Impairment of erbB1 receptor and fluid-phase endocytosis and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells

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Recently, we observed that epidermal growth factor receptor (EGFR or erbB1) endocytosis and associated mitogenic signaling occur in human prostate cancer (PCA) cells, suggesting that erbB1 endocytosis might be involved in advanced and androgen-independent PCA growth. Based on these findings, and the fact that aberrant expression of erbB family members is common in human prostatic intraepithelial neoplasia and invasive PCA, we reasoned that impairment of erbB1 endocytosis and associated mitogenic signaling might inhibit PCA growth. Inositol hexaphosphate (IP6) interacts with plasma membrane clathrin-associated protein complex 2 (AP2) and inhibits phosphatidylinositol-3 kinase (PI3K). As these are essential components of receptor-mediated and fluid-phase endocytosis, respectively, we reasoned that IP6 might impair erbB1 endocytosis and associated signaling in human PCA cells, leading to their growth inhibition. IP6 strongly and completely inhibited (26–100%; \( P < 0.05 \)) transforming growth factor \( \alpha \)-induced binding of activated erbB1 to AP2 in human PCA DU145 cells, demonstrating the impairment of the initial step in ligand-induced erbB1 endocytosis. IP6 treatment of cells resulted in a dose-dependent increase (1.8- to 7.7-fold compared with cells treated with ligand alone; \( P < 0.05 \)) in levels of activated erbB1. These two findings suggest that the inhibitory effect of IP6 on receptor endocytosis is independent of its lack of effect on ligand-induced erbB1 activation. These effects of IP6, however, were associated with strong inhibition of ligand-induced Shc phosphorylation (77–84% decrease; \( P < 0.05 \)) and its binding to erbB1 (58–100% decrease; \( P < 0.05 \)). IP6 also significantly and dose-dependently inhibited fluid-phase endocytosis (19–52%; \( P < 0.05 \)). It inhibited PI3K–AKT signaling pathway as an upstream response in its effect on the inhibition of fluid-phase endocytosis. The inhibition of erbB1 receptor and fluid-phase endocytosis, and associated signaling by IP6, was corroborated by very strong to complete inhibition (70–100%; \( P < 0.05 \)) of extracellular signal-regulated protein kinase I/2 activation by IP6. IP6 significantly (\( P < 0.05 \)) inhibited anchorage-dependent and -independent inhibition (50–100% and 30–75%, respectively) in DU145 cells. Targeting the impairment of erbB1 endocytosis and associated mitogenic signaling by IP6 in advanced and androgen-independent human PCA DU145 cells could be a useful approach for treating PCA.

Introduction

Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy, and second leading cause of cancer deaths in males in the USA (1, 2). Induction of PCA is viewed as a multistage process, involving progression from small, latent carcinomas of low histological grade, to large, metastatic carcinomas of higher grade (2–5). The widely accepted risk factors for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (2–5). Epidemiological data have revealed that environmental and behavioral factors are more important than genetic factors in determining overall cancer frequency among populations (reviewed in ref. 6). Consistent with these reports, diet and androgen have been shown to play a major role in the pathogenesis and promotion of PCA (2–5). The role of androgen in PCA is further supported by the fact that this malignancy rarely occurs in eunuchs or men with a deficiency in 5α-reductase, the enzyme that irreversibly converts testosterone to its active metabolite, dihydrotestosterone (7–9). Since the growth and development of PCA is initially androgen dependent, androgen deprivation has been extensively explored as a strategy for PCA prevention and therapy (7). PCA patients treated with androgen deprivation therapy often have remission of the disease, but tumor regrowth occurs, largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (8).

In addition to the loss of androgen dependence due to lack of androgen receptor and/or its function (10), interactions between functional autocrine or paracrine growth factors and growth factor receptors are major contributors to the multifactorial mechanisms of androgen independence in PCA (11–13). For example, advanced and metastatic human PCA cells express high levels of epidermal growth factor receptor (EGFR or erbB1) and transforming growth factor \( \alpha \) (TGF\( \alpha \)) (11–13). Furthermore, expression of erbB family members (e.g. erbB1, erbB2 and erbB3) is often abnormal in prostatic intraepithelial neoplasia (PIN) and in invasive PCA, both primary and metastatic (14–19). In fact, erbB family receptors are one of the few potential surrogate endpoint genetic markers that have been used for the screening of interventive agents against PCA in short-term phase II clinical trials (14–19). They have also been extensively explored as major potential molecular targets for PCA intervention, specifically in androgen-independent PCA (reviewed in ref. 20).

Activation of erbB1 by its ligand includes receptor dimerization, activation of intrinsic receptor tyrosine kinase activity, autophosphorylation of the receptor at the carboxyl terminus
and tyrosine phosphorylation of and/or association with intracellular signaling molecules such as Shc, and phosphatidylinositol 3-kinase (PI3K) (19–22). The binding of ligand to the receptor results in rapid disappearance of receptors from the cell surface. Receptor down-regulation is due to ligand-accelerated endocytosis and degradation of erbB1 (23,24). Morphological studies suggest that ligand increases receptor endocytosis by promoting receptor clustering into clathrin-coated pits on the plasma membrane and that this is followed by receptor internalization into clathrin-coated vesicles (25).

