated protein kinase; smooth muscle contraction; lower urinary tract symptoms

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BACKGROUND AND PURPOSE

α_1-Adrenoceptor-induced contraction of prostate smooth muscle is mediated by calcium- and Rho kinase-dependent mechanisms. In addition, other mechanisms, such as activation of c-jun N-terminal kinase (JNK) may be involved. Here, we investigated whether JNK participates in α_1-adrenoceptor-induced contraction of human prostate smooth muscle.

EXPERIMENTAL APPROACH

Prostate tissue was obtained from patients undergoing radical prostatectomy. Effects of the JNK inhibitors SP600125 (50 μM) and BI-78D3 (30 μM) on contractions induced by phenylephrine, noradrenaline and electric field stimulation (EFS) were studied in myographic measurements. JNK activation by noradrenaline (30 μM) and phenylephrine (10 μM), and the effects of JNK inhibitors of c-Jun phosphorylation were assessed by Western blot analyses with phospho-specific antibodies. Expression of JNK was studied by immunohistochemistry and fluorescence double staining.

KEY RESULTS

The JNK inhibitors SP600125 and BI-78D3 reduced phenylephrine- and noradrenaline-induced contractions of human prostate strips. In addition, SP600125 reduced EFS-induced contraction of prostate strips. Stimulation of prostate tissue with noradrenaline or phenylephrine *in vitro* resulted in activation of JNK. Incubation of prostate tissue with SP600125 or BI-78D3 reduced the phosphorylation state of c-Jun. Immunohistochemical staining demonstrated the expression of JNK in smooth muscle cells of human prostate tissue. Fluorescence staining showed that α_{1A}-adrenoceptors and JNK are expressed in the same cells.

CONCLUSIONS AND IMPLICATIONS

Activation of JNK is involved in α_1-adrenoceptor-induced prostate smooth muscle contraction. Models of α_1-adrenoceptor-mediated prostate smooth muscle contraction should include this JNK-dependent mechanism.

Abbreviations

BI-78D3, 4-(2,3-dihydro-1,4-benzodioxin-6-yl)-2,4-dihydro-5-[(5-nitro-2-thiazolyl)thio]-3H-1,2,4-triazol-3-one; BOO, bladder outflow obstruction; BPH, benign prostate hyperplasia; DMSO, dimethyl sulfoxide; LUT, lower urinary tract; LUTS, lower urinary tract symptom; SP600125, anthra[1-9-cd]pyrazol-6(2H)-one

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Introduction

α_1-Adrenoceptors are important regulators of prostate smooth muscle tone (Andersson *et al.*, 1997; Schilit and Benzeroual, 2009). In bladder outflow obstruction (BOO) caused by benign prostate hyperplasia (BPH), α_1-adrenoceptor-mediated contraction of prostate smooth muscle (dynamic factor) may contribute to lower urinary tract symptoms (LUTS) in addition to prostate growth (static factor) and extra prostatic factors (Andersson *et al.*, 1997; Schilit and Benzeroual, 2009). Consequently, α_1-adrenoceptors in the lower urinary tract (LUT) represent an important target for the pharmacological treatment of LUTS (Andersson *et al.*, 1997; Roehrborn and Schwinn, 2004; Schwinn and Roehrborn, 2008). Therefore, the mechanisms of α_1-adrenoceptor-mediated contraction are of great theoretical as well as clinical interest.

α_1-Adrenoceptor-induced contraction of prostate smooth muscle is known to involve activation of calcium- and Rho kinase-dependent mechanisms (Christ and Andersson, 2007). These mechanisms are also important contraction-mediating effectors of α_1-adrenoceptors in other types of smooth muscle, for example, rat vascular smooth muscle, where c-jun N-terminal kinase (JNK) was suggested to be involved as well (Lee *et al.*, 2006; Liu *et al.*, 2007; Zhou *et al.*, 2010). JNK represents a member of the family of MAPK (Johnson and Nakamura, 2007; Bogoyevitch *et al.*, 2010). Different functions of JNK have been described, which may be cell or organ specific. JNK-dependent functions are of relevance for cell cycle, cellular survival, cell death, inflammation and differentiation (Johnson and Nakamura, 2007; Bogoyevitch *et al.*, 2010). While the involvement of JNK in the contraction of vascular smooth muscle has been demonstrated (Lee *et al.*, 2006; Liu *et al.*, 2007; Zhou *et al.*, 2010), the role of JNK in contraction of prostate smooth muscle has, to the best of our knowledge, not been investigated previously. In the present study, we have therefore examined the possible role of JNK in α_1-adrenoceptor-mediated contraction of human prostate smooth muscle.

Methods

Human prostate tissue

Human prostate tissue was obtained from patients undergoing radical prostatectomy for prostate cancer ($n = 47$, mean age 67.4 years). Tissues for experiments were taken from the periurethral zone. Representative tissue sections did not exhibit histological signs of neoplasia, cancer or inflammation. In fact, most prostate tumours are located to the peripheral zone. In patients with prostate cancer, normal and hyperplastic tissues occur in very close proximity to each other, so that exact discrimination of these areas usually requires microscopic examination. Therefore, normal and hyperplastic areas were not separated. All procedures were approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany. The research was carried out according to the World Medical Association Declaration of Helsinki.

Measurement of prostate contraction

For isometric tension measurements, human prostate strips ($3 \times 3 \times 6$ mm) were mounted in 5 mL aerated (95% O_2 and 5% CO_2) tissue baths (37°C, pH 7.4), containing Krebs–Henseleit solution. Mechanical activity was registered with a Grass Polygraph model 7E (Grass Technologies, West Warwick, RI, USA). Preparations were stretched to 0.5 g and left to equilibrate for 45 min to attain a stable resting tone. The inhibitors of JNK, SP600125 (50 μM) and BI-78D3 (30 μM), or vehicle [dimethyl sulfoxide (DMSO)] were applied 30 min before application of phenylephrine or noradrenaline, or the second cycle of electric field stimulation (EFS). The concentration of SP600125 used in our study is in the same range of that applied previously in studies with rat aortic rings (Lee *et al.*, 2006; Zhou *et al.*, 2010). After construction of concentration-response curves, the tissue chambers were washed three times with Krebs–Henseleit solution, and viability of the preparations was assessed by exposure to 80 mM KCl.

