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Inositol Hexaphosphate Down-regulates both Constitutive and Ligand-Induced Mitogenic and Cell Survival Signaling, and Causes Caspase-Mediated Apoptotic Death of Human Prostate Carcinoma PC-3 cells

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Abstract

Constitutively active mitogenic and pro-survival signaling cascades due to aberrant expression and interaction of growth factors and their receptors are well documented in human prostate cancer (PCa). EGF and IGF-1 are potent mitogens that regulate proliferation and survival of PCa cells via autocrine and paracrine loops involving both MAPK- and Akt-mediated signaling. Accordingly, here we assessed the effect of inositol hexaphosphate (IP6) on constitutive and ligand (EGF and IGF-1)-induced biological responses and associated signaling cascades in advanced and androgen-independent human PCa PC-3 cells. Treatment of PC-3 cells with 2 mM IP6 strongly inhibited both growth and proliferation and decreased cell viability; similar effects were also observed in other human PCa DU145 and LNCaP cells. IP6 also caused a strong apoptotic death of PC-3 cells together with caspase 3 and PARP cleavage. Mechanistic studies showed that biological effects of IP6 were associated with inhibition of both constitutive and ligand-induced Akt phosphorylation together with a decrease in total Akt levels, but a differential inhibitory effect on MAPKs ERK1/2, JNK1/2 and p38 under constitutive and ligand-activated conditions. Under similar condition, IP6 also inhibited AP-1 DNA binding activity and decreased nuclear levels of both phospho and total c-Fos and c-Jun. Together, these findings for the first time establish IP6 efficacy in inhibiting aberrant EGFR or IGF-1R pathway-mediated sustained growth promoting and survival signaling cascades in advanced and androgen-independent human PCa PC-3 cells, which might have translational implications in advanced human PCa control and management.

Keywords

chemoprevention; prostate cancer; inositol hexaphosphate; phytic acid

INTRODUCTION

Prostate Cancer (PCa) is the most frequently diagnosed malignancy in elderly American men [1] and statistical estimates by the American Cancer society for the year 2008 indicated that there would have been an estimated 186,320 new cases of PCa and 28,660 associated deaths in the United States alone. This malignancy is prevalent not only in American

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population, but several epidemiological studies indicate that the PCa incidence and associated death rate are also higher in other Western countries compared to Asian countries [2,3]. This geographical distribution has been attributed to the difference in dietary pattern which is recognized as one of the major etiologic factors responsible for a variation in PCa incidence and mortality between Asian and Western male populations [2-5]. The major percentage of the dietary composition in the industrialized Western countries includes highly processed foods rich in meat, dairy products, and refined carbohydrates; however, the diets in Asian countries are more rich in fiber content, whole grain cereals, legumes, vegetables, and fruits [3,4,6]. Accordingly, over the years, several research groups worldwide have directed considerable efforts toward the identification of dietary and/or non-dietary naturally occurring chemical agents which would be beneficial for both prevention and intervention of PCa [7-9]. One such dietary agent is inositol hexaphosphate (IP6) or phytic acid, which is abundantly present in high fiber content diets, most cereals, legumes, nuts, and soybean [10,11]. The consumption of these, IP6 rich, dietary agents have been associated with reduced risk, incidence of, and mortality due to PCa in Asian countries [2,11,12].

Several *in vitro* studies have indicated that IP6 inhibits the growth of human breast, colon prostate, liver cancer cells, and rhabdomyosarcoma and erythroleukemia cells [5,13]. Further IP6 also inhibits cell transformation in mouse epidermal JB6 cells; and reverses the transformed phenotype of HepG2 liver cancer cells [5,13]. With regard to the *in vivo* anticancer efficacy of IP6, it has been shown that exogenous administration of IP6 in drinking water inhibits the azoxymethane-induced development of large intestinal cancer in F344 rats [14,15]. In other research studies, it was seen that IP6 suppresses dimethylhydrazine-induced large intestinal cancer in CD-1 mice; inhibits growth of DMBA-induced skin and mammary tumorigenesis; regresses liver cancer xenotransplant; prevents pulmonary adenomas in mice; inhibits growth of rhabdomyosarcoma tumor xenograft; inhibits the growth of mouse fibrosarcoma FSA-1 tumor xenografts; and also inhibits colon carcinogenesis [5,13].

Regarding the anticancer efficacy of IP6 against PCa, it was first reported that IP6 causes growth inhibition and induces differentiation in advanced human prostate cancer (PCa) PC-3 cells [16]. Following this, successive studies conducted by us revealed that IP6 is effective in both androgen-dependent and —independent PCa LNCaP and DU145 cells wherein, it inhibits cell growth and causes G1 cell cycle arrest and also induces their apoptotic death [17,18]. Other mechanistic studies, by our group, have revealed that IP6 impairs erbB1 receptor-associated mitogenic signaling [19] and also inhibits constitutive activation of NF- κ B in DU145 cells [20]. With regard to the *in vivo* efficacy of IP6 against PCa, we have reported that, IP6 feeding in drinking water inhibits DU145 tumor xenograft growth in athymic nude mice, which was associated with anti-proliferative, pro-apoptotic and anti-angiogenic effects of IP6 on the tumor [21]. While, growth inhibitory and pro-apoptotic effects of IP6 were also observed in mouse tumorigenic transgenic adenocarcinoma of the mouse prostate (TRAMP)-C1 cells [22]; in a recent study [23] we have also observed the effect of oral feeding of IP6 in the TRAMP animal model; this model mimics the progression of prostate cancer as it occurs in humans [24]. In this clinically relevant model we have seen that oral IP6 feeding for 24 weeks starting from the 4th week of age inhibits prostate tumor growth and progression, and this effect of IP6 is accompanied by the arrest of tumor progression at neoplastic stage with a concomitant reduction in the incidence of adenocarcinoma [23].

