Life Sciences 239 (2019) 117048

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The α1-adrenoceptor-mediated human hyperplastic prostate cells proliferation is impaired by EGF receptor inhibition

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ARTICLE INFO

Keywords:
Adrenoceptor
Benign prostatic hyperplasia
EGF
N-phenylpiperazine

ABSTRACT

Benign prostatic hyperplasia (BPH) is an aging-related and progressive disease linked to an up-regulation of α_1-adrenoceptors. The participation of EGF receptors (EGFR) in the GPCRs' signalosome has been described but so far data about the contribution of these receptors to prostatic stromal hyperplasia are scanty. We isolated and cultured vimentin-positive prostate stromal cells obtained from BPH patients. According to intracellular Ca^{2+} measurements, cell proliferation and Western blotting assays, these cultured hyperplastic stromal cells express functional α_1-adrenoceptors and EGFR, and proliferate in response to the α_1-adrenoceptor agonist phenylephrine. Interestingly, in these cells the inhibition of EGFR signaling with GM6001, CRM197, AG1478 or PD98059 was associated with full blockage of α_1-adrenoceptor-mediated cell proliferation, while cell treatment with each inhibitor alone did not alter basal cell growth. Moreover, the co-incubation of AG1478 (EGFR inhibitor) with α_{1A}/α_{1D}-adrenoceptor antagonists showed no additive inhibitory effect. These findings highlight a putative role of EGFR signaling to α_1-adrenoceptor-mediated human prostate hyperplasia, suggesting that the inhibition of this transactivation cascade could be useful to reduce BPH progression.

1. Introduction

Benign prostatic hyperplasia (BPH) is a progressive condition that affects aging men. The epithelial and stromal hyperplasia occurs in the periurethral prostatic transition zone favoring the occurrence of the lower urinary tract symptoms suggestive of BPH (LUTS/BPH). Prostate enlargement and the enhanced prostatic smooth muscle contraction are responsible for the static and dynamic components of BPH, respectively, and both components reduce bladder outflow favoring urinary retention [1–3].

The α_1-adrenoceptor belongs to the G protein-coupled receptors (GPCRs) family being composed by three receptor subtypes known as α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors [4]. The adult human prostate expresses mainly α_{1A}-adrenoceptors particularly in the stroma, and its expression is increased during BPH [5–9]. Therefore, it is well accepted that the mechanism underlying the BPH-related raise of smooth muscle contraction involves mainly the increased prostate expression of α_{1A}-adrenoceptors [5,6]. On the other hand, α_{1D}-adrenoceptors have been implicated in prostatic cell proliferation [6,10,11] and their mRNA expression is also up regulated in BPH patients [5,6]. α_{1A}-adrenoceptor blockers such as tamsulosin and silodosin are the first line drugs to treat low to moderate LUTS/BPH favoring bladder emptying, but they do not modify prostatic enlargement progression or the risk of BPH complications [12].

The etiology of prostate enlargement is complex with some evidence pointing to the involvement of metabolic and endocrine disorders, including roles of peptide growth factors, such as the epidermal growth factor (EGF) [2,13,14].

Two large families of membrane receptors, namely GPCRs and receptor tyrosine kinases, regulate cell proliferation among other cell functions. G protein-coupled receptors' signaling involves canonical and non-canonical pathways. In the case of α_1-adrenoceptor, the canonical pathway mediated by G protein involves the activation of phospholipase Cβ and the increase of intracellular Ca^{2+} [4]. On the other hand, the non-canonical receptor signaling activates other intracellular pathways including growth factor receptors signaling in a

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https://doi.org/10.1016/j.lfs.2019.117048
Received 17 June 2019; Received in revised form 24 October 2019; Accepted 5 November 2019
Available online 12 November 2019

Abbreviations

AG	AG1478
BPH	benign prostatic hyperplasia
CK	cytokeratin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GM	GM6001
GPCR	G protein-coupled receptor
HB-EGF	pro-ligand heparin-binding epidermal growth factor
LUTS	lower urinary tract symptoms
NA	noradrenaline
Phe	phenylephrine
Vim	vimentin
5-HT	serotonin

process known as transactivation [15–17].

The participation of EGF receptors in the GPCRs' signalosome is essential for physiological and disease-related conditions such as mitogenesis, inflammation, and muscle contraction [17–23]. Moreover, the pioneer work of Oganesian and colleagues [23] showed that the constitutive activity of naturally occurring variant of α_{1A}-adrenoceptor transfected to Rat-1 fibroblasts lead to cell proliferation. EGF receptors belong to the ErbB/HER protein family and are expressed in normal human prostate mainly in the epithelial cells [24,25]. However, the expression of these receptors in prostate from BPH patients expands to stromal cells [25], where α_1-adrenoceptors are mainly expressed, with EGF inducing stromal cell proliferation [26,27]. Besides, the availability of EGF receptor ligand depends on matrix metalloproteinases activity, which in turn may be activated by GPCR [15,24].

Previously we characterized two *N*-phenylpiperazine derivatives named LDT3 and LDT5 as new α_{1A}/α_{1D}-adrenoceptor antagonists, and we showed that the α_{1D}-adrenoceptor antagonist BMY7378 as well as LDT3 and LDT5 inhibit human prostatic cell proliferation *in vitro* [28,29], while another α_{1D}-adrenoceptor antagonist, naftopidil, inhibits cell proliferation *in vivo* [30]. Therefore, we explored the possible role of α_1-adrenoceptor-mediated transactivation of EGF receptor leading to mitogenesis of stromal cells from BPH patients. Our results indicate that EGF receptor transactivation is involved in the α_1-adrenoceptor-mediated proliferation of human hyperplastic prostatic cells providing insight into the understanding of BPH physiopathology, and suggesting that this transactivation cascade could be a target to reduce BPH progression.

