Benign Prostatic Hyperplasia

Immunohistochemical Distribution of Cyclic GMP-Dependent Protein Kinase-1 in Human Prostate Tissue

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Abstract

Objectives: Phosphodiesterase 5 (PDE5) inhibitors improve smooth muscle relaxation and therefore are considered for pharmacotherapy of benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS). Cyclic guanosine monophosphate (cGMP)-dependent protein kinase-1 (cGKI) has been identified as one of the downstream targets for cGMP. The aim of the present study was to evaluate, by means of immunohistochemistry and Western blot analysis, the expression and localization of cGKI isoforms in relation to smooth muscle a-actin and cGMP in the human prostate.

Methods: Cryostat sections of tissue segments excised from the transition zone of human prostates from 11 patients (aged 54–68 yr) were incubated with primary antibodies directed against smooth muscle a-actin, cGMP, cGKI, cGKI\textsubscript{\alpha}, and cGKI\textsubscript{\beta}. Visualization of double-labelled immunofluorescent staining was achieved by laser microscopy. Western blot analysis was performed to confirm the expression of cGKI isoforms.

Results: Immunoreactivities specific for cGKI, cGKI\textsubscript{\alpha}, and cGKI\textsubscript{\beta} were observed in smooth muscle cells of the fibromuscular stroma. The expression of cGKI isoforms was confirmed by Western blot analysis.

Conclusions: Our results confirm the presence of cGKI isoforms \alpha and \beta in the transition zone of human prostate tissue. In addition, the colocalization of \alpha-actin, cGMP, and cGKI isoforms provides further evidence for a significant role of the nitric oxide/cGMP pathway in the regulation of smooth muscle contractility in human prostate tissue and therefore could provide additional targets for pharmacotherapy of BPH and LUTS.

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1. Introduction

Smooth muscle hyperactivity, mediated by a variety of contractant factors, such as increased sympathetic activity and increased stimulation of smooth muscle α-adrenoceptors, might be a pathophysiological factor associated with lower urinary tract symptoms (LUTS) and benign prostatic hyperplasia (BPH). Furthermore, the nitric oxide (NO)/cyclic GMP (cGMP) signalling pathway plays an important role in the tonus regulation of human urogenital smooth musculature [1,2]. Therefore, NO/cGMP signalling could be a possible target for pharmacotherapy to counteract contraction of the smooth muscle in the human prostate. Phosphodiesterase 5 (PDE5) inhibitors have been considered for pharmacotherapy of BPH and LUTS [3]. Clinical studies indicated an influence of PDE5 inhibitors on LUTS, and a positive effect of sildenafil on LUTS from BPH while treating erectile dysfunction has been described [4,5]. Preliminary findings of a randomized, double-blind, placebo-controlled Phase 2 study suggest that tadalafil significantly improved LUTS in men with BPH, as measured by the International Prostate Symptom Score [6].

In addition to cGMP-regulated PDEs and cyclic nucleotide–gated cation channels, cGMP-dependent protein kinase-1 (cGKI) has been identified as a major downstream target for cGMP signalling in the cardiovascular system [7,8]. Two cGKI isoforms (cGKIα ≈ 76 kDa, cGKIβ ≈ 78 kDa) have been identified, and both isoforms are present in smooth muscle cells [9–11]. However, cGKIα is activated at a significantly lower cGMP concentration than is cGKIβ. In addition, defective Ca2+ regulation in cGKI-deficient vascular smooth muscle cells can be alleviated by transfection of the cGKIα isoform but not the cGKIβ isoform [12]. Studies have suggested that cGKIα-mediated smooth muscle relaxation may involve a decrease in the cytosolic Ca2+ level as well as inhibition of Ca2+ sensitization and modulation of the activity of myosin phosphatase [13]. Because most studies have been conducted on cardiovascular tissue, however, there is only limited evidence for a significant role of cGKI-mediated signal transduction in urogenital organs.

