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Short communication

Cell proliferation in human prostatic smooth muscle cells involves the modulation of protein kinase C isozymes

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Abstract

We have examined the role of protein kinase C in the regulation of foetal-calf serum-stimulated cell proliferation in human prostatic smooth muscle cells. The data showed that the proliferative effect to foetal-calf serum (10%, v/v) was partially inhibited by 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a) pyrrolo (3,4-c)-carbazole (Go-6976), a selective Ca^{2+}-dependent protein kinase C inhibitor, suggesting that Ca^{2+}-dependent protein kinase C isozymes might play roles in this proliferative regulation. Additionally, foetal-calf serum caused a significant translocation of protein kinase C-β_{II} and -ε from a cytosolic to a membrane distribution. These findings combined with the aforementioned functional experiments suggest that foetal-calf serum-stimulated cell proliferation might involve the activation of protein kinase C-β_{II} in human prostatic smooth muscle cells; however, the role of protein kinase C-ε in mediating cellular functions other than cell proliferation remains further investigation in these cells.

Keywords: Human prostate; Protein kinase C isozyme; Foetal-calf serum; Proliferation

1. Introduction

The prostate gland plays an important role in male reproduction and is also the site of benign and malignant neoplasms. Benign prostatic hyperplasia is one of the most common diseases in males and the clinical manifestations of benign prostatic hyperplasia are primarily related to bladder outlet obstruction. In addition, the stromal compartment of the prostate plays a major role in the pathogenesis of benign prostatic hyperplasia. Furthermore, anatomical and pathological studies have shown that stromal enlargement is the first event in the process of benign prostatic hyperplasia (McNeal, 1978).

Control of cellular proliferation involves a complex mechanism following the supplement of growth factors. Although there are accumulating evidences revealing that serum, insulin and basic fibroblast growth factor are mediators of stromal growth in the human prostate (Sherwood et al., 1992; Zhang et al., 1997), the proliferative mechanisms remain unclear and await detailed investigation.

Protein kinase C, which is described as a serine/threonine kinase, represents a large gene family of isozymes including Ca^{2+}-dependent (protein kinase C-α, -β_I, -β_{II} and γ) and Ca^{2+}-independent subfamilies (protein kinase C-δ, -ε, -ζ, -η, -θ and -λ) (Hug and Sarre, 1993). These isozymes show differences in their structures, tissue distribution, mode of activation, cofactor dependence, responsiveness to phospholipid metabolites and substrate selectivity (Hug and Sarre, 1993). The existence of so many isozymes exhibits the diversity of protein kinase C-mediated cell responses. However, the functional relevances and their regulation by protein kinase C isozymes appear rather complex and remain incompletely understood. The following studies were conducted to examine the role of protein kinase C in the regulation of cell proliferation in human prostatic smooth muscle cells, which constitute a major cellular component of prostatic stroma. We also determine which protein kinase C isozymes are present in these cells and characterize their translocations

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from a cytosolic to a membrane distribution following the stimulation by serum.

2. Materials and methods

2.1. Tissue explants and subcultures

Human hyperplastic prostates were obtained at operation from males by transurethral resection of the prostate. All these patients had histories of prostatism and were diagnosed to have benign prostatic hyperplasia by the combination of rectal digital examinations, transrectal sonography of prostate and urodynamic studies (including uroflowmetry, urethral pressure profile and cystometry). Prostatic tissue explants were managed and cultured cells were obtained as previously (Guh et al., 1998).

Isolated human prostatic cells were identified as previously (Guh et al., 1998) by the following criteria: the cultured cells exhibited positive immunofluorescence staining for vimentin and smooth muscle α-actin, whereas showed negative immunostaining with epithelial cytokeratins; the culture morphology was characterized by the formation of nodules of cells, that is, 'hills and valleys' (data not shown).