2. Material and methods

2.1. Cell culture maintenance

Prostate tissue samples were collected from three patients with LUTS/BPH during transurethral resection and immediately placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) and transported to the Laboratory. The inclusion criteria of the patients were IPSS greater than 8 points, 50–70 years of age and PSA less than 2.5 ng/mL. Exclusion criteria were: previous prostatic surgery, prostate cancer, use of drugs that interfere with the function of adrenoceptors such as antagonists, anti-androgens and antidepressants, herbal extract, bladder catheter or neurogenic bladder. This study was approved by the ethics committee of the Federal University of Rio de Janeiro (UFRJ; CAAE-0029.0.197.000–05; 2009) and all experiments were performed in accordance with relevant guidelines and regulations. Informed written consent was signed by all donors. Briefly, prostate tissue was washed in phosphate buffer solution (PBS) before minced. The prostate fragments (1 mm^3) were added to DMEM supplemented with 10% heat-inactivated fetal bovine serum containing 1 mg/mL type I collagenase. Tissue specimens were dissociated by magnetic bar constant stirring for 2–4 h at 37 °C. The recovered cells were seeded in 25 mm^3 flasks cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% sodium pyruvate and 1% penicillin/streptomycin (100 IU/mL and 0.1 mg/mL, respectively), and fungizone (25 μg/mL) at 37 °C and 5% CO_2. Cells were observed at phase contrast microscopy (x250) to analyze morphology and confluence. At sub-confluence (approximately 90% confluence), cells were harvested using 0.05% trypsin/EDTA, and plated. A homogeneous stromal cell culture was obtained after six passages. The subsequent steps were the sub-culture of the cells and storage at −70 °C until use. Cells were used at 9^{th} - 11^{th} passage since the initial cell harvesting [31].

Rat-1 fibroblasts stably expressing human α_{1A}- or α_{1D}-adrenoceptors were cultured in DMEM supplemented with 10% fetal bovine serum, 300 μg/mL neomycin analogue G418 sulfate, 1% penicillin/streptomycin (100 IU/mL and 0.1 mg/mL, respectively; 37 °C, 5% CO_2) until confluence. The receptor density of α_1-adrenoceptors estimated with [^{3}H]-prazosin is in the range of 1.0–1.5 pmol/mg of protein [28].

2.2. Immunofluorescence assay

To analyze BPH cell morphology we used an anti-human cytokeratin (CK) monoclonal antibody (Dako) and anti-vimentin (Vim) monoclonal antibody (Sigma) as primary antibodies. For this procedure, 2×10^4 cells (11^{th} passage) were plated and incubated for four days. Following, the culture medium was removed and cells were fixed with absolute cold ethanol for 20 min at room temperature, and then washed three times with PBS. The nonspecific binding was blocked with PBS/BSA 5% and then primary antibodies were added to the cultures and incubated for 2 h at room temperature. After that, cells were washed with PBS and incubated for an additional 2 h with goat anti-mouse Alexa 546 secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI (Santa Cruz Biotechnology). Finally, cells were washed in distilled water and mounted on histological slides with *N*-propylgallate (Sigma). Negative control conditions were performed by omitting the primary antibodies. No reactivity was observed when the primary antibody was absent. Images were captured using an inverted microscopy (Olympus IX81) and a Hamamatsu ORCA-R2 digital CCD camera using a 40× objective.

2.3. Intracellular [Ca^{2+}] measurement

In order to verify if primary stromal cell cultures from BPH patients express functional α_1-adrenoceptors we used a fluorimetric assay to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a robust marker of the canonical Gq signaling. Cells were serum-starved overnight. In the next day, cells were washed with PBS and loaded with 2.5 μM fura-2/AM in the dark for 60 min at 37 °C in Krebs-Ringer-HEPES solution containing (mM) NaCl 120, KH_2PO_4 1.2, $MgSO_4$ 1.2, KCl 4.75, glucose 10, $CaCl_2$ 1.2, HEPES 20, and 0.05% bovine serum albumin (pH 7.4). Thereafter, the cells were washed to remove the unincorporated dye, detached by gentle trypsinization, centrifuged (200 × *g*, 7 min, 4 °C), and incubated (10^6 cells/condition) for 100 s (baseline) and then stimulated with 10 μM noradrenaline (NA) or 100 μM phenylephrine (Phe). The antagonists (BMY7378, 50 nM; WB4101, 50 nM or tamsulosin, 5 nM) were pre-incubated for 100 s before addition of the agonists, and their concentrations were previously defined based on their affinities [28]. Fluorescence measurements were performed at 340 and 380 nm excitation wavelengths and 510 nm emission wavelength, with a chopper interval set at 0.5 s, using an Aminco-Bowman Series 2 luminescence spectrometer (Rochester, NY). Peak fluorescence values were used for data analysis, and the

intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated, as described by Grynkiewicz et al. [32].

2.4. Cell growth assays

Briefly, cells (3×10^3 cells/well) were plated in 96-well plates in serum-free DMEM for 24 h. Sub-confluent cells were kept in DMEM (basal) or stimulated with 3 μM phenylephrine (Phe) for 48 h in the absence or presence of GM6001 (10 μM; metalloproteinase inhibitor), CRM197 (200 ng/mL; HB-EGF inhibitor), AG1478 (5 μM; selective EGFR tyrosine kinase inhibitor) or the MEK inhibitor PD98059 (1 μM) added 30 min before addition of the agonist. EGF 100 ng/mL (48 h) was used as positive control. All concentrations were chosen based on their affinities for the targets and previously reported in the literature [19,22]. Alternatively, cells were also treated with 3 μM phenylephrine for 48 h in the absence or presence of LDT3 or LDT5 (50 nM) alone, or in combination with AG1478 (5 μM), added 30 min before. The medium was changed every 24 h with fresh dilutions of drugs. Cell growth was evaluated by counting of viable cells using Trypan blue as an exclusion dye or by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [28]. Otherwise indicated, each assay was performed in quadruplicate to calculate the mean value of one experiment. Data were expressed as the percentage of the basal condition which was considered as 100%.

