Inhibition of EGFR signaling in human prostate cancer PC-3 cells by combination treatment with β-phenylethyl isothiocyanate and curcumin

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Many naturally occurring compounds, including β-phenylethyl isothiocyanate (PEITC) and curcumin, exhibit significant anti-cancer chemopreventive effects. In this study, we investigated the combined effects of PEITC and curcumin in PC-3 human prostate cancer cells and in PC-3 cells that were stably transfected with an NF-κB luciferase plasmid (PC-3 C4). We found an additive effect of PEITC and curcumin for the induction of apoptosis. To elucidate the potential mechanisms of this effect, we studied several critical cellular signaling pathways, including the critical NF-κB cell survival signal that is hyper-activated in PC-3 cells and many other cancers. PEITC and curcumin additively inhibited NF-κB luciferase activity. Furthermore, the combined treatment significantly increased the activity of poly(ADP-Ribose) polymerase and cleavage of caspase-3 in correlation with apoptotic cell death. Studying upstream signaling events, we found that the phosphorylations of IκBα and Akt (Ser473, Thr308) were significantly attenuated by the combination of PEITC and curcumin. As these events can be downstream of the activation of epidermal growth factor receptor (EGFR), we pretreated PC-3 cells with PEITC and curcumin and then stimulated them with EGF. EGFR phosphorylations (Y845 and Y1068) were dramatically suppressed by PEITC or curcumin, and more so by the combination. Importantly, the degree of Akt and PI3K phosphorylations induced by EGF were also significantly suppressed. We conclude that the simultaneous targeting of EGFR, Akt and NF-κB signaling pathways by PEITC and curcumin could be the molecular targets by which PEITC and curcumin exert their additive inhibitory effects on cell proliferation and ultimately lead to programmed cell death of tumor cells.

Introduction

Prostate adenocarcinoma is one of the most common cancers, and it is the second leading cause of death among men in the USA (1). The major cause of death from prostate cancer is the metastasis to the bone and lymph nodes of cancer cells that ultimately resist conventional androgen-deprivation therapy (2). Because androgen ablation of advanced prostate cancer is often unsuccessful, new therapeutic strategies are needed for its treatment and prevention. Prostate cancer commonly overexpresses several growth factors and their receptors, including epidermal growth factor (EGF) receptor (EGFR). EGFR plays a critical role in tumor growth, and the prostate tissue becomes more susceptible to the growth-promoting action of EGF family growth factors during androgen withdrawal (3,4). The general inhibition of tyrosine kinase signaling pathways provides therapeutic advantage against prostate cancer metastasis (5). Therefore, inhibiting the activation of growth factor receptors, especially EGFR, may be a promising strategy for the treatment of prostate cancer.

Recent studies have shown that several dietary cancer chemopreventive agents could provide promising strategies for reducing the incidence of prostate cancer (6). In the present study, we selected two potent chemopreventive agents to investigate various critical cellular signaling pathways, including EGFR signaling, in human prostate cancer PC-3 cells. Curcumin (from turmeric) is one of the most well-known naturally occurring compounds. It has cancer chemopreventive potential against many types of cancerous cells, including prostate cancer. It is a potent nuclear factor-kappaB (NF-κB) inhibitor as well as an inducer of apoptosis. Recent data show that curcumin also has an inhibitory effect on EGFR phosphorylation in PC-3 cells (7). β-phenylethyl isothiocyanate (PEITC), from cruciferous vegetables, is also a highly potent chemopreventive agent that induces apoptosis in prostate cancer cells (8) and other cell types (9). In addition, human epidemiological studies show a statistically significant inverse correlation between dietary intake of vegetables that contain isothiocyanates (ITCs) and the risk for prostate cancer (10,11). We undertook the present study to investigate the effects of PEITC and curcumin on critical cellular signaling pathways, including the possible inhibition of EGFR phosphorylation and its downstream cell survival Akt signaling pathway.