2.2. Cell proliferation assay

The cell proliferation assay in this study was carried out using the MTT assay described by Mosmann (1983). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline at a concentration of 5 mg/ml and millipore filtered. From this stock solution, 10 μl per 100 μl of medium was added to each well, and plates were gently shaken and incubated at 37°C for 4 h. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. After the loading of MTT, the medium was replaced with 100 μl acidified (0.04 M KCl) β-isopropanol and was left for 20–30 min at room temperature for color development, and then the 96-well plate was read by enzyme-linked immunosorbent assay reader (570 nm) to obtain the absorbance density values.

2.3. Electrophoresis and Western blotting analysis

Human prostatic smooth muscle cells in each treatment group were isolated from culture dishes and suspended in buffer A (4°C), which consisted of 20 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Cells were then ultrasonically disrupted, and lysates were centrifuged ($100\,000 \times g$ for 1 h). The soluble cytosolic fraction was retained and the membrane pellet was resuspended in 0.3 ml of buffer A and sonicated. Protein concentration of each cytosolic and membrane fraction was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). All fractions were then diluted with loading buffer (0.156 M Tris, 1% sodium dodecyl sulphate (SDS), 25% glycerol, 12.5% β-mercaptoethanol, and 0.2% bromophenol blue), and boiled for 5 min. For Western blot analysis, the amounts of cytosolic (20 μg) and membrane (40 μg) protein were fractionated in 9% polyacrylamide gel electrophoresis in the presence of 0.1% SDS, according to Laemmli (1970). Proteins were then electroplotted onto nitrocellulose membranes. The membranes were incubated in phosphate-buffered saline, supplemented with 5% (w/v) nonfat milk for 1 h at room temperature and then washed with phosphate-buffered saline/0.1% Tween 20 (four times). The membranes were then incubated overnight at 4°C with anti-protein kinase C-α, -β_I, -β_{II}, -γ, -δ, -ε, -ζ, -η, -θ and -λ isozyme-specific antibodies (dilute 1:500 to 1:1000). After four washings with phosphate-buffered saline/0.1% Tween 20, alkaline phosphate-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) (dilute 1:2000) was applied to the membranes for 1 h at room temperature. The membranes were washed four times with phosphate-buffered saline/0.1% Tween 20 and then incubated in alkaline phosphatase buffer (containing 100 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, 0.33 mg/ml nitro blue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, pH 9.6) at room temperature for 10 to 20 min. Alkaline phosphatase was finally detected in visible blots.

2.4. Data analysis

Data are presented as the means ± S.E.M. for the indicated number of separate experiments. Statistical significance between drug-treated and untreated groups was evaluated by unpaired two-tailed Student's *t*-test and *P* values of less than 0.05 were considered significant.

2.5. Materials

The following materials were used: EGTA, EDTA (disodium salt), leupeptin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-isopropanol, phorbol 12-myristate 13-acetate, dithiothreitol, phenylmethylsulfonyl fluoride, nitro blue tetrazolium, staurosporine, and 5-bromo-4-chloro-3-indolyl phosphate (all from Sigma, St. Louis, MO, USA); monoclonal antibodies of protein kinase C-α, -γ, -δ, -ε, -ζ, -θ and -λ (Transduction Laboratories, Lexington, KY, USA); polyclonal antibodies of protein kinase C-β_I, -β_{II} and -η, and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-mouse IgG (Calbiochem-Novabiochem, CA, USA), and 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo (2,3-a) pyrrolo (3,4-c)-carbazole (Go-6976) (Biomol Research Laboratories, PA, USA).

3. Results

The cell proliferation was measured by MTT assay method (Mosmann, 1983) rather than [^{3}H]thymidine incorporation assay, avoiding the increasing environment risk of radioactive isotope problem. The correlation between cell number and absorbance density value for MTT assay of prostatic smooth muscle cells was measured in this study. It showed that there was a proportional increase in the absorbance density values parallel to the increase in cell number with a r value of 0.99 (data not shown). These results indicate a very good correlation between the absorbance density value and cell number and show that this MTT assay is suitable for the measurement of cell proliferation.