BPH develops mainly in the periurethral transition zone of the prostate and is histopathologically characterized by an increased number of stromal and epithelial cells. Functional and immunohistochemical studies evaluating the NO/cGMP pathway and its relevance with regard to the regulation of tone of the smooth musculature in vitro either used cultured prostatic stromal cells and segments from transurethral resections of the prostate or tissue excised from the transition zone. Because compounds used to influence smooth muscle contractility via NO/cGMP signalling (eg, NO donors, activators of soluble guanylyl cyclase, and PDE5 inhibitors) target smooth muscle cells of the fibromuscular stroma, our study focused on the fibromuscular stroma of the transition zone.

The aim of this study was to investigate, by means of immunohistochemistry and Western blot analysis, the expression and localization cGKIα and cGKIβ in relation to smooth muscle α-actin and the second messenger cGMP in the transition zone of human prostate tissue.

2. Methods

2.1. Tissue source and handling

In accordance with the regulations of the local ethical committee, human prostate tissue was obtained from 11 patients (mean: 62.4 yr, range: 54–68 yr), who had undergone radical surgery for localized carcinoma of the prostate. There were no clinical signs of LUTS, bladder outlet obstruction, or benign prostatic enlargement. The clinical stage of cancer was determined as cT1c for all patients. Routine histological assessment confirmed the unilateral localization of the carcinoma within the peripheral zone. The specimens were taken from the transitional zone of the opposite lobe. Histological and pathological examinations did not show any signs of prostatic intraepithelial neoplasia, carcinoma, inflammation, or BPH.

2.2. Immunohistochemistry

Two representative tissue blocks from the transition zone of each patient were fixed in 4% phosphate-buffered formaldehyde and embedded in Tissue-Tec (Miles Laboratories Inc., Elkhart, IN, USA). Cryostat tissue sections (10 μm; 10 consecutive sections from each representative block, 20 sections per patient) were preincubated in phosphate-buffered saline (pH 7.4) containing 0.2% Triton X-100 and 0.1% BSA for 2 h, followed by 24-h incubation with the primary antibodies (anti-cGKIα, -cGKIβ, -α-actin, and -cGMP) at the proper dilutions. After rinsing the sections in phosphate-buffered saline, Alexa Fluor secondary antibodies (1:300; Molecular Probes Europe BV, Leiden, The Netherlands) were applied for 90 min, and then sections were mounted using phenylendiamine. Sections were viewed using a laser fluorescence microscope (Olympus Corp., Osaka, Japan). Negative controls were performed for all samples by omitting the primary antibodies. Imaging was performed by Viewfinder Lite version 2.0 from Pixera Corp. (Egham, UK). To verify the reproducibility of staining intensities, the results were independently interpreted by two observers (S. Ückert, P. Hedlund) according to a score of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). There were no significant differences among the findings of the two observers (reliability coefficient: 0.87).
2.3. Antibody source

Antibodies directed against cGKIα (E-17, sc-10338; 1:100) and cGKIβ (E-20, sc-10342; 1:100) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Both antibodies were affinity-purified goat polyclonal antibodies raised against a peptide mapping within an internal region of cGKIα and cGKIβ of human origin. In Western blot analysis and immunohistochemistry, E-17 and E-20 react with cGKIα and cGKIβ of mouse, rat, and human origin [13,14]. The anti-cGKI immunofluorescence-labelled rabbit polyclonal antibody (no. 539729, Calbiochem, La Jolla, CA, USA; 1:250) recognized both isoforms cGKIα (76 kDa) and cGKIβ (78 kDa) of mouse, rat, and human origin [15]. To verify the localization of cGKIα within the smooth musculature, double-staining using the monoclonal smooth muscle α-actin antibody (AS528, Sigma Chemical Co., St. Louis, MO, USA; 1:1000) [16] was carried out. In addition, an anticycGMP rabbit polyclonal antibody (Calbiochem; 1:500) [17] was used to show the colocalization of cGKI and its effector protein cGMP.