There is increasing interest in the combined use of low doses of chemopreventive agents with differing modes of action, rather than the administration of a single agent at a higher dose, as a means of obtaining increased efficacy and minimized toxicity. This approach is extremely important when a promising chemopreventive agent demonstrates significant efficacy but may produce some untoward side effects at higher effective doses. For example, piroxicam, a non-steroidal anti-inflammatory drug (NSAID) and difluoromethylornithine (DFMO), an ornithine decarboxylase (ODC) inhibitor, were evaluated in preclinical models for their chemopreventive efficacy when administered individually at high doses and in combination at very low dose levels (12). Lowest dose levels of piroxicam and DFMO administered together were more effective in inhibiting the incidence and multiplicity of colon
adenocarcinomas than the individual compounds even at higher levels.

We therefore examined the combination of PEITC and curcumin in PC-3 cells for the potential treatment and prevention of prostate cancer. We focused on cell survival signaling pathways inhibited by PEITC and curcumin. Our results clearly show that combination treatment of PC-3 cells with PEITC and curcumin had a very potent inhibitory effect on the phosphorylation and activation of EGFR and Akt, and on the NF-κB pathway, when compared with single compound treatment. The concerted inhibitory effect of the combination on EGFR and NF-κB led to enhanced apoptotic cell death when compared with the effects of the individual compounds. The combination may thus offer therapeutic advantages in the treatment and prevention of human prostate cancer.

Materials and methods

Materials

PEITC was purchased from Sigma (St Louis, MO). Curcumin (≥98.5%) was obtained from Alexius (San Diego, CA). Rabbit polyclonal antibodies against p-EGFR (Y845, Y992 and Y1068), total EGFR, total poly(ADP-Ribose) polymerase (PARP), cleaved PARP, caspase 3, cleaved caspase 3, p-Akt (S473, S308), total Akt, p-PDK1, p-IκBα, total IκBα and p-phosphatidylinositol 3-kinase (PI3K) (p85) were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody for p-EGFR (1086) and secondary antibody were purchased from Zymed (South San Francisco, CA). Anti-actin was a product of Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines

PC-3 and PC-3 C4 (a derivative of PC-3, stably transfected with an NF-κB luciferase gene construct) were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 μl of penicillin/streptomycin mixture (Gibco BRL, Grand Island, NY), in a humidified atmosphere of 5% CO2/95% air at 37 °C. When cells were 80% confluent, they were serum starved for 24 h with MEM medium without FBS; the cells were subsequently treated with PEITC and/or curcumin.

MITT assay

The MITT assay was performed to determine cell proliferation. Briefly, PC-3 C4 cells were plated in 48-well plates at a density of 4 × 10^4/well. After incubating for 24 h, cells were serum starved overnight. Cells were then treated with different concentrations of curcumin and/or PEITC for 24 and 48 h, at which time, 20 μg of 5 mg/ml MITT solution was added to each well. After a 2 h incubation, medium was removed and dimethyl sulfoxide (DMSO) was applied to the plates. Color intensity of the solubilized formazan was measured at 570 nm with an enzyme linked immunosorbent assay (ELISA) plate reader (Bio-TEK Instruments, CA).

Luciferase assay

PC-3 C4 cells were plated overnight in 6-well plates at a density of 5 × 10^4/well and then serum starved for 24 h. The cells were then treated with the compounds for 24 h. After treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in reporter lysis buffer (Promega, Madison, WI). After brief centrifugation at 12 000 r.p.m., a 10 μl aliquot of the supernatant was assayed for luciferase activity with a luciferase kit from Promega, according to the manufacturer’s instructions. The luciferase activity was normalized for protein concentration.

Determination of DNA fragmentation

The detection of nuclear DNA fragmentation was performed as described previously (13) with some modifications. Briefly, PC-3 C4 cells cultured in 100 mm dishes were resuspended in 200 μl of hypotonic lysis buffer (0.2% Triton X-100, 1 mM EDTA, 10 mM Tris–HCl, pH 7.5) and incubated for 20 min at 4 °C. After centrifugation for 5 min at 14 000 g, the supernatant was collected and incubated with RNase A (400 μg/ml) for 30 min at 37 °C, followed by a digestion with protease K (200 μg/ml) for 30 min at 50 °C. The fragmented DNA was then precipitated overnight at −20 °C in 50% isopropanol and 0.5 M NaCl and centrifuged for 10 min at 14 000 g. Dried pellets were dissolved in 20 μl of ultra-pure water, and the DNA was separated by electrophoresis on a 2% agarose gel at 50 V for 1 h.