The ligand-dependent acceleration of receptor internalization is the rate-limiting step in receptor down-regulation and activation (26,27). The internalization process of receptor occurs through receptor-mediated endocytosis, where plasma membrane-coated pits function as sorting organelles selectively recruiting receptors that contain internalization sequences or ‘codes’ within their cytoplasmic domains (26,27). A major structural component of coated pits is the clathrin lattice anchored to cytoplasmic surface of the membrane by associated protein complexes or adaptors [plasma membrane clathrin-associated protein complex 2 (AP2)] (28). AP2 is the most widespread of the associated proteins found in coated vesicles derived from the plasma membrane (29), and has been shown to interact specifically with erbB family members (22,30). In addition to receptor-mediated endocytosis involving initial binding of activated receptor with AP2 for receptor internalization, fluid-phase endocytosis can occur, mediated via the PI3K–AKT–Rab5 pathway (31–35).

Taken together, the literature studies summarized above suggest that erbB1 endocytosis, and associated mitogenic and cell survival signaling, could be major events in the growth and proliferation of human prostate carcinoma cells. Indeed, in a recent study, we observed that both erbB1 receptor-mediated and fluid-phase endocytosis, and associated mitogenic and cell survival signaling, occur in human prostate carcinoma cells in the order of DU145 > PC3 > LNCaP (36). Based on these results, we reasoned that agents that impair receptor endocytosis and associated mitogenic/cell survival signaling could be useful for the treating human PCA.

Traditional Asian diets and those of vegetarians are not only high in starch and fiber, but are also rich in many bioactive compounds which are receiving increasing attention for the prevention and intervention of a wide variety of human cancers (20,37–43). Epidemiological data suggest that consumption of a high-fiber diet is associated with a reduction in breast, colon and prostate cancers (reviewed in ref. 44). The only types of high-fiber diet that have been consistently associated with a reduction in colon and breast cancers are cereals and legumes, which contain high levels of inositol hexaphosphate (IP6) (44). IP6 (also known as phytic acid) constitutes 0.4–6.4% (w/w) of most cereals, nuts, legumes, oil seeds and soybean; it is also a component of mammalian cells occurring at concentrations of 10–100 μM in both resting and stimulated cells (44,45). Several studies in recent years have shown the chemopreventive and anti-carcinogenic effects of IP6 against different cancers of epithelial and mesenchymal cell origin in both in vivo and in vitro models (reviewed in refs 44,46–49). It has been shown to be protective against colon (44,46,47), mammary (50,51), liver (52), lung (53) and skin (44) tumorigenesis, to inhibit the growth of mouse fibrosarcoma FSA-1 cell tumor xenograft in nude mice and to reduce the number of metastatic lung colonies and improve host survival (54). With regard to PCA, it has been shown that IP6 inhibits the growth and induces differentiation of human prostate carcinoma PC3 cells (55).

Mechanistic studies have shown that IP6 is involved in various signal transduction pathways. For example, it binds to clathrin assembly protein AP2 (56) and directly inhibits PI3K activation and activity (57); these are essential components in receptor-mediated and fluid-phase endocytosis, respectively. Based on the studies above described, we reasoned that IP6 would impair both AP2- and PI3K-mediated endocytosis, and thereby, associated cellular mitogenic responses in human PCA cells, and that this might be a novel approach for treating PCA.

Endocytosis is a carefully orchestrated process required for nutrition, down-regulation of surface receptors and maintenance of homeostasis (58). Some endocytosis proteins have been reported in human cancers, and enhanced endocytosis is associated with neoplastic transformation (59). Accordingly, we focused our efforts in this study to assess the impairment of erbB1 endocytosis and associated signaling by IP6 in human prostate carcinoma DU145 cells. Advanced and metastatic human PCA cells such as DU145 and PC3 lack functional androgen receptor and express high levels of erbB1 together with TGFα, leading to autonomous growth of cancer cells via an autocrine feedback loop (11–13,20). These cells, therefore, represent a valuable system to investigate erbB1 endocytosis and associated signaling, and in testing its impairment by potential preventive agents.

We show that IP6 inhibits (i) the binding of ligand-activated erbB1 to AP2, which may impair the erbB1–Shc–mitogen-activated protein kinase (MAPK) signaling pathway and (ii) ligand-induced activation of the PI3K–AKT signaling pathway. These effects of IP6 also resulted in the inhibition of prostate carcinoma DU145 cell growth.

Materials and methods

Materials

The human prostate carcinoma DU145 cell line was obtained from the American Type Culture Collection (Manassas, VA); IP6, horseradish peroxidase (HRP) and wortmannin were from Sigma–Aldrich Chemical Co. (St. Louis, MO). Human TGFα and all other cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Anti-erbB1 (EGFR), anti-Shc, anti–PI3K, anti-AKT, anti-phospho-AKT and anti-phosphotyrosine antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-AP2 antibody was from Affinity Bioreagents Inc. (Denver, CO). Anti-phospho-MAPK/ERK1/2 and anti-MAPK/ERK1/2 antibodies were from New England Biolabs (Beverly, MA). Secondary antibodies (rabbit antibodies against mouse immunoglobulin and goat antibodies against HRP-conjugated rabbit immunoglobulins) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The enhanced chemiluminescence (ECL) detection system was from Amersham Corp. (Arlington Heights, IL).