2.4. Western blot analysis

Fresh frozen tissue samples of the transition zone of human prostate tissue were sectioned into 20-μm slices and collected in Eppendorf tubes. Tissue was homogenized using an Ultraturrax (Janke & Kunke, Staufen, Germany) in homogenization buffer (50 mM Tris pH 7.8, containing 150 mM NaCl, and protease inhibitor cocktail tablets). Samples were centrifuged at 900 × g for 10 min to remove unhomogenized material. Western blots were executed on 5–20% gradient gels and g protein sample was loaded per lane. Blots were blocked in 5% skimmed milk in Tris-buffered saline Tween-20. Rabbit anti-cGMP-dependent protein kinase-1 antibody (cGKIα and cGKIβ, no. 539729, Calbiochem; 1:3000) was applied. In addition to the tissue taken from the transition zone of the human prostate, tissue from human cavernous arteries and corpus cavernosum served as references. For control purpose, 10 μg of a rat brain cytosolic fraction was loaded in parallel. Western blots were developed using the ECL system. Negative controls without primary antibodies were performed for all samples.

3. Results

3.1. Immunohistochemistry

Examination of the specimens revealed distinct immunoreactivities specific for all antigens (cGKI, cGKIα, cGKIβ, smooth muscle α-actin, and cGMP) examined. In the fibromuscular strata of the transition zone, bundles of smooth muscle cells exhibited strong immunoreactivities specific for α-actin and the cyclic nucleotide second messenger cGMP (Fig. 1A, C, E). Strong immunoreactivity specific for cGKIα and cGKIβ was detected in the fibromuscular strata of the prostate, where it was distributed homogenously (Fig. 1B, D, F). Double-labelled staining for either α-actin or cGMP and cGKI isoforms demonstrated that the expression of the cGKI isoforms was exclusively limited to the smooth musculature of the fibromuscular stroma, where the enzymes were found to be colocalized with its main effector, the cyclic nucleoside monophosphate cGMP (Fig. 1C–F). Moderate immunosignals indicating cGMP were also registered in the outer cellular layer (nonepithelial cells) of glandular nodes (data not shown). In contrast, no immunofluorescence indicating the presence of cGKI isoforms was registered within the epithelial and subepithelial layers of glandular structures, and slender nerve fibres transversing the sections also appeared free of staining (data not shown). No differences were detected in the staining patterns among the patients. Control staining, omitting the primary antibodies, did not reveal fluorescence signals in any of the histological components of the transition zone (nonvascular and vascular smooth muscle, collagen, glandular epithelium; Fig. 1G).

3.2. Western blot analysis

Western blot analysis for cGKI displayed clear bands without background (Fig. 2). The cGKI antibody detected two clear, characteristic bands of the correct molecular weight (cGKIα: 76 kDa, cGKIβ: 78 kDa) in the human prostate (Fig. 2, lane 5). These findings were confirmed by the results of positive controls (Fig. 2, lanes 3 and 4) and reference tissues (Fig. 2, lanes 6 and 7), although a separation of the single band at the expected size of 76–78 kDa could not be detected in our experiments. The amount of cGKIα and cGKIβ varied between the different tissue specimens.