Western blotting

PC-3 or PC-3 C4 cells were treated with dimethyl sulfoxide (DMSO; 0.2%), PEITC, curcumin or a combination of PEITC and curcumin in the absence or presence of EGF for various time periods. After treatment, the cells were washed twice with ice-cold PBS and treated with RIPA buffer [50 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4, and protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] for 40 min on ice, followed by centrifugation at 14 800 g for 15 min. The protein concentrations of the supernatants were measured by using bicinchoninic acid (BCA) solution ( Pierce, Rockford, IL). Protein (20 μg) was separated on NuPAGE 4–12% electrophoresis gels (Invitrogen, Carlsbad, CA) and transferred to polycrylilene di fluoride membranes. Membranes were probed with primary antibodies and horseradish peroxidase-conjugated secondary antibody by standard western blot procedures. The proteins were visualized with the Super Signal Chemiluminescent Substrate (Pierce). The intensity of visualized bands was measured with Quantity One software (Ver 4.4.0, Bio-Rad, Hercules, CA).

Immunofluorescence

PC-3 cells grown on cover glass were fixed in 3% paraformaldehyde in PBS (pH 7.4), for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, blocked with 10% goat serum for 1 h and then incubated with primary antibodies overnight at 4 °C. This was followed by incubation with TRITC-conjugated goat anti-rabbit (Zymed) and DAPI (Molecular Probes) for 1 h. Sample images were taken with a fluorescence microscope (Nikon, ECLIPSE E 600 system), and the images were processed using Photoshop 8.0 (Adobe Systems, San Jose, CA).

Results

Combination treatment with curcumin and PEITC has an additive inhibitory effect on cell survival signaling pathways in PC-3 C4 cells

To determine the effects of PEITC (10 μM), curcumin (25 μM) and their combination on cell viability, the MTT assay was performed on PC-3 C4 cells at two selected time points (24 and 48 h). Treatment of the cells with 25 μM curcumin for 24 or 48 h did not cause significant cell death, whereas treatment with 10 μM PEITC caused significant cell death. The viability of PEITC-treated cells was 83% and that of the control was 65% at 24 h and 48 h, respectively (Figure 1A). Notably, the combination treatment had a more potent inhibitory effect on cell viability than PEITC alone, with viability at 60 and 44% of control at 24 and 48 h, respectively (Figure 1A).

We next examined the effects of these two compounds on NF-κB signaling. We measured NF-κB luciferase activity after a 24 h incubation of PC-3 C4 cells, which are stably transfected with an NF-κB luciferase plasmid, as we have described previously (9) with the compounds. As shown in Figure 1B, NF-κB luciferase activity was inhibited down to 80, 54 and 40% of control values (P < 0.05) when the cells were treated with 25 μM curcumin, 10 μM PEITC and their combination, respectively. These data show that the combination treatment has a more potent inhibitory effect than each drug separately.

To determine the type of cell death of PC-3 C4 cells induced by the drug combination, we measured the cleavage of PARP, cleavage of caspase 3 and DNA fragmentation as markers of apoptosis. Figure 1C and D shows that the combination treatment has a more potent inhibitory effect than each drug separately.
Actin was used to ensure equal protein loading. All experiments in this study were performed at least three times.

Cleavage of PARP and caspase 3 (Fig. 1C) as apoptotic markers, were measured by western blot analysis and DNA electrophoresis, respectively, after 24 h treatment of PC-3 C4 cells with curcumin, PEITC or a combination (lower panel in C and D). The upper panel in C shows the relative fold activation of cleaved PARP or cleaved caspase 3 by densitometry. The results are presented as mean ± SD (n = 3). For t-tests, *P < 0.05 compared with control; **P < 0.05 compared with two groups. (B) NF-κB luciferase activity was measured after treatment of PC-3 C4 cells with curcumin, PEITC or combination for 24 h. Data are shown as mean ± SD (n = 3). For t-tests, *P < 0.05 compared with control (0.2% DMSO).

Cleavage of PARP and caspase 3 (C) and DNA fragmentation (D) as apoptotic markers, were measured by western blot analysis and DNA electrophoresis, respectively, after 24 h treatment of PC-3 C4 cells with curcumin, PEITC or combination for 24 h. Data are shown as mean ± SD (n = 3). For t-tests, *P < 0.05 compared with control (0.2% DMSO). Cleavage of PARP and caspase 3 (C) and DNA fragmentation (D) as apoptotic markers, were measured by western blot analysis and DNA electrophoresis, respectively, after 24 h treatment of PC-3 C4 cells with curcumin, PEITC or combination for 24 h. Data are shown as mean ± SD (n = 3). For t-tests, *P < 0.05 compared with control (0.2% DMSO).