Sampling and in vitro *stimulation*

Tissues were frozen or used for experiments directly after pathological examination of excised prostates, without any additional delay. For analysis by immunohistochemistry, samples of prostate tissue were shock frozen in liquid nitrogen after prostatectomy. For *in vitro* stimulation with adrenoceptor agonists or JNK inhibitors, samples of prostate tissue were prepared as small strips (2–3 mm × 1 mm) and allocated to three or four polyethylene tubes containing Krebs–Henseleit solution. During the experiments, tubes were kept at 37°C and continuously oxygenated with carbogen (95% O_2, 5% CO_2). Tissues were allowed to equilibrate for 20 min. For stimulation with phenylephrine or noradrenaline, 10 mM stock solutions were added at the required intervals and volumes to obtain a final concentration of 10 μM phenylephrine, or 30 μM noradrenaline. To avoid any effects due to different incubation periods, all samples were exposed to identical periods and experimental conditions. Therefore, stimulation was performed after the addition of phenylephrine or noradrenaline 20, 10 and 5 min before the end of the experiment. For incubation with SP600125 (50 μM) or BI-78D3 (30 μM), 10 mM stock solutions of inhibitors, or the equivalent volume of DMSO were added simultaneously, and incubation was performed for 2 h. At the end of each experiment, stimulated and unstimulated samples were simultaneously shock frozen in liquid nitrogen. Samples were stored at –80°C until Western blot analysis was performed.

Assessment of JNK activity

JNK is activated by phosphorylation at threonine183/tyrosine185 through MAPK kinase 4/7. For semi-quantitative assessment of JNK activity, the phosphorylation state of JNK was compared by Western blot analysis with a phospho-specific antibody. The total JNK content was compared by Western blot analysis with a non-phospho-specific antibody. After densitometric quantification, phospho-JNK, total JNK or phospho-c-Jun at 0 min or after DMSO, respectively, were set to 100%, and the contents in stimulated samples are expressed as % of the unstimulated or DMSO sample.

Western blot analysis

Frozen prostate tissues were homogenized in a buffer containing 25 mM Tris/HCl, 10 μM phenylmethanesulfonyl fluoride, 1 mM benzamidine and 10 μg mL^{-1} leupeptine hemisulfate, using a FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). After brief centrifugation, supernatants were assayed for protein concentration using the Dc-Assay kit (Biorad, Munich, Germany) and boiled for 10 min with sample buffer (Roth, Karlsruhe, Germany). Samples were subjected to SDS-PAGE (20 μg per lane), and proteins were blotted on nitrocellulose membranes. The membranes were blocked overnight, and subsequently incubated with primary antibodies. For detection, rabbit anti-phospho-stress-activated protein kinase (SAPK)/JNK (T183/Y185) antibody (98F2), rabbit anti-SAPK/JNK antibody (56G8) (Cell Signaling Technology, Danvers, MA, USA) or rabbit anti phospho-c-Jun (serine 63) antibody (sc-7980-R) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Subsequently, membranes were washed with PBS containing 0.1% Tween 20, and incubated with secondary peroxidase-coupled antibody (Calbiochem, San Diego, CA, USA). Blots were developed with enhanced chemiluminescence (ECL) using ECL Hyperfilm (GE Healthcare, Freiburg, Germany). Intensities of the resulting bands were quantified using Image J (NIH, Bethesda, MD, USA).

Immunohistochemistry

Sections (6–8 μm) from frozen tissues were stained by an indirect immunoperoxidase technique. Sections were fixed with acetone, and endogenous peroxidase activity was subsequently blocked by 0.03% H_2O_2. Thereafter, sections were blocked with goat serum diluted 1:10 in PBS and incubated with primary rabbit anti-JNK (FL) antibody (sc-572) (Santa Cruz Biotechnology), or rabbit anti-phospho-SAPK/JNK (T183/Y185) antibody (98F2) (Cell Signaling Technology). Primary antibody was diluted 1:50 in PBS at room temperature and incubated with the sections overnight. After the sections had been washed three times in PBS, biotinylated secondary goat anti-rabbit antibody and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) were sequentially applied for 30 min each. Staining was performed using the AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA). Finally, all sections were counterstained with hemalaun. Control stainings without primary antibodies did not yield any signals.

Immunofluorescence

Human prostate specimens, embedded in optimal cutting temperature compound, were snap frozen in liquid nitrogen and kept at –80°C. Three sections (8 μm) were cut in a cryostat and collected on microscope slides (Superfrost®). Sections were postfixed in methanol at –20°C and blocked in 1% BSA before incubation with primary antibody overnight at room temperature. For immunofluorescence analysis, three sections per specimen were co-labelled for α_{1A}-adrenoceptors (monoclonal mouse antibody, 1:50, sc-100291; Santa Cruz Biotechnology) and JNK (polyclonal rabbit antibody, D-2; Santa Cruz Biotechnology). Binding sites were visualized using Cy3- and Cy5-conjugated secondary antibodies (goat anti-mouse, AP124C, Millipore, Billerica, MA, USA, 1:1000; goat anti-rabbit, 1:1000, ab6564, Abcam, Cambridge, UK). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1:50 000, D1306; Invitrogen, Camarillo, CA, USA) during incubation with the secondary antibody. Slides were coverslipped with Citiflour® mounting medium. Immunolabelled sections were analysed using a laser scanning microscope (Leica SP2, Wetzlar, Germany) equipped with an argon laser, a helium-neon laser and a diode laser. Laser scanning microscopy was performed with a 40× oil-immersion objective. Fluorescence was excited at 405 nm (DAPI), 488 nm (Cy5), and 483 nm (Cy3), and recorded with separate detectors. Control stainings without primary antibodies did not yield any signals.