During initial years of disease, PCa growth is androgen-dependent, and therefore, the treatment line adopted is androgen ablation or anti androgen therapy [8,25]. However, during the period of disease progression of 1-3 years in most cases, a hormone refractory stage is reached that renders anti-androgen therapy ineffective [25]. Simultaneously,

additional genetic changes lead to epigenetic alterations, autocrine growth factor-receptor interactions accompanied by constitutively active mitogenic and cell survival signaling, and deregulated cell-cycle progression [8,25]. In coherence with this notion, an aberrant expression and activity of members of the epidermal growth factor (EGF) family of receptor tyrosine kinases (RTKs) have been reported in neoplastic and invasive PCa [8,26]. Similarly, insulin-like growth factor-I (IGF-1) mediated signaling has also been shown to be constitutively active in PCa [8]. Since EGF and IGF-1 are potent mitogens that regulate proliferation and survival of PCa cells via autocrine and paracrine loops involving both mitogen activated protein kinase (MAPK) and Akt-mediated signaling [8], here we assessed the effect of IP6 on these mitogens-stimulated signaling and associated biological responses in human PCa PC-3 cells.

The aim of the present study was, therefore, to answer the question whether IP6 had the potential to negate the growth promoting effects of the mitogens EGF and IGF-1 in the androgen-independent PC-3 cell line which represents advanced bone metastatic phenotype of PCa and has constitutively active mitogenic and pro-survival signaling pathways.

MATERIALS AND METHODS

Cell Line and Reagents

PC-3, DU145 and LNCaP human PCa cell lines were obtained from the American Type Culture Collection (Manassas, VA). RPMI 1640 media and other cell culture materials were from Invitrogen (Carlsbad, CA). IP6 was purchased from Sigma, St. Louis, MO, dissolved in water to prepare a 200 mM stock solution, and pH was adjusted to 7.5. EGF and IGF-1 were bought from Millipore, Temecula, CA. Consensus AP-1-specific oligonucleotides and the gel shift assay system was from Promega Corp., Madison, WI. The primary antibodies for phosphorylated c-Jun, c-Jun, phosphorylated c-Fos, and c-Fos were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2), JNK1/2, p38, AKT, cleaved caspase 3, cleaved PARP and goat anti-rabbit immunoglobulin horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Beverly, MA. The rabbit anti-mouse antibody and enhanced chemiluminescence detection system were from Amersham Corp., Piscataway, NJ. Antibody for β -actin was purchased from Sigma—Aldrich, St. Louis, MO.

Cell Growth Assay

All PCa cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) and plated at 3000-5000 cells/cm² in 60 mm plates under standard culture conditions. Cells were treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. After specific treatments, cells were trypsinized, collected and counted using a hemocytometer. Trypan blue dye exclusion method was used to determine cell viability.

DNA Synthesis and Cell Viability Assays

DNA synthesis was assessed by BrdU incorporation employing colorimetric ELISA. Briefly, different PCa cells were cultured in 96-well plates at 37°C for 24h and treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. After these treatments, BrdU was added and subsequently incubated for another 2 h at 37°C. Thereafter, cells were fixed and incubated with anti-BrdU antibody followed by the addition of substrate. The reaction product was quantified by measuring the absorbance at 370 and

492nm using a scanning multi-well spectrophotometer (ELISA reader). For the determination of cell viability, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was employed. Briefly, following identical treatments as detailed above for BrdU incorporation in 96-well plate, cells were washed twice with phosphate buffered saline (PBS) and incubated with 1mg MTT/ml of serum-free media for 3h. Finally, treatment medium was aspirated out and DMSO was added in each plate, and absorbance of developed color was read at 590nm using a 96-well microplate reader.

Quantitative Apoptotic Cell Death Assay

To quantify IP6-induced apoptotic death of PC-3 cells, YO-PRO-1/propidium iodide staining was performed, followed by flow cytometry. The cells were treated identically as described above for cell growth and viability studies. After desired treatments, both floating and attached cells were collected and subjected to YO-PRO-1/propidium iodide staining using Vybrant Apoptosis Assay Kit 4 (Molecular Probes, Inc., Eugene, OR) following the step-by-step protocol provided by the manufacturer. After staining, the apoptotic cells showed green fluorescence, live cells showed little or no fluorescence and dead cells showed red/green fluorescence.