Cell culture and treatments

Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard culture conditions in 100 mm dishes until they reached 70% confluency. Cells were then starved in serum-free medium for 36 h; during the last 2 h of starvation, they were treated with double-distilled water alone or with varying concentrations (0.25–2 mM) of IP6 dissolved in water or with 200 ng/ml wortmannin. The IP6 concentrations used in the present study were identical to those used in other studies (44,55,57). At the end of these treatments, cultures were treated with phosphate-buffered saline (PBS) or ligand TGFα (100 ng/ml medium) in PBS and incubated for 10 min at 37°C. Thereafter, medium was aspirated, attached cells were washed twice with cold PBS and cell lysates were prepared as described recently (20).

Immunoprecipitation and immunoblotting

For immunoprecipitation, cell lysates (200–500 μg protein) were clarified by protein A/G agarose for 1 h and then incubated with primary antibody directed against erbB1, Shc, PI3K or AKT for 4 h followed by addition of protein A/G agarose and overnight incubation at 4°C with rocking. Immunocomplexes
were washed three times with lysis buffer (20). For immunoblotting, immunocomplexes or cell lysates (20–80 μg protein) were denatured in sample buffer (2× buffer is 125 mM Tris pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% β-mercaptoethanol and 0.04% bromophenol blue), proteins were separated on SDS-polyacrylamide gels (8% or 12% gel) and transferred onto to nitrocellulose membranes. The membranes were blocked with blocking buffer (10 mM Tris pH 7.5, containing 100 mM NaCl, 0.1% Tween 20 and 5% non-fat milk powder) at room temperature for 1 h, then incubated overnight with the appropriate primary antibody (at 1–2 μg/ml dilutions) directed against AP2, phosphotyrosine, erbB1, Shc, PI3K, AKT, phospho-AKT, MAPK or phospho-MAPK followed by the appropriate secondary antibody, and developed using an ECL kit (20). Autoradiograms of the western immunoblots were scanned using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using Scion Image Program (National Institutes of Health, Bethesda, MD). In each case, only representative blots are shown with reproducible findings in three or four independent experiments. The densitometric data shown in each case are mean ± SD of three or four independent experiments.

Fluid-phase endocytosis assay

As a measure of fluid-phase endocytosis, HRP uptake was assayed as described by Li and Stahl (60). Briefly, cultures in 35 mm dishes at 70–80% confluency were washed three times with serum-free α-MEM, and treated with varying concentrations of IP6 or 200 ng/ml wortmannin in α-MEM for 2 h at 37°C. HRP endocytosis was initiated by addition of 2 mg/ml HRP and 1% (w/v) bovine serum albumin at 37°C for another 1 h. To estimate HRP uptake, the cells were washed three times with PBS, trypsinized on ice for 20 min, washed again twice with PBS and lysed in 500 μl of lysis buffer (60). Cell lysates were assayed for HRP activity (60) and protein concentration was determined using the Bio-Rad DC protein assay according to the manufacturer’s instructions. This experiment was repeated twice more, each done in duplicate.

Cell growth and soft agar colony formation assays

For cell growth, DU145 cells were plated at a density of 0.5 × 10^4 cells per 60 mm plate. After 24 h (denoted as day 0 of treatments), cells were fed with fresh medium and left untreated, or treated with IP6 dissolved at 0.25, 0.5, 1 or 2 mM (final concentration) in double-distilled water. The cultures were fed with fresh medium every third or fourth day until the end of the experiment. Each treatment and time point had four plates. After various treatment times, cells were trypsinized and counted as described (20). Cell viability was assessed using the Trypan Blue dye exclusion method. This experiment was repeated twice more. The representative data shown in this report were reproducible in three independent experiments.

For soft agar colony formation, DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin as detailed above. Soft agar colony formation assay was performed using six-well plates as described (20). Briefly, each well contained 2 ml of 0.5% agar in medium as the bottom layer, 1 ml of 0.38% agar in medium with ~1000 cells as the feeder layer, and 1 ml of 0.38% agar in medium with different concentrations of IP6 at the top layer. Three wells were used for each treatment. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a colony was counted as ≥16 cells. This experiment was repeated once more. The representative data shown were reproducible in two independent experiments.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) performed with SAS/Proc GLM (SAS Institute, Cary, NC). If the ANOVA indicated at least one significant difference (using a P-value cut-off of 0.05), pairwise comparisons were performed. The within-experiment type I error rate was controlled at 0.05 using the Bonferroni correction for multiple comparisons. For three comparisons, if a difference is to be regarded as statistically significant, the Bonferroni correction requires a P-value of <0.0167 for any given pairwise comparison. Pairs of treatment groups referred to as statistically significant in the Results section are based on these post hoc tests.