Drugs and nomenclature

SP600125 and BI-78D3 are inhibitors of JNK. SP600125 and BI-78D3 (both from Tocris, Bristol, UK) were dissolved in DMSO and kept as 10 mM stock solution at –20°C until use. Aqueous stock solutions of the α_1-adrenoceptor agonist phenylephrine (Sigma, St. Louis, MO, USA) (10 mM) were freshly prepared for each experiment. Nomenclature of receptors and enzymes conforms to BJP's 'Guide to Receptors and Channels' (Alexander *et al.*, 2011).

Statistical analysis

Data are presented as means ± SEM with the indicated number (n) of patients. Student's two-tailed t-test was used for paired or unpaired observations. P values < 0.05 were considered statistically significant.

Results

Tension measurements

Noradrenaline induced concentration-dependent contractions of human prostate strips, with a maximum at 30 μM. The JNK inhibitor SP600125 (50 μM) significantly reduced noradrenaline-induced contractions (n = 7 patients) (Figure 1A). The inhibition was observed at 1, 3, 10 and 30 μM noradrenaline (Figure 1A).

Phenylephrine also induced concentration-dependent contractions of human prostate strips, with a maximum at 10 μM. SP600125 (50 μM) significantly reduced the contractions (n = 6 patients) (Figure 1B). The inhibition was observed at 3, 10 and 30 μM phenylephrine (Figure 1B).

In a separate set of experiments, the effects of another JNK inhibitor, BI-78D3 on noradrenaline- and phenylephrine-induced contractions was tested. Similar to SP600125, BI-78D3 (30 μM) significantly reduced the contractions induced by noradrenaline (n = 6 patients) and phenylephrine (n = 12 patients) (Figure 2). Inhibition of noradrenaline-induced contraction was observed at 0.3, 1, 3 and 10 μM noradrenaline (Figure 2A). Inhibition of phenylephrine-induced contraction was observed at 1, 3, 10 and 30 μM phenylephrine (Figure 2B).

EFS induced frequency-dependent contractions of the strips, with a maximum at 32 Hz (1.9 ± 0.5 g, n = 7 patients). SP600125 (50 μM) significantly reduced the contractions (n = 7 patients) (Figure 3). This inhibition of EFS-induced contraction was observed at 8, 16 and 32 Hz (Figure 3). In contrast, contractions of the first and second cycles were not different when DMSO was applied instead of SP600125 (Figure 3).

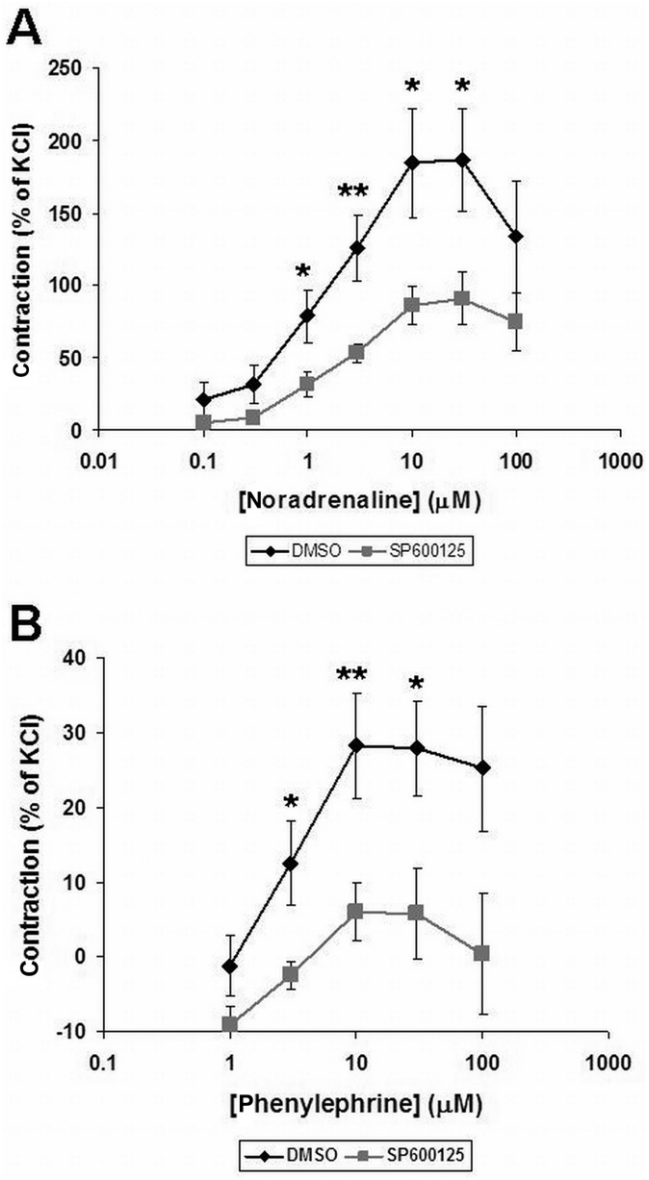

Figure 1

Effect of the JNK inhibitor SP600125 on adrenergic contraction of human prostate strips. Contraction of prostate tissue in response to noradrenaline (A) or phenylephrine (B) was determined by myographic measurements, and referred to maximal KCl-induced contraction. SP600125 (50 μM) or the vehicle DMSO was applied 30 min before the first dose of phenylephrine or noradrenaline. Shown are cumulative concentration- response curves for noradrenaline and phenylephrine. Data are means ± SEM from experiments with tissues from $n = 6$ (A) or $n = 7$ (B) patients. *$P < 0.04$, **$P < 0.02$ for DMSO versus SP600125.