To study the proliferative action in human prostatic smooth muscle cells, cultured cells were made quiescent by the deprivation of serum for 48 h. The reintroduction of foetal-calf serum (10%, v/v) induced a significant cell proliferation by MTT assay method (91.3 ± 8.7% increase over control). To investigate the involvement of protein kinase C-signaled pathway, two protein kinase C inhibitors, staurosporine and Go-6976, were used in this proliferative action to serum in this study. As shown in Table 1, both staurosporine and Go-6976 produced significant reductions in serum-stimulated human prostatic smooth muscle cell proliferation. However, at the concentration of 100 nM, staurosporine but not Go-6976 had cytotoxic effect on these cells (71.0 ± 4.9 and 95.5 ± 3.1% survival as compared with the pretreatment control, respectively).

We have examined the protein kinase C isozyme expression in human prostatic smooth muscle cells using Western blot technique. The results showed that Ca^{2+}-dependent protein kinase C-α, β_I and β_{II} were the predominant isozymes present in human prostatic smooth muscle cells. In addition, protein kinase C-δ, -ε and -ζ were also found to be present in less abundant concentrations; however, protein kinase C-γ, -η, -θ, and -λ were not detected in these cells (data not shown).

Table 1
Effects of protein kinase C inhibitors on serum-induced prostatic smooth muscle cell proliferation

Addition (nM)		Reduction (%)
Staurosporine	10	25.9 ± 6.8[a]
	30	31.0 ± 4.5[a]
	100	41.0 ± 4.6[a]
Go-6976	10	29.6 ± 6.5[a]
	30	29.8 ± 5.9[a]
	100	34.2 ± 7.9[a]

Cultured cells were made quiescent by serum deprivation for 48 h and then stimulated by 10% foetal calf serum in the various concentrations of staurosporine or Go-6976. Results are expressed as percent reduction of serum-induced cell proliferation. Each value is the mean ± S.E.M. of four to eight determinations.
[a] $P < 0.001$ as compared with foetal calf serum control.

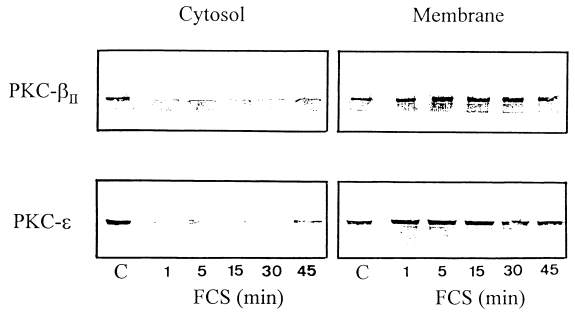

Fig. 1. Effect of serum on the translocation of protein kinase C-β_{II} and -ε isozymes in human prostatic smooth muscle cells. Cultured cells were treated with or without (C) 10% foetal-calf serum (FCS) for various time courses and then protein kinase C (PKC)-β_{II} and -ε isozymes expression in the cytosolic and membrane fractions were detected by Western blot analysis. Each lane contains 20 μg of cytosolic protein or 40 μg of membrane protein.

To investigate which protein kinase C isozyme plays a crucial role in the regulation of protein kinase C-signaled proliferation, human prostatic smooth muscle cells were treated with 10% foetal-calf serum for a different time course (1 to 45 min) to induce the redistribution of protein kinase C isozymes in these cells. As demonstrated in Fig. 1, foetal-calf serum induced a rapid translocation of protein kinase C-β_{II} and -ε from the cytosolic to the membrane fraction in these cells; by contrast, protein kinase C-α, -β_I, -δ and -ζ were also examined to remain unchanged levels in both cytosolic and membrane fractions regardless of serum treatment (data not shown).