Results

**IP6 inhibits the binding of ligand-activated erbB1 to AP2 in DU145 cells**

Using DU145 cells, we first explored the effect of IP6 on receptor endocytosis process by analyzing the binding of ligand-activated erbB1 to AP2. As shown in Figure 1A (lane 1), immunoprecipitation with anti-erbB1 antibody and immunodetection with anti-AP2 antibody showed that 36 h of serum starvation of DU145 cells resulted in diminished binding of erbB1 to AP2. This observation suggested that erbB1–AP2 binding is an activated erbB1-associated phenomenon that is reduced to very low levels after 36 h serum starvation. After starved cells had been treated with TGFα (100 ng/ml) for 10 min, however, they showed very strong erbB1–AP2 binding (Figure 1A, lane 2). Pretreatment of cultures with 0.25, 0.5 or 1 mM IP6 during the last 2 h of starvation followed by treatment with TGFα at the same concentration and for the same time resulted in a strong decrease in the binding of erbB1 to AP2 (Figure 1A, lanes 3–5). Densitometric analysis of the blots from four independent experiments (Figure 1B) showed that, compared with ligand alone, pretreatment with 0.25, 0.5 or 1 mM IP6, followed by ligand activation, resulted in a statistically significant (P < 0.05) decrease (26%, 50% and 100%, respectively) in the binding of erbB1 to AP2 (Figure 1B). Conversely, treatment of serum-starved cultures with a PI3K inhibitor, wortmannin, at 200 ng/ml followed by TGFα did not show any change in erbB1 binding to AP2
Fig. 2. Impairment of erbB1 receptor-mediated endocytosis by IP6 does not affect erbB1 activation in DU145 cells. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. ErbB1 was immunoprecipitated using anti-erbB1 antibody and immunoprecipitates were then subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting. (A) Membrane was probed with anti-phosphotyrosine (upper panel) or anti-EGFR (lower panel) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (B) Densitometric analysis data (mean ± SD) from three independent experiments.

We next assessed the involvement of erbB1 activation in this ligand-induced binding of erbB1 to AP2. As shown in Figure 2A (top panel), serum starvation of cells for 36 h completely inactivated erbB1 (lane 1). However, treatment of starved cultures with TGFα for 10 min resulted in a marked activation of erbB1 receptor (Figure 2A, lane 2). Pretreatment of cultures with 0.25, 0.5, 1 or 2 mM IP6 for 2 h followed by ligand treatment under identical conditions resulted in a strong increase in erbB1 tyrosine phosphorylation (Figure 2A, lanes 3–6). Densitometric analysis of the blots from three independent experiments (Figure 2B) showed that, compared with ligand alone, pretreatment with IP6 at 0.25, 0.5, 1 or 2 mM followed by ligand stimulation resulted in a statistically significant ($P < 0.05$) increase (~1.8-, 4.7-, 4.6- and 7.7-fold, respectively) in the levels of tyrosine-phosphorylated erbB1 (Figure 2B). Immunoblotting the membrane with anti-erbB1 antibody (Figure 2A, lower panel) and densitometric analysis of the blots (data not shown) did not reveal any differences in erbB1 protein following different treatments. These results suggested that the observed increase in erbB1 activation by IP6 was not due to an increase in erbB1 protein. Treatment of starved cultures with IP6 alone at 1 mM did not affect tyrosine phosphorylation or the level of erbB1 protein (data not shown), suggesting that the observed increase in erbB1 activation following treatment with IP6 plus ligand is not due to a direct receptor activation by IP6 alone. When erbB1 activation data were compared with the binding of erbB1 to AP2, they were inversely related (compare Figure 2 with Figure 1). This suggests that the observed increase in erbB1 activation by IP6 could have been due to impairment of erbB1 endocytosis where a lack of binding with AP2 leaves the activated erbB1 receptor at the cell surface instead of being internalized and degraded by a stepwise process (22,23). These results also suggest that IP6 impairs receptor endocytosis by targeting its effect on AP2 binding with erbB1 in an erbB1 activation-independent manner.