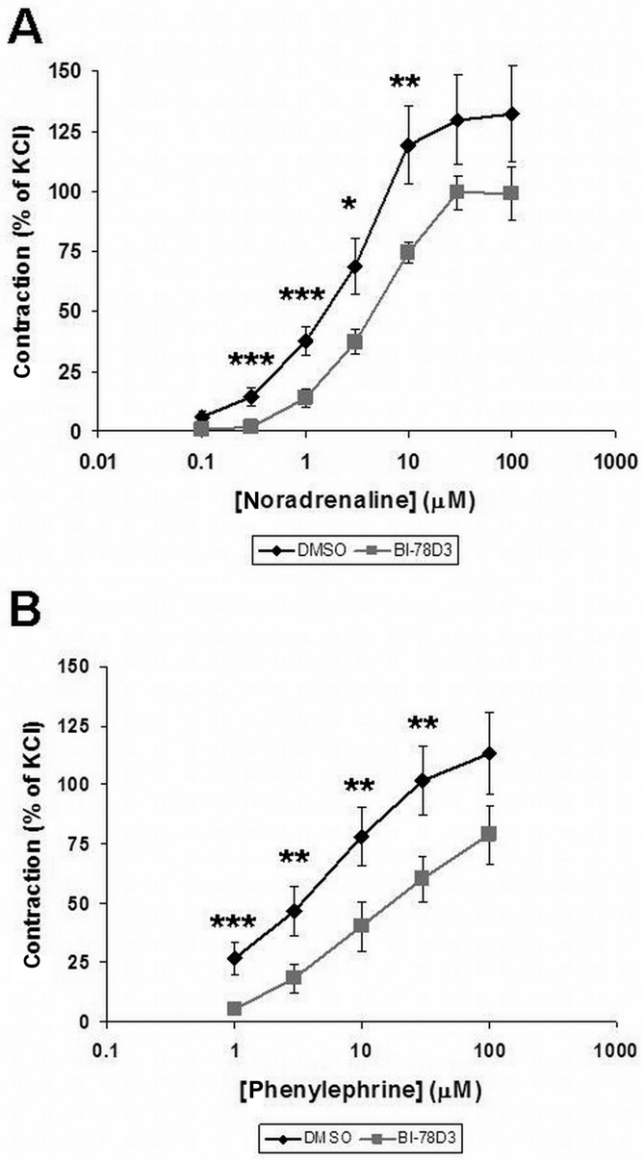

Figure 2

Effect of the JNK inhibitor BI-78D3 on adrenergic contraction of human prostate strips. Contraction of prostate tissue in response to noradrenaline (A) or phenylephrine (B) was determined by myographic measurements, and referred to maximal KCl-induced contraction. BI-78D3 (30 μM) or the vehicle DMSO was applied 30 min before the first dose of phenylephrine or noradrenaline. Shown are cumulative concentration response curves for noradrenaline and phenylephrine. Data are means ± SEM from experiments with tissues from $n = 6$ (A) or $n = 12$ (B) patients. *$P < 0.04$, **$P < 0.03$, ***$P < 0.01$ for DMSO versus BI-78D3.

JNK activity

Stimulation of human prostate tissue with noradrenaline (30 μM) increased the phosphorylation of JNK, reflecting activation of JNK (Figure 4). This phosphorylation was observed 5, 10 and 20 min after stimulation (Figure 4). In contrast, the total JNK content in prostate tissue did not change during the stimulation experiments (Figure 4).

Stimulation of human prostate tissue with phenylephrine (10 μM) increased the phosphorylation of JNK, reflecting activation of JNK (Figure 5). The phosphorylation was observed 10 min after stimulation (Figure 5). In contrast, the total JNK content in prostate tissue did not change during the stimulation experiments (Figure 5).