Immunoblotting

PC-3 cells were plated in 100 mm plates under standard culture conditions. At 50% confluency, Cells were treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. At the end of specific treatments, cell lysates were prepared in non-denaturing lysis buffer (10 mM Tris—HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenyl methyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, 5 U/ml aprotinin). For lysate preparation, medium was aspirated and cells were washed two times with ice-cold PBS followed by incubation in lysis buffer for 10 min on ice. Then cells were scraped and kept on ice for 30 min, and finally cell lysates were cleared by centrifugation at 4°C for 30 min at 14 000 r.p.m. Cytosolic and nuclear extracts were prepared as described earlier [27]. Protein concentrations in lysates were determined using Bio-Rad DC protein assay kit (Bio-Rad laboratories, Hercules, CA) by the Lowry method. Cell lysates (60-80 µg protein) were denatured in sample buffer, and equal amount of protein was resolved by SDS-PAGE. The separated proteins were then transferred on to nitrocellulose membranes followed by blocking with 5% non-fat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) overnight at 4°C and then incubated with desired primary antibody followed by secondary antibody, and developed by enhanced chemiluminescence kit. In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. The blots were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody (Sigma, St Louis, MO).

Electrophoretic Mobility Shift Assay

For electrophoretic mobility shift assay (EMSA), AP-1-specific oligonucleotides (3.5 pmol) were end-labeled with [γ -³²P]ATP (3,000 Ci/mmol at 10 mCi/mL) using T4 polynucleotide kinase in 10x kinase buffer as per the manufacturer's protocol (Promega Corp., Madison, WI). Labeled double-stranded oligo probe was separated from free [γ -³²P] ATP using a G-25 Sephadex column. The consensus sequences of the oligonucleotides used were 5'-CGCTTGATGAGTCAGCCGAA-3' and 3'-GCGAACTACTCAGTCGGCCTT-5' for AP-1. For EMSA, 8 µg protein from nuclear extracts was first incubated with 5x gel shift binding buffer [20% glycerol, 5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly (deoxyinosinic-deoxycytidylic acid)-poly(deoxyinosinic-deoxycytidylic acid)] and then with ³²P-end-labeled consensus

oligonucleotide for 20 min at 37°C. DNA-protein complexes thus formed were resolved on 6% DNA retardation gels (Invitrogen). In supershift assay, samples were incubated with anti-c-Jun, anti-c-Fos, and anti-c-Jun together with anti-c-Fos antibodies before the addition of ^{32}P end-labeled AP-1 oligo. DNA-protein or DNA-protein-antibody complexes thus formed were resolved on 6% DNA retardation gels. The specificity of DNA binding was also confirmed by adding 100-fold excess of cold probe in complete reaction incubation. In each case, the gel was dried and bands were visualized by autoradiography.

Statistical Analyses

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific, San Rafael, CA), and P values <0.05 were considered significant. The difference between the IP6 treated group versus the control or the respective ligand group was analyzed by unpaired two-tailed Student's t -test.

RESULTS

IP6 Inhibits Cell Growth, Viability and DNA Synthesis in Human PCa PC-3, DU145 and LNCaP Cells

First we assessed an overall efficacy of IP6 against PCa where PC-3, DU145 and LNCaP cells under 10% serum conditions were treated with IP6 (2mM) for 24 and 48h. At each time point we analyzed cell growth (Trypan blue dye exclusion method), viability (MTT assay) and DNA synthesis (BrdU incorporation assay) in all the three prostate carcinoma cells. Treatment of IP6 resulted in 37% and 47% ($P<0.001$) decrease in PC-3, 33% and 30% ($P<0.001$) decrease in DU145, and 22% and 27% ($P<0.001$) decrease in LNCaP cell growth compared to untreated controls after 24 and 48 hours, respectively (Table 1). The results in cell count assay were further confirmed by MTT assay, where we observed a decrease the viability with IP6 treatment by 63% and 69% ($P<0.001$) in PC-3 cells, 25% and 37% ($P<0.05$) in DU145 cells, and 17% and 30% ($P<0.01$) in LNCaP cells as compared to the respective controls after 24 and 48 hours, respectively (Table 1). Furthermore, IP6 decreased DNA synthesis in PC-3 cells by 51% and 47% ($P<0.001$) after 24 and 48 hours of treatment, respectively, compared to controls; significant decrease in DNA synthesis by 52% ($P<0.001$) was observed only at 48h in DU145 cells. Similar effects of IP6 treatment were observed in LNCaP cells with a decrease of 30% and 49% ($P<0.001$) at 24 and 48h, respectively as compared to the untreated controls.

IP6 Inhibits Constitutive and Mitogen-induced Cell Growth and DNA Synthesis in Human PCa Cells

In Trypan blue dye exclusion method, we observed that treatment of PC-3 cells with 2mM IP6 not only results in growth inhibition of serum starved control cells but also results in strong inhibition of EGF- and IGF-1-stimulated growth at both 24 and 48 hours of treatment (Fig. 1A). Specifically, treatment with IP6 resulted in 41% and 59% ($P<0.05$) decrease in PC-3 cell growth compared to untreated controls after 24 and 48 hours, respectively (Fig. 1A). While treatment with EGF and IGF-1 resulted in increase in cell growth at both 24 and 48 hours of treatment ($P<0.05$, for both mitogens); simultaneous treatment with 2mM IP6 resulted in 52% and 69% ($P<0.05$) growth inhibition at 24 hours and 74% and 88% ($P<0.05$) inhibition at 48 hours, respectively (Fig. 1A). The effect of IP6 treatment on DNA synthesis as determined by BrdU incorporation assay indicated that IP6 decreased DNA synthesis in PC-3 by 65% and 54% ($P<0.05$) after 24 and 48 hours of treatment, respectively, compared to untreated serum starved controls (Fig. 1B). A higher inhibition in EGF- or IGF-1-induced DNA synthesis was also observed with IP6 treatment (Fig. 1B). Specifically, DNA synthesis induced by EGF was decreased by 55% and 68% ($P<0.05$) after 24 and 48 hours of IP6 treatment, respectively; while IGF-1-induced DNA synthesis

was also reduced by 30% ($P < 0.001$) and 71% ($P < 0.05$) after 24 and 48 hours of IP6 treatment, respectively (Fig. 1B).

