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Phosphorylation of the PKG substrate, vasodilator-stimulated phosphoprotein (VASP), in human cultured prostatic stromal cells

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Abstract

Nitric oxide (NO) is known to regulate contractility and proliferation of cells within the prostate, however, the mechanism by which this occurs is unknown. The cGMP-dependent protein kinase (PKG) signalling pathway may be involved, and recent work has shown that activation of this pathway can be assessed by analysis of phosphorylation of vasodilator-stimulated phosphoprotein (VASP). The aim of the current study is to characterise the expression of VASP in the human prostate and human cultured prostatic stromal cells (HCPSCs), and to investigate whether NO activates PKG in these cells. Our studies revealed that VASP is expressed, and that incubation of HCPSCs with PKG-activating cGMP-analogues or the NO-donor, SNP, caused a significant PKG-dependent increase in VASP serine-239 phosphorylation. In addition, SNP elicited a reduction in intracellular K^+ in a time frame consistent with the phosphorylation of VASP and activation of PKG. These data demonstrate that VASP can be used to assess the NO/cGMP/PKG signalling pathway in HCPSCs. In addition, we demonstrate for the first time that SNP, probably via NO release, leads to phosphorylation of VASP in a manner consistent with PKG activation.

Keywords: Prostate; Prostatic stromal cells; cGMP-dependent protein kinase; cGMP; Vasodilator-stimulated phosphoprotein; Nitric oxide

Nitric oxide (NO) plays an important, if somewhat vague, role in normal prostatic physiology and has been implicated in the pathophysiology of benign prostatic hyperplasia [1–8]. There is rich nitrergic innervation of human prostatic stromal and glandular tissue [1–3,7], and either electrical stimulation of nitrergic nerves, application of exogenous NO, or inhibition of phosphodiesterase 5 (PDE; a cGMP-specific PDE) results in relaxation of prostatic stromal smooth muscle [4,7,9]. Despite this evidence demonstrating a clear role for NO in normal prostatic function, little is known of the signal transduction pathways activated by NO in the prostate.

Generally, NO activates soluble guanylate cyclase, which in turn leads to the production of cGMP and the activation of cGMP-dependent protein kinase (PKG) [10,11]. The functional consequences of PKG activation are many and varied ranging from regulation of intestinal chloride secretion [12], to proliferation [13], apoptosis [14] and smooth muscle relaxation [15]. Consistent with these observations is previous work from our laboratory demonstrating that PKG plays a role in the regulation of contraction and proliferation in human cultured prostatic stromal cells (HCPSCs) [16,17]. In general, investigation into the NO/cGMP/PKG signalling pathway has been quite difficult since PKG substrates were poorly characterised. Recently, however, a protein known as vasodilator-stimulated phosphoprotein (VASP) [18–20] was identified as a substrate for PKG activity. VASP is one of the founding members of a new family of proline-rich proteins known as Ena/VASP proteins [21] that are crucial regulators of actin dynamics and associated processes, such as cell adhesion, motility [21–24] and possibly also proliferation [25]. There are three major cAMP-dependent protein kinase (PKA) and PKG

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phosphorylation sites within the VASP molecule; serine-157, serine-239 and threonine-278 [19,21]. Kinetic analysis of these phosphorylation sites has revealed that serine-239 is phosphorylated by PKG more rapidly than serine-157, and that the converse is true for PKA [19,21]. Phosphorylation of serine-157 by either PKG or PKA leads to a shift in the apparent molecular weight from 46 to 50 kDa (this shift is not observed upon phosphorylation of either serine-239 or threonine-278; [19]). Thus phosphorylation of VASP serine-239 can be used to assess NO/cGMP/PKG signalling pathway activity [22,26–28]. As a result, new roles for VASP and PKG are constantly emerging, such as the involvement of phosphorylated VASP serine-239 in the growth inhibitory effects of NO on smooth muscle cells [25].

In the current study we establish the presence of PKG and VASP in both human prostate tissue and also in HCPSCs, and show that phosphorylation of VASP can be used to demonstrate activation of the NO/cGMP/PKG signaling pathway in the human prostate. In previous work we have shown that activation of PKG with cGMP-analogues in HCPSCs leads to the opening of ATP-sensitive K^+ channels (K_{ATP} channels) and a subsequent efflux of K^+ [16]. In the current work we demonstrate that SNP, via activation of PKG, also leads to cellular K^+ efflux.