**IP6 inhibits Shc activation and Shc–erbB1 binding in DU145 cells**

We next assessed the effect of erbB1 endocytosis impairment by IP6 on the erbB1-mediated immediate downstream signaling pathway involving Shc activation. In contrast to erbB1 activation data, treatment of cultures with different concentrations of IP6 for 2 h, before the addition of TGFα, showed a strong decrease in the tyrosine phosphorylation of Shc protein (Figure 3A, lanes 3–6). As a control, serum starvation of cells for 36 h led to complete absence of tyrosine phosphorylated 46 and 52 kDa Shc proteins (Figure 3A, lane 1); however, treatment of starved cultures with TGFα showed strong phosphorylation of the 46 kDa Shc protein and weak phosphorylation of the 52 kDa Shc protein as evidenced by a reactivity of immunoprecipitated Shc to anti-phosphotyrosine antibodies (Figure 3A, lane 2). Densitometric analysis of the blots from three independent experiments showed that the levels of phosphorylated 52 kDa Shc protein were changed inconsistently following different treatments (data not shown). However, in the case of the phosphorylated 42 kDa Shc protein, 0.5, 1 and 2 mM IP6 pretreatment followed by ligand, resulted in a statistically significant ($P < 0.05$) decrease ($77$–$84\%$) in its levels compared with treatment with ligand alone (Figure 3D). The observed inhibitory effect of IP6 on Shc activation was not due to a change in Shc protein levels (Figure 3B). Shc proteins contain a Src homology 2 (SH2) domain, which binds to phosphotyrosine-containing sequences, including erbB1, upon ligand activation. As shown in Figure 3C, there was strong binding in the sample treated with TGFα alone (lane 2), whereas IP6 treatment at various concentrations resulted in a very strong decrease in the binding of Shc to erbB1 (Figure 3C, lanes 3–6). Densitometric analysis of the blots from three independent studies showed that, compared with ligand alone, IP6 treatments resulted in a statistically significant decrease (58–100%; $P < 0.05$) in the binding of Shc to erbB1 (Figure 3D). This observation further suggests an inhibition of erbB1-mediated downstream signaling following impairment of ligand-activated erbB1 binding to AP2 and thereby inhibition of receptor endocytosis by IP6. Studies are in progress to test this hypothesis and rule out any possibility that IP6 has a direct effect on Shc phosphorylation independent of an inhibitory effect on activated erbB1 endocytosis.
IP6 also inhibits fluid-phase endocytosis in DU145 cells

To determine the effect of IP6 on fluid-phase endocytosis in DU145 cells, HRP uptake experiments were carried out as described (60). As shown in Figure 4, compared with control, IP6 treatment resulted in a dose-dependent decrease in HRP uptake in DU145 cells as measured by HRP activity in terms of its binding with cellular proteins (60). The lowest concentration (0.25 mM) of IP6 used in this study caused only 19% inhibition ($P < 0.05$) of fluid-phase endocytosis (Figure 4). Much higher inhibition was observed at 0.5, 1 and 2 mM IP6, accounting for 38%, 42% and 52% inhibition ($P < 0.05$), respectively (Figure 4). Treatment of cells with a PI3K inhibitor, wortmannin, also showed similar decrease in fluid-phase endocytosis (Figure 4; 44% inhibition, $P < 0.05$). From these results with wortmannin, showing inhibition of fluid-phase endocytosis, as compared with the findings that it did not change the binding of AP2 to erbB1 in the receptor-mediated endocytosis study (Figure 1A, lane 6), it can be concluded that fluid-phase endocytosis involves a PI3K-mediated pathway in DU145 cells, whereas receptor-mediated endocytosis does not.

Inhibition of fluid-phase endocytosis by IP6 is mediated by impairment of the PI3K–AKT pathway in DU145 cells

As IP6 and the PI3K inhibitor wortmannin inhibited fluid-phase endocytosis, further studies were performed to delineate the involvement of PI3K–AKT pathway in this process. DU145 cells serum starved for 36 h did not show any tyrosine phosphorylation band for the 110 or 85 kDa PI3K subunits (Figure 5A, top panel, lane 1). However, immunoprecipitation of PI3K and blotting with an anti-phosphotyrosine antibody clearly showed that treatment of serum-starved cultures with TGfα results in a marked tyrosine phosphorylation of 110 kDa PI3K protein (Figure 5A, top panel, lane 2). This band could be an erbB family member since activated erbB receptors have been shown to bind and activate PI3K (19–22, 31–35). Studies are in progress to characterize this band further. In the studies assessing the effect of IP6 on PI3K phosphorylation, pretreatment of cells with IP6 resulted in a very strong inhibition of ligand-induced tyrosine phosphorylation of the 110 kDa PI3K band (and the ~170 kDa protein) (Figure 5A, top panel, lanes 3–5). Densitometric analysis (Figure 5B) of the blots from three independent studies showed that IP6 caused a statistically significant decrease (56%; $P < 0.05$) in tyrosine phosphorylation of the 110 kDa PI3K band at 0.25 mM and 100% inhibition ($P < 0.05$) at $\geq 0.5$ mM (Figure 5A, top panel, lanes 4 and 5; Figure 5B). Wortmannin (at 200 ng/ml) also completely inhibited tyrosine phosphorylation of a band at ~170 kDa in this sample (Figure 5A, lane 2). This band could be an erbB family member since activated erbB receptors have been shown to bind and activate PI3K (19–22, 31–35). Studies are in progress to characterize this band further. In the studies assessing the effect of IP6 on PI3K phosphorylation, pretreatment of cells with IP6 resulted in a very strong inhibition of ligand-induced tyrosine phosphorylation of the 110 kDa PI3K band (and the ~170 kDa protein) (Figure 5A, top panel, lanes 3–5). Densitometric analysis (Figure 5B) of the blots from three independent studies showed that IP6 caused a statistically significant decrease (56%; $P < 0.05$) in tyrosine phosphorylation of the 110 kDa PI3K band at 0.25 mM and 100% inhibition ($P < 0.05$) at $\geq 0.5$ mM (Figure 5A, top panel, lanes 4 and 5; Figure 5B). Wortmannin (at 200 ng/ml) also completely inhibited tyrosine phosphorylation of the 110 kDa PI3K band (Figure 5A, top panel, lane 2). The observed activation of the 110 kDa PI3K band by TGfα and its inhibition by IP6 was not due to a change in either the amounts of the 110 and 85 kDa PI3K proteins (Figure 5A, bottom panel).