2.5. Western blot assays

BPH cells (1×10^6) seeded on 6-well plate were incubated with 100 μM phenylephrine (Phe) or 10 μM noradrenaline (NA; in the presence of 1 μM propranolol added 2 min before) for 2, 5, 15, and 30 min in serum free medium. After treatment, cells were lysed with 200 μL of RIPA buffer (150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM NaF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 50 mM Tris-HCl, pH 7.4) under agitation, centrifuged at $13,000 \times g$ for 5 min (4 °C) and the supernatants were used for evaluation of activation (phosphorylation) of extracellular signal-regulated kinases 1 and 2 (ERK 1/2). Protein content was determined according to the method of Lowry et al. Twenty μg of proteins were boiled for 5 min and resolved using 10% SDS/PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated for 1 h with Tris buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Then, membranes were washed 3 times in TBS-T and incubated overnight with the primary monoclonal antibodies phospho-p44/p42 MAPK (p-ERK 1/2 Thr202/Tyr204, #9101; 1:1000), or p44/p42 MAPK Total (ERK 1/2, #4695; 1:1000) diluted in TBS-T with 5% non-fat milk at 4 °C with gentle shaking. The peroxidase-conjugated anti-rabbit IgG secondary antibody (#7074; 1:10000) was diluted in cold TBS-T and incubated for 1 h. Membranes were washed in TBS-T and detection of immunoreactivity was performed by enhanced chemiluminescence (ECL, Westar Cyanagen). Protein blot images were scanned and the optical density was obtained using ImageJ software (NIH, USA). The relative expression of p-ERK was normalized in relation to total ERK (also used as loading control).

2.6. Human androgen receptor binding assay

Filtration based radioligand binding assays for human prostate androgen receptors (LNCap cells; catalogue number 0933) using 1 nM [^{3}H]-methyltrienolone (4 °C, 24 h) were performed by Eurofins Cerep SA, France (Study 100018032). Mibolerone (1 μM) was used as positive control and showed an IC_{50} value of 1.9 nM. LDT3 and LDT5 were tested at the final concentration of 1 μM and the results were expressed as the percentage of the specific binding of [^{3}H]-methyltrienolone (considered as 100%).

2.7. Reagents and antibodies

LDT3 and LDT5 were synthesized as previously described [28]. The following reagents were acquired from the sources indicated between parenthesis: (R)-(−)-phenylephrine hydrochloride, (±)-propranolol hydrochloride, (−)-noradrenaline, fungizone, EGF, CRM197 and reagents for RIPA buffer (Sigma Aldrich, USA). Fura 2/AM (Molecular Probes, USA). Dulbecco's modified Eagle's medium (DMEM), fetal

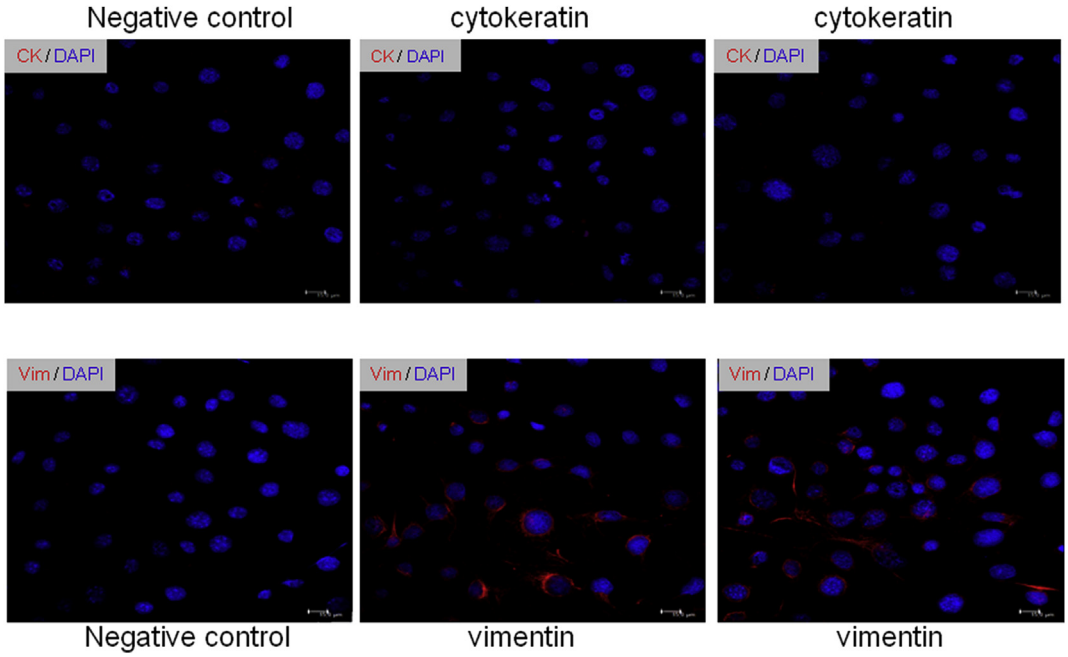

Fig. 1. BPH cells are positive for vimentin and negative for cytokeratin staining. Cells were incubated with anti-human primary monoclonal antibodies against cytokeratin (upper panels, CK) or vimentin (lower panels, Vim, red). In all immunostaining-negative controls, reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was absent. Blue staining = DAPI. Bars = 150 μm, objective 40×. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bovine serum (South America), trypsin, penicillin (10000 IU/mL)/streptomycin (10 mg/mL), PD98059 and other reagents for cell culture (Life Technologies, USA). AG1478 (Calbiochem, USA). GM6001 (Merck Millipore, USA). Rabbit monoclonal primary antibodies against extracellular signal-regulated kinases (ERK) 1/2 (#4695), and phospho-ERK 1/2 (#9101) (Cell Signaling, USA). Monoclonal anti-human cytokeratin antibody (#0821, Dako, USA). Monoclonal anti-human vimentin antibody (#V2258, Sigma Aldrich, USA). Anti-rabbit IgG secondary antibody (#7074, Cell Signaling, USA) and anti-mouse Alexa 546 secondary antibody (#A11003, Invitrogen, USA).