Based on the results in PC-3 cells showing strong effect of IP6 on cell growth and DNA synthesis under constitutive and ligand-induced conditions, we next asked the question whether these effects of IP6 are specific to PC-3 or exist in other human PCa cell lines. For this, we extended our studies to DU145 and LNCaP cells, which represent advanced but androgen-independent and androgen-dependent stages of PCa, respectively. Importantly, the IP6 treatment of these cell lines showed comparable efficacy to that observed in PC-3 cells (Fig. 1). In case of DU145 cells, as observed by Trypan blue dye exclusion method, treatment with IP6 resulted in 44% ($P < 0.05$) and 38% ($P < 0.001$) decrease in DU145 cell growth compared to untreated controls after 24 and 48 hours, respectively (Fig. 1A). Similarly, combination of EGF and 2mM IP6 decreased DU145 cell growth by 68% ($P < 0.01$) and 59% ($P < 0.05$) at 24 and 48 hours of treatment, respectively, compared to treatment with EGF only (Fig. 1A). Simultaneous treatment with IGF-1 and 2mM IP6 resulted in 60% ($P < 0.01$) and 43% ($P < 0.001$) growth inhibition at 24 hours and 48 hours, respectively, compared to treatment with IGF-1 alone (Fig. 1A). In case of LNCaP cells, treatment with IP6 resulted in 39% -40% ($P < 0.001$) decrease in cell growth compared to untreated controls after 24 and 48 hours, respectively (Fig. 1A). Inhibition in EGF- or IGF-1-induced LNCaP cell growth was also observed with IP6 treatment (Fig. 1A). Specifically, cell growth induced by EGF was decreased by 60% and 51% ($P < 0.001$) after 24 and 48 hours of IP6 treatment, respectively; while IGF-1-induced cell growth was reduced by 82% and 51% ($P < 0.001$) after 24 and 48 hours of IP6 treatment, respectively (Fig. 1A).

In other studies, IP6 decreased DNA synthesis in DU145 by 53% ($P < 0.01$) and 47% ($P < 0.001$) after 24 and 48 hours of treatment, respectively, compared to untreated serum starved controls. DNA synthesis induced by EGF was decreased by 35% ($P < 0.001$) and 61% ($P < 0.001$) after 24 and 48 hours of IP6 treatment, respectively; while IGF-1-induced DNA synthesis was reduced by 31% ($P < 0.01$) and 41% ($P < 0.001$) after 24 and 48 hours of IP6 treatment, respectively (Fig. 1B). In case of LNCaP cells, IP6 decreased DNA synthesis under serum starved conditions by 47% ($P < 0.001$) and 35% ($P < 0.01$) after 24 and 48 hours of treatment, respectively, compared to untreated controls. Further, DNA synthesis induced by EGF was decreased by 35% ($P < 0.05$) and 17%; while IGF-1-induced DNA synthesis was reduced by 27% ($P < 0.05$) and 43% ($P < 0.01$) after 24 and 48 hours of IP6 treatment, respectively (Fig. 1B).

IP6 Decreases Cell Viability and Induces Apoptotic Death of PC-3 Cells

The results in cell count assay were corroborated by MTT assay, where simultaneous treatment of serum starved PC-3 cells with EGF and 2mM IP6 decreased cell viability by 63% - 73% ($P < 0.05$) at 24 and 48 hours of treatment, respectively, compared to treatment with EGF only (Fig. 2A). Similarly, combination of IGF-1 and 2mM IP6 decreased PC-3 cell viability by 61% - 68% ($P < 0.01$ - < 0.05) at 24 and 48 hours of treatment, respectively, compared to treatment with IGF only (Fig. 2A). Comparable effects of IP6 were also observed when serum starved PC-3 cells were treated with this agent alone (Fig. 2A). A loss of apoptotic response due to constitutively active cell survival and anti-apoptotic events has been recognized as the major hallmarks of various human malignancies including PCa [8]. In PC-3 cells, constitutively active survival factor Akt plays an important role in their survival even in the absence of growth factors [28]. Based on the results on cell viability showing strong efficacy of IP6 in PC-3 cells, we next assessed whether IP6 induces apoptotic death of PC-3 cells in the absence/presence of growth factors. Treatment of PC-3 cells under 10% serum conditions with IP6 at 2mM dose resulted in 1.8 ($P < 0.05$) and 1.9 ($P < 0.05$) fold increase in apoptotic cell death after 24 and 48 hours, respectively (Fig. 2B).