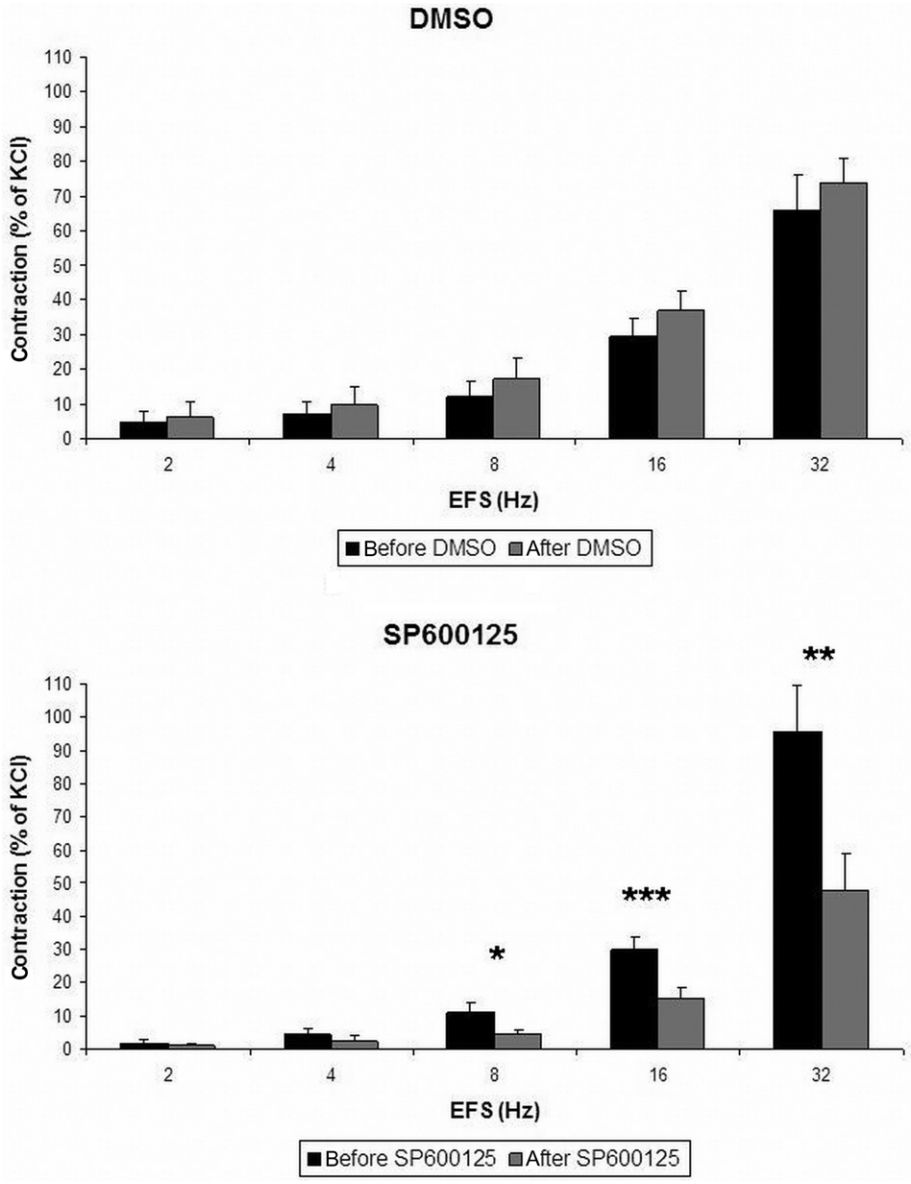

Figure 3

Effect of the JNK inhibitor SP600125 on EFS-induced contraction of human prostate strips. Contraction of prostate tissue in response to EFS was determined by myographic measurements, and referred to maximal KCl-induced contraction. SP200125 (50 μM) or the vehicle DMSO was added between two cycles of EFS (30 min before the second cycle). Shown are frequency-dependent concentrations. Data are means ± SEM from experiments with tissues from $n = 7$ patients. *$P < 0.05$, **$P < 0.03$, ***$P < 0.01$ before and after SP600125.

Incubation of human prostate tissue with SP600125 (50 μM) or BI-78D3 (30 μM) for 2 h reduced the phosphorylation state of the JNK substrate, c-Jun at serine 63 (Figure 6). This reflects inhibition of JNK activity by SP600125 and BI-78D3.

Immunohistochemistry

JNK staining was found in perinuclear and nuclear regions of prostate smooth muscle cells, and in the perinuclear regions of glandular cells (Figure 7). Faint immunoreactivity after staining with a phospho-specific JNK antibody was observed in smooth muscle cells (Figure 7). Control stainings, where the primary antibody was replaced by PBS, did not show any immunoreactivity (Figure 7).

Immunofluorescence

Fluorescence staining revealed immunoreactivity for JNK and α_{1A}-adrenoceptors in prostate smooth muscle cells (Figure 8). Overlaid images showed regions with co-localization of JNK and α_{1A}-adrenoceptors, as indicated by yellow colour in merged pictures (Figure 8). Control stainings, where the primary antibodies were replaced by PBS, did not show immunoreactivity (Figure 8).

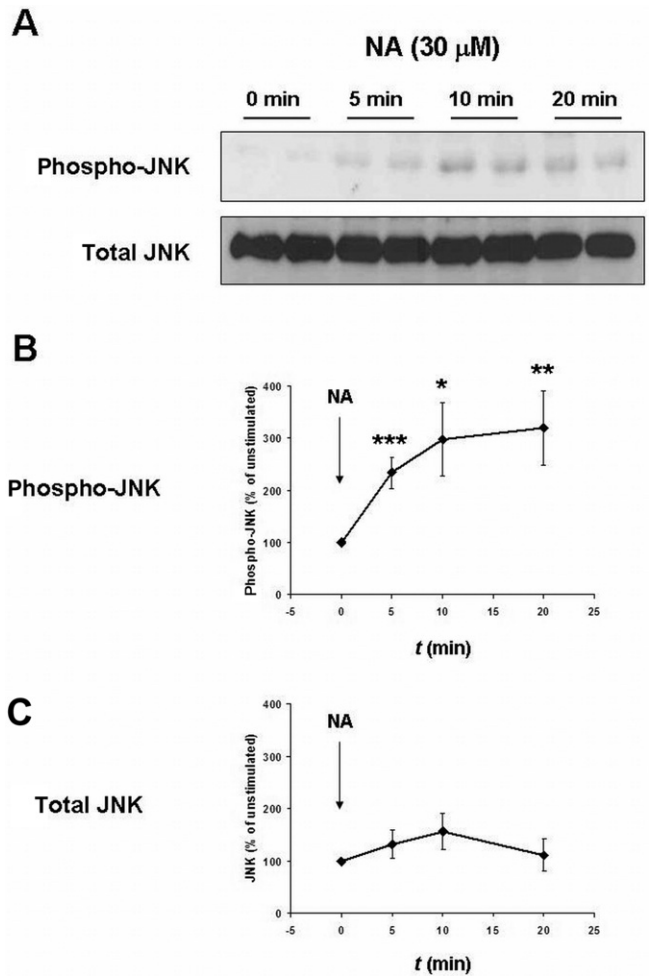

Figure 4

JNK activation by noradrenaline in human prostate tissue. Samples of human prostate tissue were stimulated *in vitro* with noradrenaline (30 μM) for 0, 5, 10 or 20 min. All samples including the unstimulated ('0 min') were exposed for identical periods to experimental conditions and simultaneously shock frozen. The phosphorylation state of JNK was assessed by Western blot analysis with a phospho-specific antibody, and reflects JNK activity. (A) Representative Western blots for phosphorylated JNK and total JNK. (B and C) Results from densitometric quantification of all experiments (data are means ± SEM from independent experiments with tissues from $n = 8$ patients). *$P < 0.02$, **$P < 0.01$, ***$P < 0.001$ versus '0 min'.