2.8. Statistical analysis

Otherwise indicated, data are expressed as mean and S.E.M. of 3–5 independent experiments. Independent experiments were performed using different primary cell cultures. The significance of the differences among two or more conditions was determined by two-tailed Student's *t*-test or one-way analysis of variance (one way ANOVA), respectively. ANOVA was followed by Dunnett's multiple comparisons test using the software GraphPad Prism 6.0 (Graphpad, La Jolla, CA, USA). Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. BPH stromal cells express functional α_1-adrenoceptors

The cultured hyperplastic cells are positive for vimentin and negative for cytokeratin staining which is a signature of stromal cells (Fig. 1). The canonical α_1-adrenoceptor signaling is linked to the increase of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. As shown in Fig. 2, the stimulation of BPH cells expressing native α_1-adrenoceptors with either 100 μM phenylephrine or 10 μM noradrenaline induced similar increments of $[Ca^{2+}]_i$ in relation to basal, which indicates that the receptors are functional. We chose the selective agonist of α_1-adrenoceptor phenylephrine to perform the subsequent experiments.

The agonist specificity for α_1-adrenoceptor was confirmed by measuring the intracellular Ca^{2+} concentration in Rat-1 fibroblasts stably expressing α_{1A}- or α_{1D}-adrenoceptors subtypes, the two adrenoceptor subtypes relevant for BPH. Cell pre-incubation with the antagonists WB 4101 (50 nM; α_{1A}-adrenoceptor antagonist) and tamsulosin (5 nM, $\alpha_{1A/1D}$-adrenoceptor antagonist), but not with BMY7378 (50 nM, α_{1D}-adrenoceptor antagonist), blocked the effect of 100 μM phenylephrine in cells expressing α_{1A}-adrenoceptors, while BMY7378 (50 nM) blocked the agonist effect only in cells expressing α_{1D}-adrenoceptors (Fig. 3A and B).

3.2. The EGF receptor modulates the α_1-adrenoceptor-mediated BPH cell growth

The presence of EGF receptors in the stromal cells was evaluated by cell proliferation. Cells were stimulated with EGF (100 ng/mL) in the absence and presence of an antagonist. As shown in Fig. 4A, the EGF-induced BPH cell proliferation was blocked by the EGF receptor tyrosine kinase inhibitor (AG1478, 5 μM). AG1478 *per se* did not alter basal cell growth discarding a putative cytotoxicity. Interestingly, phenylephrine (3 μM) increased BPH cell proliferation similarly to EGF. Then, in order to evaluate a possible dependence on the EGF receptor for the α_1-adrenoceptor-mediated BPH cell proliferation, we investigated if the blockage of downstream EGF receptor signaling could alter phenylephrine-mediated cell growth.

GM6001 (10 μM) is a pan inhibitor of the matrix metalloproteinases involved in pro-ligand heparin-binding epidermal growth factor (HB-EGF) cleavage, and consequently EGF receptor signaling [23]. GM6001 treatment did not alter the basal cell proliferation but prevented cell proliferation induced by phenylephrine (Fig. 4B), and the specific inhibitor of human HB-EGF, CRM197 (200 ng/mL) [18], had the same effect. The selective EGF receptor tyrosine kinase inhibitor AG1478 (5 μM) also prevented completely the effect of phenylephrine, without altering the basal cell proliferation. Cells were treated with the MEK inhibitor PD98059 (1 μM) which also inhibited cell proliferation in response to phenylephrine (Fig. 4B). Similar qualitative results were obtained using the MTT assay (Fig. 4C). Moreover AG1478, GM6001 and PD98059 blunted the EGF-mediated cell growth (Fig. 4D). None of the inhibitors *per se* inhibited cell growth. Altogether, the inhibition of sequential steps of EGF receptor signaling impaired the proliferative effect of the α_1-adrenoceptor agonist phenylephrine.

3.3. α_1-adrenoceptor agonists induce ERK phosphorylation in BPH cells

Since BPH cell proliferation induced by activation of α_1-adrenoceptor was prevented by the inhibition of EGF receptor signaling, which usually involves activation of the mitogen-activated protein kinase pathway, we then evaluated the impact of phenylephrine on ERK 1/2 activation by quantifying the ratio of phospho-ERK (p-ERK) to total ERK protein. Due to BPH cell limitation, we used only one concentration of phenylephrine (100 μM) which has been used elsewhere to activate ERK 1/2 pathway [33,34]. Moreover, previous data obtained from different cell systems showed that this concentration usually induces the maximal effect of the agonist [33,34].