Similarly, treatment of serum starved PC-3 cells with IP6 at 2 mM dose resulted in a ~2 fold ($P < 0.01$) and 1.3 fold ($P < 0.05$) increase in apoptotic death after 24 and 48 hours, respectively (Fig. 2B). Treatment with either EGF or IGF-1 for 24 and 48 hours resulted in a decrease in apoptotic cell death compared to untreated serum starved PC-3 controls; however co-treatment with EGF and IP6 resulted in a significant increase [~ 7 fold ($P < 0.01$) and ~ 3 fold ($P < 0.01$)] in apoptotic death after 24 and 48 hours, respectively (Fig. 2B). Under same treatment conditions, co-treatment with IGF-1 and IP6 also increased the apoptotic death by ~ 11 fold ($P < 0.01$) and ~ 6 fold after 24 and 48 hours, respectively (Fig. 2B). The results obtained by YOPRO staining assay were also corroborated by western blot analysis (Fig. 2C), where a very strong increased expression of cleaved caspase-3 and cleaved—PARP was observed in treatment conditions where IP6 was used either alone or in combination with the mitogens.

IP6 Inhibits Constitutive and Mitogen-induced Levels of Phospho- and Total Akt and MAPKs in PC-3 Cells

Based on our results showing that IP6 has an inhibitory effect on both growth and survival promoting effects of mitogens EGF and IGF-1, we next assessed the effect of IP6 on the pro-survival signaling pathways known to be induced by these mitogens. It has been reported earlier that PC-3 cell lines have constitutively activated form of Akt, which is attributed to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) accumulation resulting from the inability to degrade this compound due to the lack of the lipid phosphatase activity of phosphatase and tensin homolog (PTEN) [28]. This deregulation of the phosphoinositide 3-kinase (PI3K)—Akt signaling axis contributes to survival and deregulated proliferation in this cell line [28]. In the present study, treatment of PC-3 cells with IP6 not only strongly decreased the levels of phosphorylated Akt but also strongly inhibited the activation of Akt induced by mitogens EGF and IGF-1, even after 24-48 hours of co-treatment (Fig. 3). The modulatory effect on Akt phosphorylation by IP6 treatment was also accompanied by a decrease in total Akt levels (Fig. 3).

Regarding the effect of IP6 on MAPKs ERK1/2, JNK1/2 and p38, EGF and IGF-1 treatment strongly induced the phosphorylation of ERK1/2 in starved cells which was almost completely inhibited by co-treatment with IP6 without any effect on total ERK1/2 protein levels (Fig. 3). Interestingly, serum starvation alone also showed an increase in pERK1/2 levels specifically at 48 hours when compared to the cells under serum conditions; however, IP6 treatments under these conditions also showed strong decrease in pERK1/2 levels (Fig. 3). We next assessed the effect of IP6 on the stress associated proteins JNK 1/2 (c-jun N-terminal protein kinase, also known as stress-activated protein kinase, SAPK) and p38 (Fig. 3). Expression of phospho JNK1/2 was observed in untreated controls; however the levels of phospho JNK1/2 did not seem to alter much following prolonged exposure (24-48 hours) with the mitogens, in which case, the expression was lesser than the untreated controls (Fig. 3). Even though the levels of phosphorylated JNK 1/2 altered only little with EGF or IGF-1, they were still reduced with IP6 treatment (Fig. 3). Similar to pERK1/2, we also observed an increase in pJNK1/2 when cells were serum starved for 24 or 48 hours compared to those under serum conditions and IP6 reversed this induction in pJNK1/2 (Fig. 3). The expression of another stress related MAPK, phospho p38, was also modulated with IP6 treatment. Not only were the levels of phospho p38 decreased when cells were treated with IP6 alone, the decrease in the phosphorylation of this molecule was also strong when the cells were co-treated with the growth factor EGF along with IP6 for 24 and 48 hours; no effect however was observed with IP6 in IGF1 co-treatment studies for p38 phosphorylation (Fig. 3). On the other hand, no change in the expression of phospho p38 was observed when cells were treated with EGF or IGF-1 for 24-48 hours as compared to untreated control (Fig. 3). These results suggest that while IP6 was able to strongly inhibit Akt phosphorylation (both

constitutive and ligand-induced); the MAPK signaling molecules showed differential effect when exposed to prolonged treatment with growth factors and IP6. This differential effect on MAPK molecules can be attributed to the varying activity of these MAPK molecules as induced by the growth factors; where some signaling molecules were activated even after prolonged treatment, the others were not affected that strongly.