AKT, a downstream target of PI3K, becomes serine–threo-
Fig. 5. Inhibition of fluid-phase endocytosis by IP6 is mediated by impairment of the PI3K–AKT pathway in DU145 cells: effect on PI3K activation. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. PI3K was immunoprecipitated using an anti-PI3K antibody, and immunoprecipitates or total cell lysates were then subjected to SDS-PAGE followed by western blotting as described in Materials and methods. (A) Immunoprecipitated PI3K were blotted and then probed with an anti-phosphotyrosine antibody (upper panel), and cell lysates were blotted and then probed with an anti-PI3K (lower panel) antibody. Membranes were then incubated with a peroxidase-conjugated appropriate secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (B) Densitometric analysis data (mean ± SD) from three independent experiments.

nine phosphorylated in vivo in a PI3K-sensitive manner (35,61,62). It has been linked to diverse cellular processes including cell survival by suppressing apoptosis via phosphorylation of BAD (63). Based on our data showing that IP6 inhibits ligand-induced activation of PI3K, we next assessed its effect on AKT phosphorylation. As shown in Figure 6A (top panel), immunoprecipitated samples from serum-starved cells showed no reactivity towards an antibody against phospho-AKT, whereas treatment of starved cultures with TGFα resulted in a strong activation of AKT (lanes 1 and 2, respectively). Pretreatment of cells with IP6 resulted in strong to complete inhibition of TGFα-induced AKT activation (Figure 6A, top panel, lanes 3–5). The PI3K inhibitor wortmannin completely inhibited ligand-induced AKT activation (Figure 6A, top panel, lane 6). Densitometric analysis of the blots (Figure 6B) from three independent studies showed that IP6 inhibited on ligand-induced AKT phosphorylation (25–100% decrease in the levels of phospho-AKT; Figure 6B) was statistically significant (P < 0.05). The observed changes in the levels of activated AKT were not due to a change in AKT protein levels in different treatment samples (Figure 6A, bottom panel).

IP6 also inhibits MAPK/ERK1/2 phosphorylation in DU145 cells

Via several different cytoplasmic signaling pathways, activation of erbB1 ultimately activates MAPK/ERK1/2, which localizes to nucleus and activates transcription factors for cell growth and proliferation (19–22). Besides inhibiting Shc activation, Shc binding to erbB1 and PI3K–AKT activation, IP6
IP6 impairs endocytosis in prostate cancer cells

Fig. 7. Inhibition of erbB1 receptor-mediated and fluid-phase endocytosis by IP6 impairs MAPK/ERK1/2 activation in DU145 cells. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. Total cell lysates were subjected to SDS-PAGE followed by western blotting as described in Materials and methods. (A) Membranes were probed with anti-phospho-MAPK (upper panel) or anti-MAPK (lower panel) antibody. They were then incubated with an appropriate peroxidase-conjugated secondary antibody and visualized by the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (B) Densitometric analysis data (mean ± SD) from three independent experiments.

IP6 also inhibited TGFα-induced MAPK/ERK1/2 phosphorylation with no change in protein levels (Figure 7A). Densitometric analysis of the blots (Figure 7B) from three independent studies showed that 0.25 mM IP6 resulted in a ~40% increase in phospho-ERK1/2 levels. Higher concentrations of IP6 (0.5, 1 and 2 mM) resulted in 100% inhibition (P < 0.05) of ligand-induced ERK1 phosphorylation and 70% inhibition (P < 0.05) of ERK2 phosphorylation (Figure 7B). Together, these data provide convincing evidence that IP6 treatment of DU145 cells results in the impairment of receptor-mediated and fluid-phase endocytosis and associated signaling which leads to the inhibition of MAPK signaling pathway as a downstream effect.

IP6 inhibits both anchorage-dependent and -independent growth of DU145 cells

To assess whether impairment by IP6 of erbB1 endocytosis and the mitogenic signaling associated with it, produces biological effects that occur at similar concentrations, we next assessed the effect of IP6 on anchorage-dependent and -independent growth of DU145 cells. As shown by data in Figure 8A, the treatment of cells with IP6 resulted in a significant inhibition of anchorage-dependent cell growth in both concentration- and