Phenylephrine stimulated ERK 1/2 activity in stromal BPH cells in a time-dependent manner, and its effect peaked after 15 min. A similar stimulatory effect was also observed with the α_1-adrenoceptor agonist noradrenaline (10 μM, in the presence of propranolol 1 μM). In these experiments, EGF (100 ng/mL, 5 min stimulation) was used as a positive control and it stimulated ERK 1/2 activation (Fig. 5A–C). In isometric contraction assays, 100 μM phenylephrine added in the plateau of the contraction induced by 1 μM 5-HT, and in the presence of 1 μM prazosin, failed to relax rat aorta therefore discarding a putative β-adrenergic effect (*data not shown*). Previously we showed that the selective α_{1D}-adrenoceptor antagonist BMY7378, as well as the α_{1A}-/α_{1D}-adrenoceptor antagonists LDT3 and LDT5, inhibited human hyperplastic prostate stromal cell growth mediated by phenylephrine [28]. Current data showed that AG1478 (5 μM) inhibited the proliferative effect of phenylephrine, and the association with the α_{1A}/α_{1D}-adrenoceptor antagonists LDT3 or LDT5 (50 nM) resulted in similar inhibition suggesting that in this model the EGF receptor pathway is necessary for α_1-adrenoceptor-mediated BPH cell proliferation (Fig. 6). Of note, this inhibitory effect of LDT3 or LDT5 (50 nM) did not involve the inhibition of prostate androgen receptors (AR) since even at a much higher

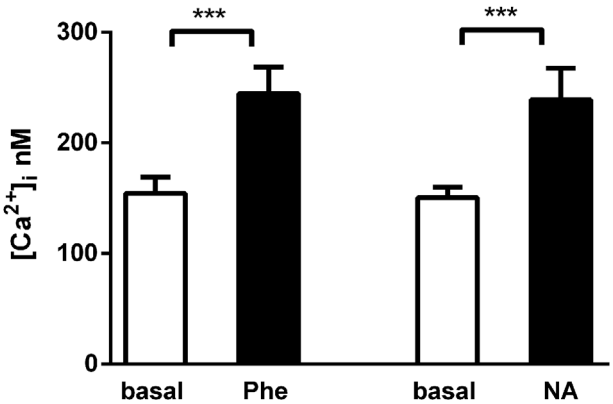

Fig. 2. Phenylephrine and noradrenaline increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in hyperplastic prostatic stromal cells obtained from BPH patients. Cells were loaded with 2.5 μM fura-2 AM for 60 min before stimulation with the agonists (10 μM NA, 100 μM Phe). Data were expressed as mean and S.E.M. (n = 7–10 replicates from 3 individual experiments). ***P = 0.007 vs. basal (two-tailed Student's *t*-test). Phe = phenylephrine; NA = noradrenaline.

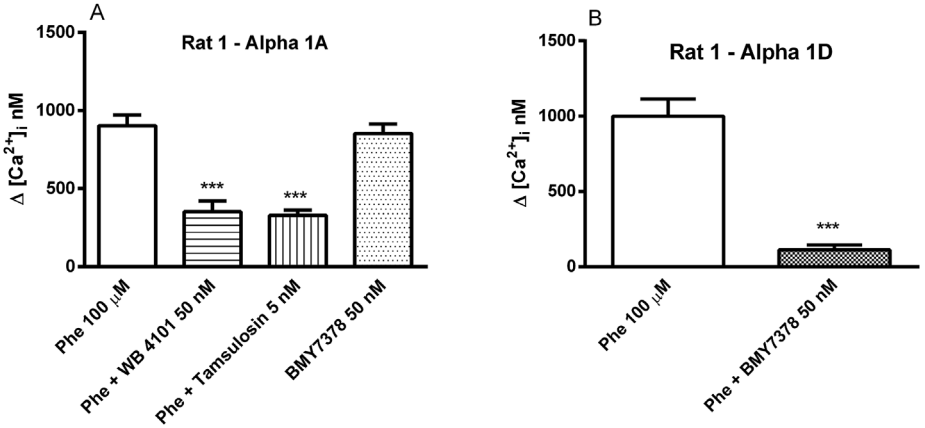

Fig. 3. The increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Rat-1 cells transfected with human α_{1A}- or α_{1D}-adrenoceptors induced by phenylephrine is inhibited by selective antagonists added 100 s before. Cells were loaded with 2.5 μM fura-2 AM for 60 min before stimulation with phenylephrine (100 μM Phe). WB 4101 (50 nM): α_{1A}-adrenoceptor antagonist; tamsulosin (5 nM): $\alpha_{1A/D}$-adrenoceptor antagonist; BMY7378 (50 nM): α_{1D}-adrenoceptor antagonist. Data represent the difference (Δ) between the basal and agonist-induced increase of fluorescence, and were expressed as mean and S.E.M. (n = 6–8 replicates from 3 individual experiments). ***P < 0.001 vs. Phe (A, one way ANOVA followed by Dunnett's multiple comparisons test; B, two-tailed Student's *t*-test).

concentration (1 μM) they did not inhibit the specific binding of the AR agonist (LDT3: 6.9 ± 4.9%, n = 2; LDT5: 17.7 ± 8.1%, n = 2).

4. Discussion

BPH is an aging-related disease linked to an imbalance between prostate cell proliferation and apoptosis, favoring hyperplastic stromal cell growth [35]. Mounting evidence points to the importance of GPCRs for cell proliferation in pathological conditions [17,22,23]. Here we showed that the α_1-adrenoceptor-mediated proliferation of human hyperplastic prostatic stromal cells is fully inhibited by EGF receptor and MEK inhibitors suggesting the transactivation of the EGF receptors by α_1-adrenoceptors as an important event for BPH cell proliferation. To the best of our knowledge this is the first report about the stromal α_1-adrenoceptor-EGF receptor signaling in BPH.