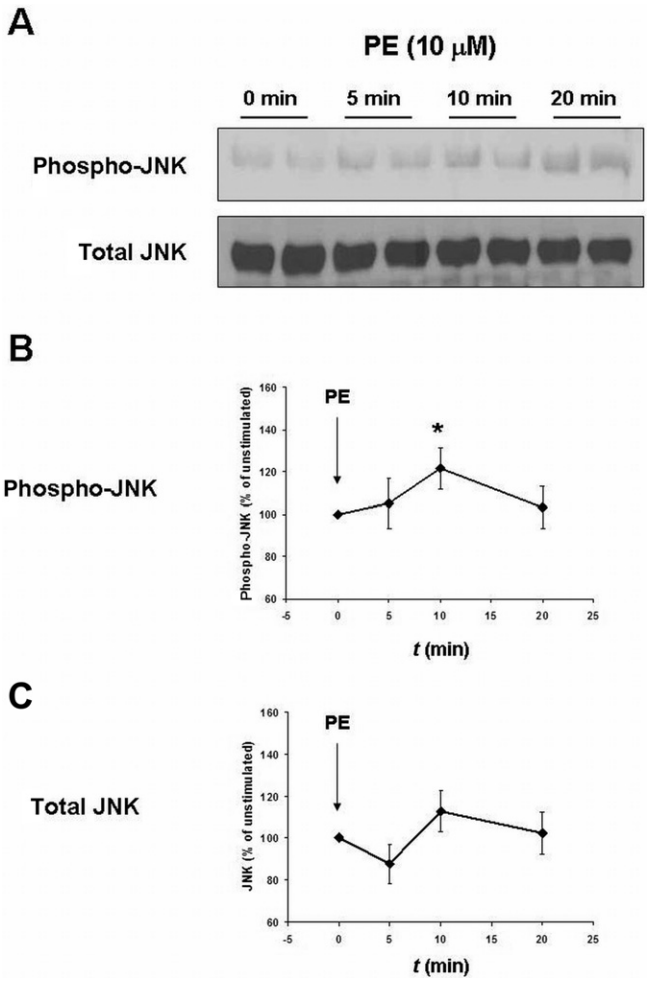

Figure 5

JNK activation by phenylephrine (PE) in human prostate tissue. Samples of human prostate tissue were stimulated *in vitro* with phenylephrine (10 μM) for 0, 5, 10 or 20 min, and assessed for JNK activation as described in Figure 4. (A) Representative Western blots for phosphorylated JNK and total JNK. (B and C) Results from densitometric quantification of all experiments (data are means ± SEM from independent experiments with tissues from $n = 9$ patients). *$P < 0.05$ versus '0 min'.

Discussion

As mentioned in the Introduction, it is widely accepted that α_1-adrenoceptor-induced contraction of prostate smooth muscle is caused by activation of calcium- and Rho kinase-dependent pathways (Christ and Andersson, 2007). In the present study, we identified an additional mechanism contributing to α_1-adrenoceptor-mediated prostate smooth muscle contraction. This is based on two main findings. Firstly, the JNK inhibitors SP600125 and BI-78D3 inhibited contractions of human prostate strips induced by α_1-adrenoceptors and EFS. Secondly, stimulation with noradrenaline or the α_1-adrenoceptor agonist, phenylephrine, resulted in activation of JNK in the tissue. Together, this suggests that JNK activation is involved in α_1-adrenoceptor-induced contraction, in addition to the established mechanisms (Christ and Andersson, 2007). α_1-Adrenoceptor-mediated tone represents an important target for the pharmacological therapy of LUTS in patients with BOO secondary to BPH (Andersson *et al.*, 1997; Roehrborn and Schwinn, 2004; Schwinn and Roehrborn, 2008). Treatment with α_1-adrenoceptor blockers causes smooth muscle relaxation in the LUT, including the prostate (Andersson *et al.*, 1997; Schilit and Benzeroual, 2009). Reduced prostate smooth muscle tone may improve urinary flow and symptoms due to reduced urethral resistance (Andersson *et al.*, 1997; Schilit and Benzeroual, 2009). As SP600125 and BI-78D3 prevented α_1-adrenoceptor-mediated contraction of prostate tissue, future *in vivo* studies in animals

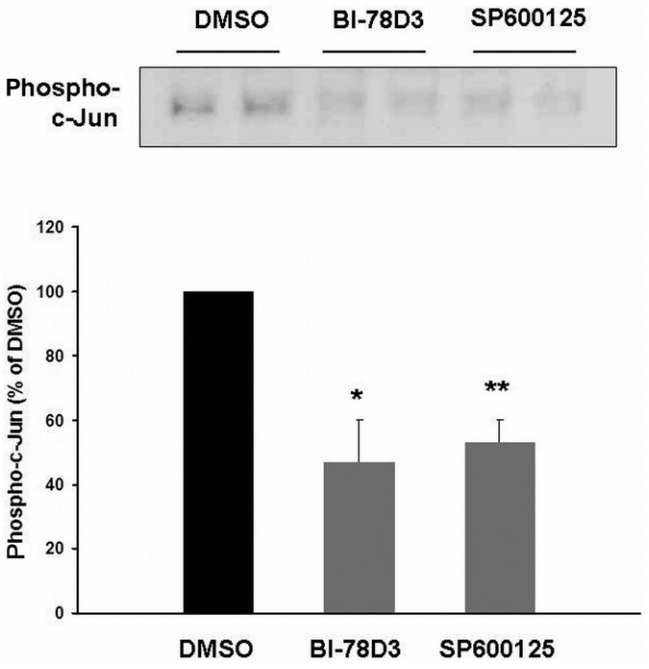

Figure 6

Effect of the JNK inhibitors BI-78D3 and SP600125 on phosphorylation of the JNK substrate, c-Jun at serine 63. Samples of human prostate tissue were stimulated *in vitro* with SP600125 (50 μM) and BI-78D3 (30 μM) for 2 h, assessed for phospho-JNK by Western blot analysis. Shown is a representative Western blot for phospho-JNK, and densitometric quantification of all experiments (data are means ± SEM from independent experiments with tissues from $n = 5$ patients). $^*P < 0.01$, $^{**}P < 0.001$ versus DMSO.