Fig. 8. IP6 inhibits both anchorage-dependent and -independent growth of DU145 cells. To monitor anchorage-dependent cell growth (A) and cell death (C), cells were plated at 0.5 × 10⁵ cells per 60 mm plate and, on day 2, treated with water or the indicated concentrations of IP6. The total number of cells was counted for varying time periods and cell viability was assessed by Trypan Blue dye exclusion assay. The cell growth data shown are mean ± se of four independent plates; each sample counted in duplicate. The cell death data shown are mean ± se of three independent plates; each sample counted in duplicate. Representative data from one experiment are shown; data from three independent studies were reproducible. To investigate anchorage-independent cell growth (B), a soft agar colony formation assay was performed using six-well plates as detailed in Materials and methods. The number of colonies was determined under an inverted phase-contrast microscope at ×100 magnification; a group of ≥16 cells was counted as a colony. The data shown are means ± se of three independent wells at optimum time (10 days) from the start of cell seeding; the experiment was repeated once with similar results.
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Fig. 9. Proposed mechanism for anti-proliferative action of IP6 on DU145 cells. IP6 impairs TGF\(\alpha\)-induced erbB1 receptor endocytosis leading to inhibition of cell proliferation by two mechanisms: (i) by inhibiting erbB1 recruitment of AP2 and Shc and (ii) by inhibiting PI3K–AKT activation. These events result in impairment of both receptor-mediated and fluid-phase endocytosis, and inhibition of mitogenic signaling such as MAPK/ERK1/2 activation, explaining the anti-proliferative action of IP6 in DU145 cells. The numbers shown in black as 1, 2 and 3 represent the steps for erbB1 endocytosis, and associated signaling pathways and cell proliferation.

Time-dependent manner. Compared with controls, treatment of cells with 0.25 mM IP6 showed >50% inhibition (statistically significant; \(P < 0.05\)) of cell growth at day 4 of treatment (Figure 8A). Much greater inhibition of cell growth occurred with 0.5 mM IP6 throughout the treatment time; by day 4 of treatment there was almost 80% inhibition (\(P < 0.05\)) (Figure 8A). At 1 and 2 mM IP6, no cell growth was observed after 1 day of treatment throughout the study (Figure 8A).

We next assessed the effect of IP6 on anchorage-independent growth of DU145 cells using soft agar colony formation assay. As many as 54.5 ± 3.6 (mean ± se of three independent plates) colonies/1000 cells (per plate) were counted in controls after 10 days of initial seeding (data not shown). Treatment of cells with IP6 resulted in a concentration-dependent inhibition in soft agar colony formation by DU145 cells (Figure 8B). The lower concentrations of IP6 (0.1 and 0.2 mM) showed almost no inhibition, whereas ~30% and 50% inhibition (statistically significant; \(P < 0.05\)) was evident at 0.4 and 0.5 mM, respectively (Figure 8B). The highest concentration of IP6 (4 mM) used in this assay caused a reduction by almost 75% (statistically significant; \(P < 0.05\)) in the number of colonies per plate.

Trypan Blue exclusion assays were used to assess the viability of IP6-treated cells; as shown in Figure 8C, 0.5, 1 and 2 mM IP6 caused statistically significant (\(P < 0.05\)) cell death, compared with controls, accounting for 6–40% death in a concentration- and time-dependent manner. These data suggest that the observed growth-inhibitory effects of IP6, shown in Figure 8A and B, could be due to its inhibitory effects on mitogenic signaling as well as cytotoxicity. Studies are currently in progress to assess and define apoptotic effects of IP6 in human prostate carcinoma cells.

Discussion

As summarized in Figure 9, the central finding of the present study is that IP6 impairs both receptor-mediated and fluid-phase endocytosis, resulting in the inhibition of mitogenic signals associated with growth and proliferation of human prostate carcinoma DU145 cells. The results obtained suggest a novel molecular pathway to be further explored for the intervention of advanced and androgen-independent human PCA by IP6. The major thrust in PCA control has been to design and develop intervention approaches based on molecular mechanisms (14,15). For example, the major emphasis of the National Cancer Institute, NIH, has been to develop surrogate
endpoint biomarkers for early detection, risk assessment and treatment of PCA (14,15). Special emphasis has also been placed on those markers which relate to the progression of microscopic to clinically relevant PCA that could be explored in intervention trials (14,15). As found in a significant number of routine needle biopsies without cancer, high-grade PIN is the most likely precursor of PCA, so PIN has been extensively used as a suitable endpoint biomarker for PCA treatment in clinical trials (14,15). It is important to emphasize here that erbB family members are one of the few potential surrogate endpoint genetic markers which are being employed for the screening of agents for treating PCA in short-term phase II clinical trials (14–18). ErbB family members are also being extensively explored as potential molecular targets for PCA intervention, specifically in the case of androgen-independent PCA (20). In view of these efforts, the results of the present study showing that a naturally occurring phytochemical, IP6, impairs erbB1 receptor endocytosis and associated mitogenic signaling in advanced and androgen-independent human PCA DU145 cells, could have direct implications in the treatment of advanced and androgen-independent human PCA.