4. Discussion

In order to define a role of protein kinase C in the regulation of cell growth in human prostatic smooth muscle cells, we first examined the influence of protein kinase C inhibitors on serum-induced proliferation in these cells. As a result, staurosporine, a potent protein kinase C inhibitor, caused a significant reduction on serum-induced cell proliferation at nontoxic concentrations (10 and 30 nM) demonstrating the involvement of protein kinase C-dependent mechanism. Additionally, a selective Ca^{2+}-dependent protein kinase C inhibitor, Go-6976 (Martiny-Baron et al., 1993), was employed to elucidate the roles of Ca^{2+}-dependent protein kinase C isozymes on the regulation of this protein kinase C-signaled cell proliferation. As demonstrated by Martiny-Baron et al. (1993), nanomolar concentrations of Go-6976 inhibited the Ca^{2+}-dependent protein kinase C isozymes, whereas even micromolar concentration of this compound had no effect on the kinase activity of the Ca^{2+}-independent protein kinase C isozymes δ, ε, and ζ. Based on a high degree of selectivity, nanomolar concentrations of Go-6976 caused a similar reduction

as that did by staurosporine on serum-induced prostatic smooth muscle cell proliferation suggesting that Ca^{2+}-dependent protein kinase C isozymes might be the key enzymes in this protein kinase C-dependent mechanisms.

There are accumulating evidences suggest that a variety of protein kinase C isozymes involve in the signal transduction pathway of the proliferative effect in several types of cells (Guizzetti et al., 1996; Huwiler and Pfeilschifter, 1994; Levy et al., 1994). In order to confirm which protein kinase C isozymes involve in human prostatic smooth muscle cell proliferation, we next investigated the expression of protein kinase C isozymes and their translocation following the serum treatment in these cells. Using Western blot technique we determined that human prostatic smooth muscle cells used here expressed protein kinase C-α, -β_I, -β_{II}, -δ, -ε and -ζ but did not express protein kinase C-γ, -η, -θ and -λ. After confirming that human prostatic smooth muscle cells express the above six protein kinase C isozymes, we examined the effect of serum on the translocation of these isozymes from a cytosolic to a membrane distribution. As shown in Fig. 1, the treatment with 10% foetal-calf serum for several time courses caused the significant associations of both protein kinase C-β_{II} and -ε with the membrane fractions in human prostatic smooth muscle cells. These results suggest that activation of protein kinase C-β_{II} and -ε isozymes might be the crucial mechanism in this protein kinase C-mediated pathway. However, in the aforementioned functional experiment, the Ca^{2+}-dependent protein kinase C isozyme (i.e., Go-6976-sensitive protein kinase C isozyme) might be the key enzyme in this protein kinase C-dependent mechanism demonstrating that growth-promoting effects of serum on human prostatic smooth muscle cells are mediated through activation of protein kinase C-β_{II}. Additionally, protein kinase C-ε also showed a marked translocation to the membrane fraction in these cells following serum treatment in this study. However, the role of protein kinase C-ε in mediating cellular functions other than cell proliferation remains further investigation in these cells although it plays a role in the regulation of cell proliferation in some kinds of cells (Gomez et al., 1995).

The involvement of protein kinase C-β_{II} in mediating human prostatic smooth muscle cell proliferation was supported by the observation that prolonged treatment with high concentrations of phorbol 12-myristate 13-acetate for 20 h, which downregulates most protein kinase C isozymes, completely eliminated protein kinase C-β_{II} in these cells (data not shown); furthermore, in parallel experiments, prolonged exposure (20 h) to high concentrations of phorbol 12-myristate 13-acetate also significantly reduced serum-stimulated cell proliferation ($31.4 \pm 3.5\%$ reduction as compared with the serum treatment control) in these cells.

In summary, the results of the present study demonstrate that the proliferative effects of serum on human prostatic smooth muscle cells involve the activation of protein kinase C. Furthermore, the present investigation characterizes the expression of various protein kinase C isozymes in these cells. The association of protein kinase C-β_{II} with the membrane fraction significantly increased during serum-induced cell proliferation. These findings suggest that protein kinase C-β_{II} may play a crucial role in serum-evoked proliferative signaling in human prostatic smooth muscle cells. Further studies are required to determine the exact role of protein kinase C-ε during serum-induced functional expression in these cells.

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