The prostatic stroma makes up a large percentage of prostate volume during BPH, and the use of human stromal cells is considered valuable for BPH studies [35,36]. However, the isolation of stromal

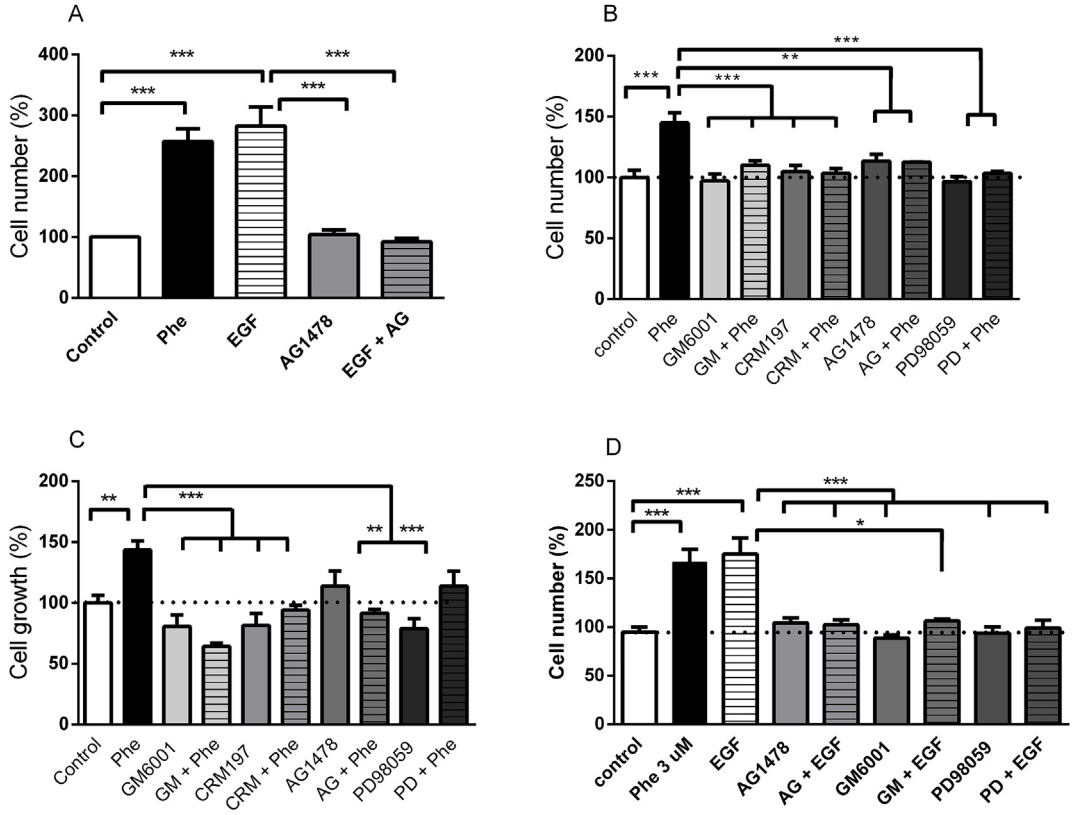

Fig. 4. The BPH cell proliferative effect of phenylephrine depends on the activation of EGF receptors. A) Hyperplastic stromal cells in culture were treated with 3 μM phenylephrine (Phe) or 100 ng/mL EGF for 48 h in the absence (control, white bar) or presence of the EGF receptor tyrosine kinase inhibitor AG1478 5 μM ***P < 0.001 Phe and EGF vs. control; AG1478 and EGF + AG vs. EGF (one way ANOVA followed by Dunnett's multiple comparisons test).
B and C) Hyperplastic stromal cells were treated with 3 μM phenylephrine (black bar) for 48 h in the absence (control, white bar) or presence of the following inhibitors of EGF receptor signaling: GM = GM6001 10 μM; CRM = CRM197 200 ng/mL; AG = AG1478 5 μM; PD = PD98059 1 μM. Cell proliferation was accessed by cell counting using Trypan Blue exclusion dye (B) and MTT assays (C). D. The EGF-mediated cell growth (100 ng/mL) is fully inhibited by AG1478, GM6001 and PD98059. The inhibitors alone did not alter basal cell proliferation (P > 0.05). Data were expressed as mean and S.E.M. of 3 experiments (performed in duplicate (A) or quadruplicate (B)), or 4 experiments performed in quadruplicate (C, D). B and C: **P < 0.01 and ***P < 0.001 vs. Phe; D: *P < 0.05 and ***P < 0.001 vs. control or EGF (one way ANOVA followed by Dunnett's multiple comparisons test).