IP6 Inhibits Constitutive and Mitogen-induced Activation of AP-1 in PC-3 Cells

The transcription factor, activator protein-1 (AP-1), is involved in the regulation of various essential processes including cell proliferation, cell death and transformation [29]. Because AP-1 is composed of either homodimers or heterodimers between members of Jun and Fos families, and the activation of AP-1 is differentially regulated in response to various stimuli [29], we next assessed the effect of IP6 on the phosphorylation AP-1 subunits under identical treatment conditions. The levels of phospho and total c-Fos and c-Jun proteins were determined in nuclear lysates by western blot analysis. It was observed that IP6 treatment results in a strong decrease in the levels of phosphorylated c-Fos and c-Jun under both serum or serum starved and ligand (EGF and IGF1)-induced conditions (Fig. 4A). Importantly, IP6 treatment was also able to decrease the levels of total c-Fos and c-Jun under identical treatment conditions (Fig. 4A). Based on these results, we next assessed the effect of IP6 on AP-1 activation under these treatment conditions, where IP6 initially increased the DNA-binding activity of AP-1 after 24 hours of treatment of serum starved cells, which was followed by significant inhibition in this activity after prolonging the treatment time with IP6 to 48 hours (Fig. 4B). Also the DNA-binding activity of AP-1, which was induced by exposure to either of the mitogens EGF and IGF1, decreased significantly by co-treatment with IP6 (Fig. 4B). To determine the specificity of AP-1 band in EMSA, gel super shift assay was performed (Fig. 4C). Nuclear extracts of untreated control were incubated with anti-c-Jun, anti-c-Fos or both the antibodies, followed by EMSA. This showed a strong super-shift in case of anti-c-Jun as well as when lysates were incubated with anti-c-Jun and anti-c-Fos together (Fig. 4C). For further authentication of AP-1 bands, 100-fold excess of unlabeled AP-1 probe (cold oligo) was added in complete reaction incubation, which did not show AP-1 band verifying that the AP-1 band in EMSA is indeed the case (Fig. 4C).

Discussion

IP6 is a naturally occurring hexaphosphorylated carbohydrate, which is ubiquitously present in most of the plants and mammalian cells [11] and has shown anticancer efficacy against various *in vitro* and *in vivo* cancer models, including PCa [5,11,30]. IP6 is also available over the counter as a dietary supplement owing to its anti-oxidant property and known beneficial effects such as prevention against the formation of kidney stone, high cholesterol, and heart and liver diseases [10,31]. The novel findings in the present study are: (i) IP6, an essential component of high fiber diet, inhibits growth factor induced PCa cell proliferation, viability and DNA synthesis and in turn increases their apoptotic death, (ii) IP6 efficacy is accompanied by strong inhibition of both constitutive as well as mitogens-induced activated form of the pro-survival factor Akt and differential modulation of the MAPKs, and (iii) that IP6 inhibits AP-1 activation which was consistent with its observed biological effects in human PCa cell lines.

Several *in vitro* and *in vivo* studies have reported a constitutively active mitogenic and pro-survival signaling pathways in human PCa cells as well as tumor tissue [8,28,32]. It has been observed in PCa, that, there is an aberrant expression and interaction, via an autocrine/paracrine loop, of growth factors and their receptors [8]. EGF is one of the most essential mitogens for many epithelial cells, and typically it promotes cell growth and proliferation via a well-characterized EGFR—Shc—Grb2/SOS—Ras—Raf—ERK pathway [26]. The

associated receptor, EGFR, is a ligand-activated receptor tyrosine kinase that helps transduce mitogenic signals to the nucleus via MAPK family members, which are well recognized to play an important role in mediating the intracellular events essential for cell growth, proliferation, and apoptosis [26]. Since, regulation of MAP kinase activity is essential for normal cell growth and differentiation, it is obvious that disruption in MAPK signaling pathway would lead to pathophysiological changes including oncogenesis and apoptosis [26].

Recent epidemiological studies have shown that higher circulating levels of the mitogen IGF-1 and/or lower IGFBP-3 level are strongly and positively correlated with increased risk of many cancers including PCa [33]. With regard to IGF-1 mediated activation of IGF-1R, studies indicate that bioactive forms of IGFs are tightly controlled by the presence of their binding proteins-IGFBPs [33]. IGFBP-3 sequesters and prevents the interaction of IGF with IGF receptors and thereby inhibits the mitogenic and survival activities of IGFs [33,34]. IGF-1 interaction with its receptor activates tyrosine kinase activity of IGF-1 receptor which triggers down-stream signaling either via Ras/Raf/MAPK or PI3K/Akt mediated signaling, which has been reported to be deregulated in PCa and is a predictor of the advanced stage of this malignancy [34]. Furthermore, it is also known that some of the PCa cell lines (LNCaP, PC-3) also have constitutively activated form of Akt, which is attributed to the loss of functional PTEN [28]. This deregulation of the PI3K—Akt signaling axis also contributes to survival and deregulated proliferation in these cell lines [28].

MAPKs are also known to play a dual role in regulating specific functions associated with both proliferation and apoptosis [35,36]. Inhibition or sustained activation of MAPKs' response to stress and other signals can result in either a proliferative or an apoptotic response [36]. JNK and p38 stress kinase pathways are strongly implicated in the induction of growth arrest/ apoptosis by a wide variety of signals such as ultraviolet irradiation, oxidative stress, DNA-damaging agents, inflammatory cytokines, growth factors etc [37]. While high levels of JNK and p38 activities have been correlated with the induction of apoptosis, other studies have shown that JNK and/or p38 activation occurs without influencing cell death [35,36]. In the present study, we observed a sustained increase in the pERK1/2 and pJNK1/2 levels after starvation which led to a slight increase in the apoptotic death in serum starved controls compared to cells under serum conditions. In the presence of mitogens such as EGF or IGF1 though a strong increase in the levels of pERK1/2 was observed, it was not accompanied with an induction of apoptosis but on the other hand an increase in proliferation was observed, suggesting that ERK1/2 phosphorylation (and possibly activation) has a differential biological fate depending on the cellular environment where starvation causing apoptosis but mitogen stimulation leads to proliferation. Interestingly, we observed that IP6 not only decreased the activated levels of ERK1/2, JNK1/2 and p38 but at the same time was able to cause a further induction of apoptosis together with a decrease in cell proliferation.