4. Discussion

Large epidemiological studies presented strong evidence for an association between LUTS/BPH and male sexual dysfunction. Efforts have been made to identify putative common denominators for both entities, emphasizing the issue that common pathophysiological mechanisms could be targeted by combined treatments. A variety of different mechanisms have been proposed, including an increased sympathetic activity, a dysregulation of the expression of α1-adrenergic receptor subtypes, alterations in the Rho/Rho kinase signaling pathway, an age-related imbalance of sex hormones, and a dysregulation of the NO/cGMP pathway [19–21].
Fig. 1 – Cryostat sections from the fibromuscular stroma of the transition zone of the human prostate. Immunohistochemical distribution of cGKIα and cGKIβ in the smooth musculature of the fibromuscular stroma.
There is increasing evidence for a significant role of the NO/cGMP pathway in the control of the smooth musculature of human prostate tissue. The fibromuscular stroma is densely supplied by NO synthase–containing nerve terminals [22]. Cyclic GMP-regulated PDEs and cGKs are considered to be major cGMP effectors. The presence of mRNA transcripts encoding for cGMP-hydrolyzing PDEs, including PDE1A, PDE5, PDE9, and PDE11 (dual substrate PDE), has been described, and immunohistochemistry confirmed the expression of PDE5 and PDE11 in the transition zone of the human prostate [23,24]. Moreover, in vitro experiments have demonstrated that PDE5 inhibitors reverse norepinephrine-induced tension of prostatic strips isolated from the transition zone. The potential clinical relevance of the NO/cGMP/cGKI signalling is supported by studies indicating a positive effect of PDE5 inhibitors on LUTS in men with BPH [4–6].

High levels of cGKI have been detected in vascular and intestinal smooth muscle cells. The mammalian cGKI family consists of two isoforms. Although cGKIα and cGKIβ differ in tissue distribution and functional properties, both isoforms are present in the smooth musculature of cardiovascular tissue [8]. The present study provides evidence that both isoforms of cGKI are expressed in the smooth musculature of the fibromuscular stroma of the human prostate. The expression of the enzymes was confirmed by Western blot analysis and accords well with the detection of mRNA encoding for both isoforms in transurethral resections of the prostate tissue [25]. Conventional cGKI-knockout mice have been used to investigate the in vivo relevance of cGKI as a mediator of NO/cGMP signalling. Cyclic GKI-deficient mutants demonstrated impaired relaxation of penile smooth muscle as well as bladder hyperactivity and a significant reduction of NO/cGMP-dependent relaxation of urethral smooth muscle [26,27]. Using a genetic deletion/rescue strategy, cell culture studies of vascular smooth muscle cells suggested that NO/cGMP-dependent smooth muscle relaxation is mediated via activation of cGKIα but not cGKIβ [12]. It has been suggested that the cGKIβ isoform might regulate cell growth and differentiation rather than smooth muscle tone [28]. Therefore, the expression of both cGKIα and cGKIβ isoforms supports the hypothesis that activators of the NO/cGMP/cGKI signalling cascade may interfere with the regulation of stromal smooth muscle tone and tissue proliferation in human prostate tissue. Both factors are considered to be associated with the voiding symptoms of LUTS, which can be divided into dynamic (smooth muscle tone of the fibromuscular stroma) and static (benign prostate gland enlargement) components.

In vitro organ bath studies have been conducted to further elucidate the functional role of cGKI in human urogenital tissues. The relaxing effects of sodium nitroprusside, sildenafil, and tadalafil on isolated strips of human corpus cavernosum were significantly reduced by Rp-8-pCPT-cGMPS, an inhibitor of cGK [29]. Contractility studies on tissues from transurethral resections of the prostate demonstrated that the cGKI activators ATP-cGMP and PET-cGMP both significantly inhibited phenylephrine-induced contractions. Studies have suggested that cGKI-dependent smooth muscle relaxation is mediated by the activation of ATP-sensitive potassium channels [25].

Based on animal experiments, there is also evidence for an interaction between prostatic stromal and epithelial cells, which might partially