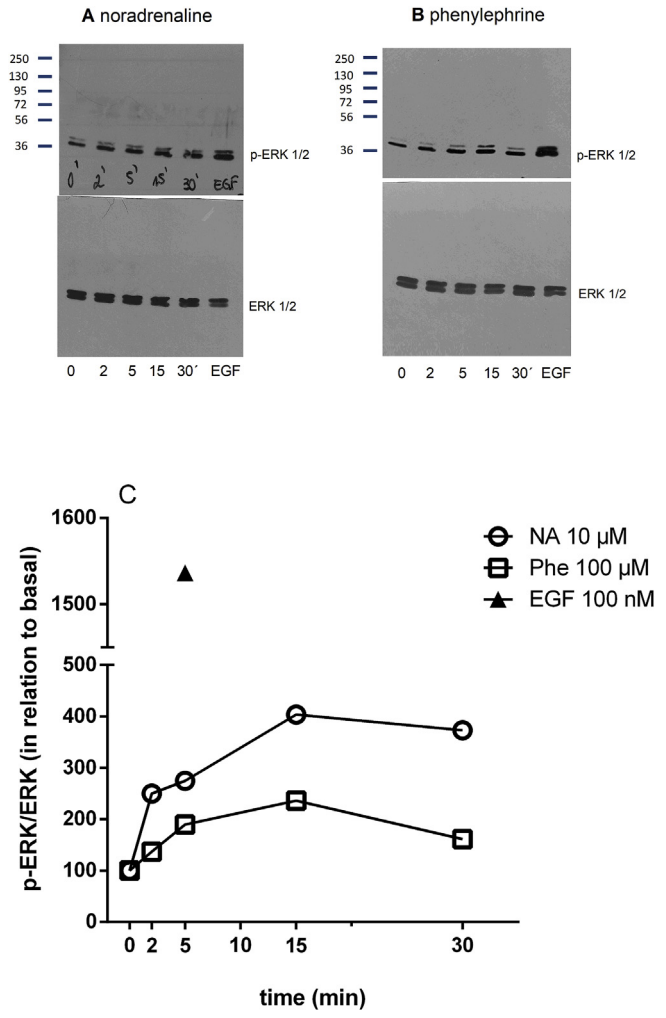

Fig. 5. Time-dependent activation of ERK 1/2 by phenylephrine and noradrenaline in hyperplastic stromal cells obtained from BPH patients (full-length representative blots). Cells were treated with 100 µM phenylephrine (Phe; A), 10 µM noradrenaline (NA) in the presence of 1 µM propranolol (B), for the indicated times or 100 ng/mL EGF (5 min, A-C). C. Densitometric analysis. Data were expressed as mean of 2 individual experiments.

cells from human prostate yields a small quantity of material. The cell immortalization frequently causes the loss of cell characteristics, making the screening of immortalized cell clones for lines that keep the phenotypic characteristics needed. For instance, immortalized human prostate stromal cells show an increased expression of α_{1B}-adrenoceptors which is not observed in human prostate or primary cultured cells [37]. Therefore we used primary cell culture obtained from BPH patients (*i.e.*, non-transformed cells). In our model, cultured cells stained positively for vimentin, and negatively for the epithelial cell marker cytokeratin, indicating the predominance of stromal cells.

The proposed ratio of α_{1A}:α_{1B}:α_{1D}-adrenoceptors mRNA in normal human prostate is approximately 63:6:31%, respectively [6]. In support to these data real time RT-PCR assays corroborate the predominance of α_{1A}- and α_{1D}-adrenoceptors mRNA [5]. Moreover, an increased expression of α_{1A}- and α_{1D}-adrenoceptors mRNA has been reported in BPH, and the α_{1D}-adrenoceptor mRNA has a more pronounced increased expression (as high as three times) [5,6]. Therefore these upregulated receptors are supposed to participate in the pathophysiology of BPH. However, most, if not all, commercially available antibodies against each subtype of α_1-adrenoceptor lacks selectivity, which limits the quantification of the protein at cellular level [38], and the identification of functional α_1-adrenoceptor subtypes relies on the use of

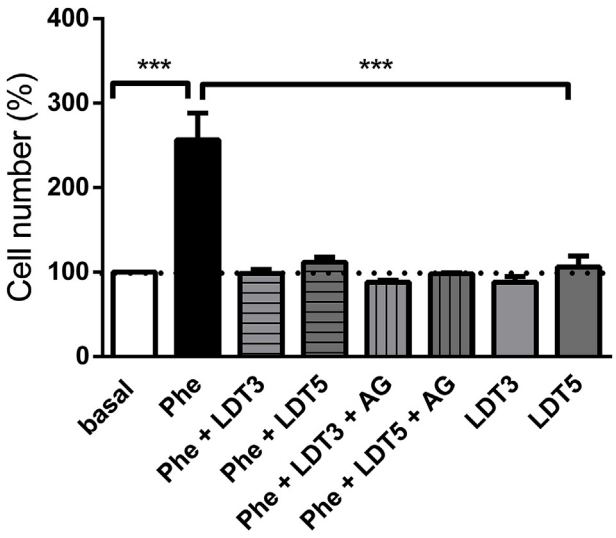

Fig. 6. The BPH cell proliferative effect of phenylephrine is completely inhibited by α_1-adrenoceptor antagonists (LDT3 and LDT5) or AG1478, an EGF receptor tyrosine kinase inhibitor, alone or in association. The co-incubation of AG1478 (5 µM) with the α_1-adrenoceptor antagonists did not potentiate the inhibitory effect of LDT3 and LDT5. Phe = phenylephrine. Data were expressed as mean and S.E.M. of 2 individual experiments performed in triplicate. ***P < 0.001 all conditions vs. Phe (one way ANOVA followed by Dunnett's multiple comparisons test).

selective drugs.

Our data showed that BPH stromal cells express functional α_1-adrenoceptors since phenylephrine, a selective α_1-adrenoceptor agonist, activated the canonical signaling pathway involving the increase of $[Ca^{2+}]_I$. Similar data were obtained using the endogenous non-selective adrenoceptor agonist noradrenaline, when tested in the presence of propranolol for blocking its β–adrenoceptor effects. Moreover, functional data suggest that α_{1D}-adrenoceptor is involved in the BPH cell proliferative effect of phenylephrine since BMY7378, a selective α_{1D}-adrenoceptor antagonist, blocked the agonist effect [28].

EGF receptor is a membrane-bound glycoprotein expressed in normal and hyperplastic human prostate [39,40] including stromal cells [27,35, and present data]. One of the proposed mechanisms leading to EGF receptor transactivation by GPCR includes the action of metalloproteinases promoting the shedding of members of EGF family such as HB-EGF, and consequently the binding to its cognate receptor [15,18,20]. As a consequence, metalloproteinase inhibition is reported as a strategy to interrupt EGF receptor transactivation. Here we showed that inhibition of metalloproteinase prevented BPH cell growth mediated by phenylephrine, which could suggest that the activation of α_1-adrenoceptor induces EGF receptor transactivation. In support of these data it was previously shown in rat lacrimal gland epithelial cells that α_{1D}-adrenoceptor mediates the shedding of EGF and EGF receptor activation [41].