Based on the studies discussed above, the results of the present study showing the inhibitory effects of IP6 on these signaling pathways and associated biological responses in human PCa PC-3 cells are highly significant. Specifically, our findings showed a comparison among the pro-survival and mitogenic signals inhibited by IP6 and related biological effects in terms of inhibiting the growth and proliferation and inducing apoptotic death of PC-3 under prolonged exposure to the growth factors like EGF and IGF1. Furthermore, the experimental conditions of our study mimic advanced human PCa situation where various pro-survival and mitogenic signals are constitutively active including those involving EGF and IGF1 as elaborated above. Under these situations, the effect of IP6 in inhibiting both constitutive as well as growth factor (EGF and IGF1)-induced signaling pathways suggest that this agent could be effective in blunting both pro-survival and mitogenic signaling in

PCa cells irrespective of the nature of the stimuli, and that such an effect of IP6 eventually leads to inhibition of growth and proliferation and induction of apoptotic death of PCa cells under both constitutive and ligand activated conditions. Further, the ability of IP6 to induce apoptotic death even in the presence of the mitogens, suggests that IP6 had a profound effect on the anti-apoptotic pathways stimulated by both EGF and IGF1. Importantly, the effect of IP6 in inhibiting various biological responses was not specific to PC-3 cells as we also observed a similar kind of growth inhibitory effect of IP6 in two additional human PCa cell lines namely DU145 and LNCaP, which represent advanced but androgen-independent and —dependent PCa, respectively.

The 2 mM dose of IP6 showing strong efficacy in the present study was based on earlier *in vitro* studies where a dose of up to 5 mM IP6 was employed by others and us and was found to be optimal [11,16-19]. The physiological and/or pharmacological significance of IP6 level in the present *in vitro* studies needs to be studied and established in future clinical studies. However, when the *in vitro* concentration of IP6 used in the present study (with only once treatment) is compared with published literature showing its anti-cancer activity in animal models, it is much lower than those (up to 15 mM) used in the chronic *in vivo* animal studies [11,21] without any toxicity, which clearly establishes the relevance present findings in defining the molecular mechanism of IP6 efficacy at least in pre-clinical anti-cancer studies.

Overall, we observed pleiotropic molecular alterations by IP6 suggesting down-regulation of Akt- and MAPK-mediated signaling culminating in the induction of pro-apoptotic caspase-3 cascade and inhibition of the activity of the transcription factor AP-1. Since, the modulations of these molecules are implicated in both mitogenic and survival effects [8], as expected, IP6 also caused a marked decrease in cell proliferation, and strongly enhanced apoptosis in PC-3 cells. In this regard, it is likely that potential mechanisms of IP6 could be the inhibition of EGFR/IGFR-PI-3K (phosphatidylinositol-3 kinase)-Akt signaling, differential modulation of EGFR—MAPK pathway, and induction of caspase-dependent apoptosis which have been reported to be deregulated in PCa cells [8]. The other possible mechanism could be that IP6 is targeting the IGF-1 (insulin-like growth factor-1)-IGFBP-3 (IGF binding protein-3) axis for its antiproliferative and pro apoptotic effects as has been observed in the xenograft study using DU145 PCa cell line [21]. Whereas more studies are needed in future to further address these questions, nevertheless, the findings of the present study are both novel and highly significant in establishing IP6 efficacy in inhibiting aberrant EGFR or IGF-1R pathway-mediated sustained growth promoting and survival signaling cascades in advanced and androgen-independent human PCa PC-3 cells, which might have translational implications in advanced human PCa control and management.

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Abbreviations

PCa	prostate cancer
IP6	inositol hexaphosphate
EGF	epidermal growth factor
EGFR	EGF receptor
RTKs	receptor tyrosine kinases
IGF-1	insulin-like growth factor-1

IGF-1R	IGF-1 receptor
IGFBP-3	insulin-like growth factor binding protein-3
MAPK	mitogen activated protein kinase
ERK1/2	extra cellular signal-regulated kinase 1/2
JNK 1/2	c- <i>jun</i> N-terminal protein kinase
AP-1	activator protein-1
EMSA	electrophoretic mobility shift assay
NF-κB	nuclear factor-kappa B
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	phosphatase and tensin homolog
PI3K	Phosphoinositide 3-kinase
TRAMP	transgenic adenocarcinoma of the mouse prostate

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Figure 1. IP6 Inhibits Constitutive and Mitogen-induced Cell Growth and DNA Synthesis in PC-3, DU145 and LNCaP Cells. The cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) under standard culture conditions. After 24 h, cells were switched to serum-free medium for 12 h and then treated with IP6 alone or with EGF/ IGF (100ng/mL) along with 2mM IP6 in fresh serum-free medium for 24 and 48 h. The effects of different treatments on cell growth in terms of total cell number by Trypan blue dye exclusion method (A) and DNA synthesis by BrdU incorporation assay (B) were assessed as detailed in Methods. The data shown are the mean $\pm$ SE of two independent experiments each done in triplicate. The difference between the IP6 treated group versus the control or the respective ligand group was analyzed by unpaired two-tailed Student's *t*-test. *P* values <0.05 were considered significant. *, *P*<0.001; #, *P*<0.01; \$, *P*<0.05. IP6, inositol hexaphosphate; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1.