The phosphorylation of EGFR, PI3K (p85) and Akt were detected by western blotting with antibodies specific to phosphorylated proteins. As shown in Figure 2, all of these proteins were activated after EGF treatment. Two EGFR phosphorylations (Y845 and Y1068) were detected at 2 min after EGF administration and gradually decreased with time. However, EGFR phosphorylated at Y992 was constitutive even in

(IkBα). The combined effect of 25 μM curcumin and 10 μM PEITC was the most potent compared with combinations of different concentrations tested.

**EGFR and NF-κB signaling are activated by EGF**

To investigate the effects of EGF on PC-3 C4 cells, we analyzed signaling molecules related to the EGFR pathway.
control cells not treated with EGF. PI3K (p85), a functional subunit of PI3K, was activated within 2 min of EGF administration and gradually decreased by 30 min. One of the downstream targets of EGFR-PI3K is Akt, and phosphorylated Akt (S473) gradually increased and peaked at 30 min. Previously, it has been shown that NF-κB is activated by EGFR signaling (14,15). Consistent with this observation, we found that treatment of PC-3 cells with EGF activated the phosphorylation of IκBα as early as 2 min after treatment, and the increased phosphorylation persisted for up to 30 min.

PEITC has an enhanced inhibitory effect on EGFR phosphorylation when it is combined with curcumin in EGF-stimulated PC-3 C4 cells

EGF signaling might be one of the most critical signaling mechanisms for cancer cells, including prostate cancer cells (3,5,7,16). We therefore focused on PEITC and its combination effect with curcumin on EGFR signaling in PC-3 C4 cells. The cells were pretreated with the single agents or with their combination for 5 min. Then, EGF (100 ng/ml) was added for 10 min. As shown in Figure 3A, curcumin had an inhibitory effect on EGFR phosphorylation as reported previously (7). However, PEITC and the combination treatments show a much greater inhibitory effect than curcumin alone on the inhibition of EGFR phosphorylation (Y845, Y992, Y1068 and Y1086). Among the four types of phosphorylated EGFR, p-EGFR (Y1086) shows the largest additive inhibition by combination treatment in PC-3 C4 cells. Similar results were also obtained in parental PC-3 cells (data not shown). We next examined a downstream target of the EGFR signaling pathway, Akt, and found that EGF-stimulated Akt phosphorylation was dramatically inhibited by the combination treatment (Figure 3B).

Combination treatment with PEITC and curcumin significantly inhibits EGFR signaling in PC-3 cells

To corroborate the above observations obtained in PC-3 C4 cells and to further examine the inhibitory effects of the drugs
Fig. 4. Additive inhibition of EGFR signaling in PC-3 parental cells by combination of PEITC and curcumin. (A) PC-3 cells were pretreated with compounds (curcumin, 25 μM; PEITC, 10 μM; or their combination) for 5 min and then stimulated with EGF (100 ng/ml). For evaluation of the EGFR signaling proteins, phospho-specific antibodies against the phosphorylated tyrosine residues (Y845, Y992 and Y1068) of EGFR were used. Phospho-p85, a subunit PI3K, was detected using a specific antibody, which can detect p-YXXM motif (Y, tyrosine; X, amino acid; M, methionine). Actin was used as an equal loading control. Relative fold of activation of p-EGFR (Y1068) was measured by densitometry analysis (right). Con, control; C, curcumin; P, PEITC. (B) PC-3 cells were pretreated with compounds for 5 min and then incubated with EGF (100 ng/ml) for 2 h. Phosphorylation of Akt was measured using specific antibody detecting phosphorylation of S473 or T308, by western blotting. Relative fold activation of p-Akt was measured by densitometry (right). Total protein levels of Akt and actin were used for the Akt expression level and protein equal loading controls, respectively. Phosphorylation of IκBα was also measured by western blotting using a phospho-specific antibody. Con, control; C, curcumin; P, PEITC. (C) PC-3 cells were treated with 0.2% DMSO or 10 μM PEITC for 5 min followed by a 10 min incubation with EGF (100 ng/ml). Phosphorylated EGFR (red) and nuclei (blue) were detected by immunofluorescence analysis using anti-p-EGFR (Y1086) and DAPI dye, respectively. Pictures with red and blue color were merged. Magnitude: 400-fold; Con, control.
on EGFR signaling pathway, parental PC-3 cells were treated with PEITC alone or in combination with curcumin. Parental PC-3 cells were pretreated for 5 min, followed by EGF (100 ng/ml) treatment for 10 min. As shown in Figure 4A, EGF-stimulated EGFR activation was attenuated by treatments with PEITC or curcumin. Importantly, the combination treatment showed much more significant inhibition of EGFR phosphorylation than the single treatment, especially with respect to the p-EGFR (Y1068) protein. Densitometry analysis of p-EGFR (Y1068) protein levels (right panel of Figure 4A) shows that EGF-stimulated EGFR phosphorylation was inhibited by 19, 53 and 86% when the cells were treated with curcumin, PEITC and the combination, respectively.