In our model EGF induced stromal cell proliferation. As shown by Duque and colleagues [27], both HB-EGF and EGF (100 ng/mL) induce a similar mitogenic effect upon human prostate stromal cell suggesting a role of these agonists in BPH. Moreover, stromal cells express HB-EGF mRNA and the HB-EGF inhibitor CRM197 inhibits in a concentration-dependent manner the stromal cell growth [27].

There is evidence that a naturally occurring human α_{1A}-adrenoceptor genetic variant is constitutively coupled to EGFR transactivation [23]. In support of our hypothesis that α_1-adrenoceptors might induce transactivation of EGF receptor in BPH cells, the HB-EGF inhibitor CRM197, as well as the EGF tyrosine kinase receptor inhibitor AG1478, prevented hyperplastic cell growth induced by phenylephrine. Of note, the concentration of AG1478 used (5 µM) fully inhibited the

phenylephrine- and the EGF-induced cell proliferation. As ERK 1/2 activation is important for cell proliferation, and it may be activated by EGF receptor signaling, we investigated the effect of the MEK inhibitor PD98059, which also inhibited the proliferative effect of phenylephrine. Accordingly, in Western blotting assays, besides EGF (positive control), phenylephrine (and noradrenaline) also stimulated ERK 1/2 phosphorylation corroborating functional data. Therefore, our results suggest that α_1-adrenoceptors (most probably α_{1D}-subtype) transactivate EGF receptors in stromal cells from BPH patients leading to cell proliferation. However, present data do not rule out that the canonical signaling of α_1-adrenoceptor (*i.e.*, Gq canonical signaling) could also activate ERK pathway. On the other hand, as the inhibition of EGF receptor signaling did not reduce cell proliferation in the absence of phenylephrine, we could suggest that α_1-adrenoceptor-mediated EGF receptor transactivation is mainly agonist-dependent, rather than constitutively active as previously shown in cardiomyoblasts [42].

Different sets of evidence obtained by others indicate that transactivation of EGF receptors is involved in α_1-adrenoceptor signaling. For instance, it has been shown that α_{1D}-adrenoceptor activation induces the shedding of biologically active EGF and subsequent EGF receptor transactivation, and an EGF neutralizing antibody reduces phenylephrine-induced ERK activation in rat lacrimal gland [41]. Moreover, α_1-adrenoceptor activation induces ERK 1/2 activity in rat aorta myocytes (mainly α_{1D}-adrenoceptor type) [19], and in human epithelial prostatic cells where ERK 1/2 activity was related to cell volume regulation [43]. In good accordance with our data, the metalloproteinase inhibitor GM6001 [41] and the EGF receptor inhibitor AG1478 [19] blocked ERK 1/2 activation in response to phenylephrine linking α_1-adrenoceptor to EGF receptor transactivation. On the other hand, the β-adrenoceptor-mediated EGF receptor transactivation may involve (COS-7) or not (brown adipocytes) ERK 1/2 pathway [44,45]. Therefore ERK 1/2 activation during EGF receptor transactivation depends both on GPCR and cell type.

It is noteworthy that the co-incubation of EGF receptor inhibitor AG1478 with the α_{1A}-/α_{1D}-adrenoceptor antagonists LDT3 or LDT5 resulted in full inhibition of cell proliferation, which was similar to the inhibition promoted by each drug alone. A putative nonspecific inhibition of androgen receptors by these α_1 adrenoceptor antagonists was ruled out by binding assays. Moreover, as previously shown by our group or by others, the α_{1D}-adrenoceptor selective antagonists BMY7378 and naftopidil also fully inhibited the proliferative effect of phenylephrine in BPH cells [28,30]. However, in these models tamsulosin, a benzenesulfonamide derivative, showed no effect [28,30]. Furthermore, tamsulosin alone did not inhibit the increase of volume of the cell line BPH-1 in response to phenylephrine [43]. In common, BMY7378, naftopidil, LDT3 and LDT5 are α_{1D}-adrenoceptors antagonists that share the *N*-phenylpiperazine moiety which could shape some inhibitory functional selectivity upon BPH cell proliferation, and therefore other experiments would be welcome. Patients' adherence to current pharmacotherapy is low, a fact that favors BPH progression [46] and stimulates the search for new drugs. Understanding the mechanisms involved in prostate cell proliferation may help the development of new drugs.

In conclusion, our findings demonstrate that α_1-adrenoceptor activation in human hyperplastic prostate cells induces canonical and non-canonical signaling. The α_1-adrenoceptor non-canonical signaling involved in mitogenesis of BPH cells depends on EGF receptor transactivation. This mechanism could contribute to prostate enlargement and to the development of LUTS/BPH, and therefore our data give new insight into the physiopathology of BPH. We therefore propose that blockage of this transactivation cascade could be a putative non-hormonal pharmacological strategy to reduce concomitantly LUTS and BPH progression.

Author contributions

Conducted experiments: JBNV, RAH, LACB.

Contributed with reagents or cell culture: LASR, LEN, EOB, JAGS.

Participated in research design and coordinated experiments: CLMS, JAGS.

Performed data analysis, discussion, revised the manuscript: JBNV, RAH, PRF, FN, JAGS, CLMS.

Wrote the manuscript with important contributions by all authors: CLMS. All authors read and approved the final version of the manuscript.

Declaration of competing interest

None.

Acknowledgements

Supported by The Brazilian National Council for Scientific and Technological Development (CNPq, Brazil; Grants #306431/2015-7, #455436/2014-2, #312709/2017-0). JBNV was a PDJ fellow of CNPq (#166215/2015-5). CLMS, FN, LEN, and LASR are senior fellows of CNPq (Brazil). The Study 100018032 was sponsored by Biozeus Desenvolvimento de Produtos Biofarmacêuticos S.A. (Brazil). The funding source had no role in the study design, collection, analysis or interpretation of data, writing or submission of the manuscript. Orlando R. Moreira (UFRJ) for technical assistance.

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