demonstrated by laser fluorescence microscopy. Double-labelled staining indicates the presence of immunofluorescence specific for smooth muscle α-actin (A, goat anti-mouse IgG, green-fluorescent, 20×) and cGKI (B, goat anti-rabbit IgG, red-fluorescent, 20×). (C–F) The colocalization of cGMP (C and E, goat anti-rabbit IgG, green fluorescent, 20× and 40×, respectively) and its effector proteins cGKIα (D, rabbit anti-goat IgG, red fluorescent, 20×) and cGKIβ (F, rabbit anti-goat IgG, red fluorescent, 40×). (G) Control staining, omitting the primary antibodies, did not reveal fluorescence signals in any of the histological components of the transition zone (nonvascular and vascular smooth muscle, collagen, glandular epithelium; 10×).
regulate cell differentiation and proliferation, and thus may contribute to the manifestation of BPH. A reduction of NO synthase-containing nerves and a change in the distribution pattern of NO synthase isoforms in BPH tissue has been reported, although evidence for BPH-related alterations in the NO/cGMP pathway is still limited [2,3]. Therefore, additional experimental studies are needed to compare BPH to normal prostate tissue to further evaluate the expression and functional relevance of the different components of NO/cGMP/cGKI signalling.

5. Conclusions

Our results confirm the presence of cGKn and cGKn in the transition zone of human prostate tissue. In addition, the colocalization of smooth muscle α-actin, the second messenger cGMP, and cGKI as one of the effector proteins of cGMP provides further evidence for a significant role of the NO/cGMP/cGKI pathway in the regulation of smooth muscle contractility in human prostate tissue. These findings accord well with clinical studies indicating that PDE5 inhibitors significantly improve LUTS in men with BPH. Therefore, the NO/cGMP/cGKI signalling pathway may provide additional targets for the pharmacotherapy of BPH and LUTS using NO donors, activators of soluble guanylyl cyclase, or PDE5 inhibitors [2,3,30].

Conflicts of interest

The authors have nothing to disclose.

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Editorial Comment on: Immunohistochemical Distribution of Cyclic GMP-Dependent Protein Kinase-1 in Human Prostate Tissue

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Although benign prostatic hyperplasia (BPH) is one of the most common diseases affecting the elderly male population, its etiology and relation to clinical symptoms remain poorly understood [1]. The clinical manifestations of BPH are thought to be primarily due to bladder outlet obstruction (BOO). However, the mechanism of bladder outlet obstruction in BPH is unclear. The specific features of the hyperplastic prostate predisposing to the development of BOO remain essentially unknown [1,2]. A poor correlation between the histologic prevalence of prostatic hyperplasia, prostate volume, and the clinical syndrome of lower urinary tract symptoms (LUTS) has been found in many studies. These observations suggest that specific changes in prostate tone independent of its size may contribute to LUTS. Interestingly, patients taking phosphodiesterase 5 (PDE5) inhibitors for erectile dysfunction report significant improvement in LUTS [3]. In view of similar neuroeffectors regulating prostatic and erectile tissue tone, it is logical to hypothesize that PDE5 inhibitors may relax the two tissues simultaneously.

The article by Waldkirch et al introduces a new concept in the pathophysiology and treatment of BPH and LUTS [4]. The colocalization of smooth muscle $\alpha$-actin, cyclic guanosine monophosphate (cGMP), and cGMP-dependent protein kinase-1 (cGK1) isoforms in the stroma of the transition zone, reported in this article, indicates a NO/cGMP/cGK1 signaling pathway in human prostate. This mechanistic finding is of great clinical relevance.

The possible role of NO/cGMP in human prostate tone, reported by Waldkirch et al, is supported by findings in experimental models showing that supersensitivity of ischemic prostatic tissue to contractile stimuli occurs concurrently with a marked decrease in cGMP production [5]. It was shown that treatment with the $\alpha$-adrenoceptor blocker doxazosin significantly increased prostatic cGMP levels [5]. This study also showed that sildenafil diminishes neurogenic contraction of prostatic tissue and augments the inhibitory effect of $\alpha$-adrenoceptor inhibitors [5]. Studies with cultured human prostate smooth muscle cells have shown that sildenafil efficiently inhibits lysophosphatidic acid-induced BPH-like smooth muscle growth. Further insight into the role of PDE5 and the NO/cGMP/cGK1 signaling pathway in prostate may lead to newer therapeutic strategies for BPH and LUTS.

References


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