The erbB and other receptor- and non-receptor-mediated signaling cascades activate MAPKs, a family of signaling molecules which are the ultimate cytoplasmic targets in signaling cascades (reviewed in refs 64–68). Following their activation, MAPKs are translocated to the nucleus where they activate transcription factors for cell growth, proliferation and differentiation (64–68). These studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, it has been shown recently that MAPK/ERK1/2 is constitutively active in human PCA DU145 cells (69). This study also showed that epidermal growth factor, insulin like growth factor I (IGF-1) and protein kinase A activator significantly activate MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via the erbB1 receptor (69). An increase in the activation of MAPK/ERK1/2 signaling has also been reported recently as human PCA progresses to a more advanced and androgen-independent malignancy (70). Consistent with the involvement of activated MAPK/ERK1/2, possibly via an erbB1 autocrine loop, in the progression of advanced and androgen-independent human PCA, in the present study, we observed that impairment of erbB1 endocytosis by IP6 also results in the inhibition of MAPK/ERK1/2 activation (Figure 9). The observed inhibitory effect of IP6 on MAPK/ERK1/2 activation could be via impairment of erbB1–She–Ras/Raf and/or erbB1–PI3K pathways (Figure 9); more studies are in progress to define further the specific signaling pathway affected by IP6 in inhibiting MAPK/ERK1/2 activation. The specificity of IP6 in inhibiting PI3K followed by AKT activation and fluid-phase endocytosis also needs to be further explored. For example, whereas it can be argued that the inhibitory effect of IP6 on PI3K activation observed in the present study is due to the impairment of ligand-induced erbB1 receptor endocytosis in DU145 cells, direct inhibition of PI3K by IP6 has also been reported in a recent in vitro assay (57). This study (57) also showed that IP6 significantly inhibits tumor promoter-induced cell transformation, AP-1 activity, PI3K activity and MAPK/ERK1/2 activation in JB6 cells. Consistent with the effects of IP6 reported in previous study (57), we have identified additional upstream molecular signaling events which are impaired by IP6 as a plausible cause for its downstream inhibition of PI3K and MAPK/ERK1/2 followed by AP-1 activation and cell transformation (this study).

The results obtained in the present study for the activation of the 110 kDa PI3K subunit by TGFα differ from those observed by us in another study showing that treatment of serum-starved DU145 cells with IGF-1 or neu differentiation factor (NDF) results in the activation of the 85 kDa PI3K subunit (36). PI3K can be activated by (i) its 85 kDa subunit, which binds to tyrosine kinase autophosphorylated at the sequence YXXM, a specific SH2 domain phosphotyrosine-binding sequence or (ii) its 110 kDa subunit, which binds to the Ras effector domain in a GTP-dependent manner (33,61). Differential ligand-induced activation of PI3K 110 and 85 kDa subunits observed by us in a recent study (36) suggests that, in DU145 cells, TGFα activates the PI3K class which does not interact with SH2 domain-containing adaptors but contains an amino-terminal Ras-binding site and therefore interacts with Ras proteins in a GTP-dependent manner. More detailed mechanistic studies are in progress to address this pathway further. However, to our knowledge, ours is the first report showing that TGFα activates the 110 kDa PI3K subunit of PI3K in DU145 cells and that IP6 inhibits this activation.

Several recent studies have shown a direct involvement of the PI3K–AKT pathway in fluid-phase endocytosis by regulating Rab5, which is active in its GTP-bound form and is a rate-limiting factor for endocytosis (33,34); specifically, Ras–PI3K is connected to the activation of AKT, which is a key regulator of fluid-phase endocytosis (33,34). In accordance with our present finding that TGFα activates the 110 kDa PI3K subunit in DU145 cells, as compared with the 85 kDa PI3K subunit by IGF-1 and NDF found in another study by us (36), TGFα also showed strongest effect in terms of fluid-phase endocytosis of HRP compared with that by IGF-1 and NDF (36). Together, these results further support the arguments that the Ras–PI3K–AKT pathway is involved in fluid-phase endocytosis and that IP6 inhibits fluid-phase endocytosis via inhibition of the PI3K–AKT pathway (Figure 9).

Based on the data reported here, together with earlier findings showing that IP6 has cancer-preventive and anticarcinogenic effects in several tumor models, we suggest that the role of dietary IP6 in preventing human PCA can be explored. Diet varies significantly from country to country, and has been estimated to account for up to 35% of differences in overall cancer rates (71). For example, the incidence of prostate, breast and colon cancers is lower in Asian countries than in the West, including the USA; people living in Japan, China, Korea and other Asian countries are four to ten times less likely to be diagnosed with and die from prostate and breast cancers than those in the USA (1). In the specific case of PCA, the clinical incidence of malignancy is low in Asian men and highest in African-Americans and Scandinavians (1,72). However, the incidence and mortality rate due to PCA in Asian men who have moved to the USA are approximately the same as those in Americans (72). Epidemiological studies suggest that dietary and environmental factors are the major causes of increase in PCA in the USA men as well as in migrating Asians (1,72). Low-fat and/or high-fiber diets significantly affect sex hormone metabolism in men (73), so despite the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and the mortality rate associated with it is low in Japan and some other Asian countries (72). This could, at least partly, be explained by a diet-related reduction in this malignancy (73,74). IP6 is also a ubiquitous plant component.
constituting 0.4–6.4% of most cereals, nuts, legumes, oil seeds and soybean, and its levels are high in certain dietary fibers. Diets rich in IP6 could be beneficial for preventing PCA in particular and other human malignancies in general.

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References


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