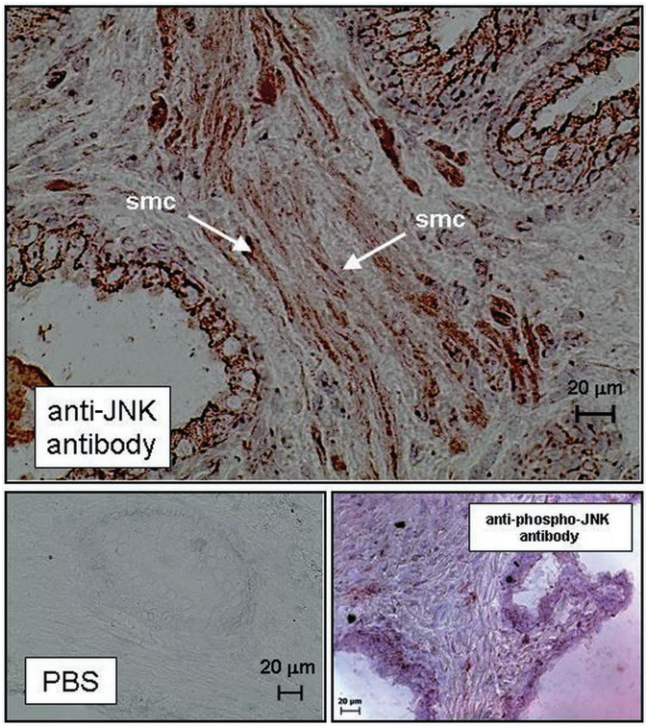

Figure 7

Immunohistochemical detection of JNK expression and phospho-JNK in human prostate tissue. Sections of human prostate tissue were stained by the peroxidase technique using antibodies for JNK or phospho-JNK. In control stainings, the primary antibody was replaced by 'PBS' (lower panel). Shown are representative stainings from experiments with tissues from $n = 5$ patients with similar results. Examples of smooth muscle cells ('smc') are indicated by arrows.

are required to find out whether JNK represents a reasonable target for therapy of LUTS.

A similar role of JNK for α_1-adrenoceptor-mediated contraction has been previously assumed for vascular smooth muscle. Thus, the JNK inhibitor SP600125 blocked the noradrenaline-induced contraction of rat aortic rings (Lee *et al.*, 2006; Zhou *et al.*, 2010). This was confirmed by *in vivo* studies, where JNK inhibitors caused hypotension and decreased peripheral vascular resistance in anaesthetized rats (Liu *et al.*, 2007; Zhou *et al.*, 2010). We speculate that JNK is of similar relevance for the contraction of prostate smooth muscle as in vascular smooth muscle. In fact, SP600125 and BI-78D3 blocked the contraction of human prostate strips, regardless of whether the contraction was elicited by phenylephrine, noradrenaline or EFS. Reduction of prostate smooth muscle tone is an important strategy for the treatment of LUTS in patients with BOO and LUTS.

Although SP600125 has been described as an inhibitor of JNK, its specificity may be limited (Bennett *et al.*, 2001; Bain *et al.*, 2003). To confirm the involvement of JNK in α_1-adrenoceptor-mediated prostate contraction, we tested the effect of BI-78D3 on noradrenaline- and phenylephrine-induced contraction of prostate strips. BI-78D3 is a JNK inhibitor, which is structurally unrelated to SP600125 (Stebbins *et al.*, 2008). Similar to SP600125, BI-78D3 inhibited both noradrenaline- and phenylephrine-induced contractions. This supports the conclusion that JNK is involved in α_1-adrenoceptor-mediated contraction of prostate smooth muscle. Finally, inhibition of JNK by both inhibitors was confirmed by Western blot analyses using a phospho-specific antibody for the JNK substrate, c-Jun. Application of SP600125 or BI-78D3 to prostate tissues resulted in reduced phosphorylation of c-Jun.

In vitro kinase assays using recombinant enzymes showed that SP600125 inhibits the three isoforms JNK1, -2 and -3 with similar potency (Bennett *et al.*, 2001). IC_{50} values during its application in intact tissues or cultured cells may differ considerably from those in biochemical assays. In a study investigating the effects of SP600125 on noradrenaline-induced contraction of rat aortic rings, 1 μM of SP600125 was without effect, while 10 and 100 μM dose-dependently inhibited contraction (Lee *et al.*, 2006). Therefore, we used an intermediate concentration from that range (i.e. 50 μM).

The present study shows that the current models of intracellular α_1-adrenoceptor signalling in the human prostate have to be extended. To the best of our knowledge, our findings show for the first time that a JNK-dependent mechanism may be involved in α_1-adrenoceptor-mediated prostate smooth muscle contraction, in parallel with the calcium- and Rho kinase-dependent mechanisms. Substrates, which are used by JNK to mediate prostate contraction, remain to be identified. In vascular smooth muscle, JNK may lead to con-