Figure 2.

IP6 Decreases Cell Viability and Induces Apoptotic Death of Human PCa PC-3 Cells. PC-3 cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) under standard culture conditions, and treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. The effect of different treatments on cell viability by MTT assay (A) and apoptotic cell death in terms of percentage of apoptotic cells by YOPRO staining (B) was determined as detailed in Methods. The data shown are the mean $\pm$ SE of two independent experiments each done in triplicate. The difference between the IP6 treated group versus the control or the respective ligand group was analyzed by unpaired two-tailed Student's *t*-test. *P* values <0.05 were considered significant. *, *P* <0.001 ; #, *P* <0.01 ; \$, *P* <0.05 . (C) The effect of different treatments on cleaved caspase-3 and cleaved PARP was determined by western blot analysis. In each case, membranes were stripped and probed with β -actin as loading control. IP6, inositol hexaphosphate; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1.

Figure 3.

IP6 Inhibits Constitutive and Mitogen-induced Levels of Phospho- and Total Akt and MAPKs in PC-3 Cells. PC-3 cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) under standard culture conditions, and treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. After specific treatment times, cell lysates were prepared and Western immunoblot analysis was done as detailed in Methods. Reactive protein bands for the expression of phosphorylated and total Akt, ERK1/2, JNK1/2 and p38 were visualized by enhanced chemiluminescence detection system, and membranes were stripped and probed with β -actin as loading control. The data shown are representative of at least two independent experiments. IP6, inositol hexaphosphate; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1.

Figure 4.

IP6 Inhibits Constitutive and Mitogen-induced Activation of c-Jun, c-Fos and AP-1 in PC-3 cells. PC-3 cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) under standard culture conditions, and treated with IP6 (2 mM) in 10% serum condition or serum-starved for 12 hrs and treated with IP6 without or with ligand (EGF or IGF-1, both 100 ng/ml) for 24 and 48 hrs. (A) After specific treatments, nuclear lysates were prepared and Western blot analysis was done as detailed in Methods. Reactive protein bands for the expression of phosphorylated and total c-Jun and c-Fos were visualized by enhanced chemiluminescence detection system. (B) To determine the effect on the DNA-binding activity of AP-1, EMSA was performed as described in Methods. (C) To determine AP-1 band specificity in EMSA, gel super shift assay was performed as detailed in Methods. Untreated control nuclear extracts were incubated with anti-c-Jun, anti-c-Fos or both these antibodies, followed by EMSA. The specificity of DNA binding was also confirmed by adding 100-fold excess of cold probe in complete reaction incubation. The data shown are representative of at least two independent experiments. IP6, inositol hexaphosphate; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1.

Table 1

IP6 inhibits cell growth, viability and DNA synthesis in human PCa PC-3, DU145 and LNCaP cells*

PCa cells	Treatment	Total no. of cells ($\times 10^4$)		MTT assay (OD 570 nm)		BrdU incorporation assay (OD 370-492 nm)	
		24h	48h	24h	48h	24h	48h
PC-3	Control	44.8 $\pm$ 1.55	53.6 $\pm$ 1.6	0.58 $\pm$ 0.02	0.73 $\pm$ 0.03	1.86 $\pm$ 0.08	2.57 $\pm$ 0.22
	2mM IP6	27.9 $\pm$ 1.7*	28.2 $\pm$ 1.5*	0.21 $\pm$ 0.03*	0.22 $\pm$ 0.01*	0.91 $\pm$ 0.02*	1.34 $\pm$ 0.33*
DU145	Control	55.57 $\pm$ 1.85	68.24 $\pm$ 1.2	0.27 $\pm$ 0.02	0.29 $\pm$ 0.06	0.57 $\pm$ 0.06	0.68 $\pm$ 0.5
	2mM IP6	36.87 $\pm$ 1.02*	47.7 $\pm$ 1.6*	0.20 $\pm$ 0.02\$	0.18 $\pm$ 0.08*	0.55 $\pm$ 0.07	0.32 $\pm$ 0.03#
LNCaP	Control	68.5 $\pm$ 1.2	90.7 $\pm$ 3.4	0.62 $\pm$ 0.08	0.76 $\pm$ 0.03	2.5 $\pm$ 1.2	0.61 $\pm$ 0.1
	2mM IP6	53.2 $\pm$ 1.4*	65.4 $\pm$ 1.8*	0.51 $\pm$ 0.05#	0.53 $\pm$ 0.04#	1.75 $\pm$ 0.12*	0.31 $\pm$ 0.1*

* Human PCa cells were cultured in RPMI 1640 medium (containing 10% fetal bovine serum and 1% penicillin—streptomycin) under standard culture conditions, and the effects of IP6 treatment on PCa cell growth (in terms of total cell number by Trypan blue dye exclusion method), cell viability (by MTT assay) and DNA synthesis (by BrdU incorporation assay) were assessed as detailed in Methods. The data shown are the mean $\pm$ SE of two independent experiments each done in triplicate. The difference between the IP6-treated groups versus the controls was analyzed by unpaired two-tailed Student's *t*-test. *P* values <0.05 were considered significant.

* *P*<0.001

P<0.01

\$ *P*<0.05.