Materials and methods

Ethics approval

Human tissues were obtained with the approval of the Southern Healthcare Network Ethics and Experimentation Committee (Reference number 02066A).

Human prostatic tissue

Explants of human prostate from patients (mean age 68 yrs) undergoing transurethral resection of the prostate (TURP) to treat benign prostatic hyperplasia were used to generate primary HCPSC cultures.

Primary explant cell culture

Primary cells were grown as described previously [17] on tissue culture dishes in MCDB 131 media (Sigma-Aldrich Chemical Company Castle Hill, Australia) supplemented with charcoal stripped horse serum (10%), Hepes (10 mM), insulin (5 μg ml^{-1}), MEM-EAGLE solution of non-essential amino acids (2%), penicillin (50 IU ml^{-1}) and streptomycin (50 μg ml^{-1}) at a pH of 7.4. All media supplements were purchased from Sigma-Aldrich Chemical Company Castle Hill, Australia. This growth stimulatory media (growth media) has been suggested to preferentially promote the growth of smooth muscle cells [29]. Confluent cells were detached from tissue culture flasks and split as described previously [17]. In order to minimise the effect of decreased PKG expression during long term culture [30–32], only cells between passage 3 and 6 were used.

Immunohistochemistry

TURP tissue was placed in fixative solution containing formaldehyde (4% in PBS) for 2 h at room temperature. Fixed TURP tissue was then washed four times (10 min each) in PBS containing sucrose (7%) and sodium azide (0.01%) and then stored in this solution for 48 h at 4 °C. Tissues were placed in a plastic mould, embedded in Tissue Tek® O.C.T (Sakura, Finetechnical Co. Ltd, Japan) and snap frozen in liquid nitrogen, prior to storage at −80 °C. Sections (8 μm thick) were cut using a Leica CM1850 cryostat at −20 °C and thawed onto gelatin-coated slides.

Sections were washed in PBS, blocked with PBS containing BSA (5%) for 1 h at room temperature and then washed in PBS. All antibodies were diluted in PBS containing BSA (5%) and Triton-X100 (0.5%), and used at a 1:250 dilution. Sections were incubated overnight at room temperature with primary antibodies to VASP (Alexis Biochemicals, USA). Unbound primary antibody was removed by washing four times in PBS containing BSA (1%). Following this, sections were incubated with fluorescently labelled secondary antibodies (Alexa-488; Molecular Probes, USA) at room temperature for 1 h; unbound secondary antibody was removed by washing four times in PBS containing BSA (1%). In negative control studies, primary antibodies were excluded. Sections were then mounted in PROLONG® mounting media and coverslipped. Sections were later visualized with a Nikon TE2000 microscope equipped with a Coolsnap fx camera (Roper Scientific, USA), and illuminated at 488 nm (Alexa-488) with a lambda-DG4 lamp and filter set (Sutter Instrument Company, USA).

Immunocytochemistry

Approximately 15,000–20,000 cells were grown on glass coverslips in growth media for 24 h and then serum starved as described above. Cells were fixed in methanol: acetone (1:1) for 30 min at room temperature, and then incubated in PBS containing Triton-X100 (0.5%; Sigma Chemical Company St. Louis, USA) for 30 min. Cells were then washed four times in PBS, blocked with PBS containing BSA (5%) for 30 min and washed in PBS. Primary antibodies were diluted in PBS containing BSA (5%) and Triton-X100 (0.5%), and used at a 1:30 dilution. Cells were incubated overnight at room temperature with primary antibodies to VASP (Alexis Biochemicals, USA). Unbound primary antibody was removed by washing four times in PBS containing BSA (1%), and incubated with fluorescently labelled secondary antibody (Alexa-488; Molecular Probes, USA) for 1 h; unbound secondary antibody was removed by washing four times in PBS containing BSA (1%). In negative control studies, primary antibodies were excluded. Cells were visualized with a Nikon TE2000 microscope equipped with a Coolsnap fx camera (Roper Scientific, USA), and illuminated at 488 nm (Alexa-488) with a lambda-DG4 lamp and filter set (Sutter Instrument Company, USA).