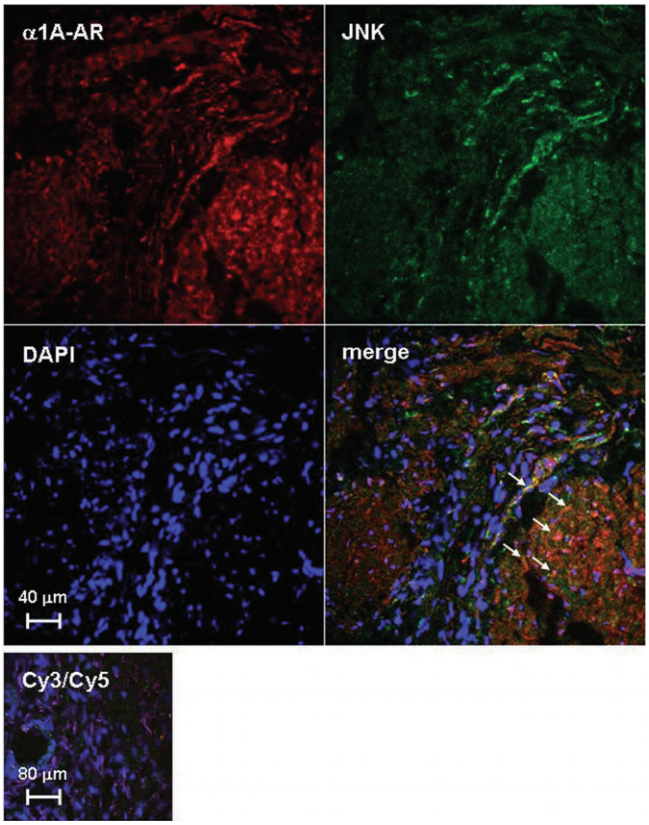

Figure 8

Co-localization of α_{1A}–adrenoceptors (α_{1A}-ARs) and JNK in human prostate smooth muscle cells. Using an immunofluorescence technique, prostate sections were double stained for α_{1A}-adrenoceptors and JNK. In control stainings ('Cy3/Cy5', lower panel), the primary antibodies were replaced by PBS. Arrows indicate examples of areas with strong signals for co-localization. Shown are representative stainings from experiments with tissues from $n = 5$ patients with similar results.

traction by phosphorylation of caldesmon (Zhou *et al.*, 2010). In fact, caldesmon is expressed and regulated by α_1-adrenoceptors in the human prostate (Walther *et al.*, 2012). Together, these findings demonstrate that intracellular signalling induced by prostate α_1-adrenoceptors is still not completely understood to date, despite the fact that expression and subtype distribution of α_1-adrenoceptors in the prostate have been widely studied (Michel and Vrydag, 2006).

JNK is a member of the MAPK family (Maroni *et al.*, 2004). Non-motoric JNK functions may differ between cell types and organs. The function of JNK in prostate cells has been investigated using different cell lines with diverse results (Maroni *et al.*, 2004). For non-malignant, epithelial human prostate cells, not only a pro-apoptotic, antiproliferative role of JNK activation but also JNK-dependent survival has been described (Uzgare and Isaacs, 2004; Wadsworth *et al.*, 2004). Proliferation after JNK activation has been reported from non-malignant, human stromal prostate cells (Koochekpour *et al.*, 2005). Several studies using different lines of prostate tumour cells have suggested a pro-apoptotic role in these cells (Sanchez *et al.*, 2007; Ho *et al.*, 2009; Liou *et al.*, 2009; Zhang *et al.*, 2009). What these studies have in common is that they all support the role of JNK in survival and growth of prostate cells.

We speculate that JNK may be of relevance for further functions besides contraction in prostate smooth muscle cells. In non-prostate smooth muscle cells, JNK participates in the regulation of growth and differentiation. In vascular and airway smooth muscle cells, JNK activation is involved in proliferation (Kim and Iwao, 2003; Zhai *et al.*, 2004). Interestingly, a role for JNK activation in vascular neointimal hyperplasia and in hyperplasia of airway smooth muscle has been proposed (Kim and Iwao, 2003; Xie *et al.*, 2007). In the bladder, mechanical stress leads to JNK-mediated hypertrophy (Yamaguchi, 2004). Together with our finding that α_1-adrenoceptors in the human prostate activate JNK, this suggests that α_1-adrenoceptor-mediated JNK activation in prostate smooth muscle cells may be of relevance for prostate hyperplasia. In fact, previous studies in rodents or using cultured prostate cells proposed that α_1-adrenoceptors represent one of many regulators of prostate growth (Golomb *et al.*, 1998; Kanagawa *et al.*, 2003; Marinese *et al.*, 2003). However, any clinical effect of α_1-blockers on prostate volume may be prevented by other important regulators such as androgens, growth factors or cytokines, which may cover the α_1-adrenoceptor-dependent component of growth (Royuela *et al.*, 1998; Lucia and Lambert, 2008).

In our experiments, we assessed agonist-induced changes in phospho-JNK by semi-quantitative comparisons between bands of the same blot in each experiment. We did not perform any comparisons between bands of different blots or films. Therefore, any variations in intensities of '0 min' samples, due to different exposure times of films or different levels of constitutive phospho-JNK, did not influence our measurements. Similar procedures were applied to examine agonist-induced phosphorylation of other targets in recent studies (Hennenberg *et al.*, 2011).

Our immunohistochemical stainings using a peroxidase technique demonstrated the expression of JNK in smooth muscle cells of human prostate tissue. This confirms results from previous studies (Royuela *et al.*, 2002; Ricote *et al.*, 2003), and supports the idea that α_1-adrenoceptor-mediated JNK activation is at least partially located in smooth muscle cells. This was in fact confirmed by double stainings using an immunofluorescence technique, where immunoreactivity for α_{1A}-adrenoceptors and JNK co-localized in smooth muscle cells. JNK was shown to be expressed in cultured prostate smooth muscle and stroma cells (Kanagawa *et al.*, 2003). However, in contrast to our study, adrenergic regulation was not observed in cultured cells (Kanagawa *et al.*, 2003). Thus, important cellular functions may get lost during cell culture. In our stainings, we focussed on α_{1A}-adrenoceptors, as this subtype is responsible for smooth muscle contraction in the human prostate (Marshall *et al.*, 1995; Roehrborn and Schwinn, 2004; Michel and Vrydag, 2006; Schwinn and Roehrborn, 2008).

Conclusion

Our findings provide evidence for a JNK-dependent mechanism contributing to α_1-adrenoceptor-mediated human pros-

tate smooth muscle contraction. Current models of intracellular signalling in prostate smooth muscle contraction should be extended to include this JNK-dependent pathway, which may represent a potential target for the treatment of LUTS in patients with BPH.

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Conflicts of interest

None.

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