To analyze the downstream signaling events of the EGFR pathway, we next examined EGF-stimulated phosphorylation of PI3K (p85), which also showed significant inhibition by the combination treatment (Figure 4A). In addition, when we examined the downstream signaling molecules Akt and IкBα, levels of p-Akt (S473), p-Akt(T308) and IкBα were all significantly inhibited by the combination treatment in PC-3 cells (Figure 4B). Densitometry analysis shows that p-Akt (S473) protein levels are decreased by curcumin, PEITC and combination by 7, 53 and 69%, respectively, as compared with EGF alone. Immunofluorescence studies after PEITC treatment confirmed that PEITC significantly inhibits EGF-stimulated EGFR phosphorylation (Figure 4C).

These data corroborate very well with that of the PC-3 C4 cells and suggest that both PEITC and curcumin can inhibit EGFR-mediated cell survival signals such as PI3K, Akt and NF-κB. Furthermore, the combination of PEITC and curcumin demonstrated additive inhibitory effects on these critical cell survival signaling proteins and, importantly, in cell survival.

Discussion

Prostate cancer is the second leading cause of death among men in the USA. While the incidence of prostate cancer has increased steadily over the years, its etiology is still not completely understood. Factors contributing to prostate cancer may involve genetic changes, activated oncogenes, growth factors, hormones and/or dietary factors. Both the EGFR and NF-κB signaling pathways are implicated in the survival of androgen insensitive human prostate cell lines such as PC-3 and in advanced prostate cancer in humans (3). Recent studies have indicated that naturally occurring compounds, such as PEITC and curcumin, are potential therapeutics for the prevention and treatment of human prostate cancer (10,11,17). Therefore, in the present study we asked whether PEITC and curcumin could interfere with the EGFR and NF-κB pathways.

We utilized both parental PC-3 cells as well as PC-3 C4 cells, which have a stably transfected NF-κB luciferase gene (9). These cell lines provide a model system to study signaling mechanisms and pharmacological effects. Our results demonstrate that PEITC significantly inhibits EGFR activation and, moreover, PEITC displays additive effects in combination with curcumin in both PC-3 C4 and its parental PC-3 cells.

It is well known that EGFR and other growth factor receptors are frequently overexpressed in several types of cancers, including prostate. In fact, the progressive and metastatic
growth of prostate cancer has been associated with a significant increase in the expression of EGFR and one of its ligands (18,19). Similarly, NF-κB activation in cancer cells has been intensively studied by many groups, and constitutive or improper activation of NF-κB in prostate cancer cells in vitro and in vivo has been recently recognized (20). Moreover, it has recently been shown that the enzyme NF-κB inducing kinase (NIK), which preferentially phosphorylates I(B)/kinase (IKK) during the activation of the IKK complex, can regulate the function of NF-κB through IκBα phosphorylation following activation of EGFR (14,15). Importantly, isothiocyanates such as PEITC and sulforaphane can directly inhibit NF-κB via the direct inhibition of IKKa/β/γ, as we have shown recently (9).

Akt is another important regulator of cell survival and cell proliferation that significantly contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis. Overexpression of Akt has been reported in a variety of human cancers including prostate cancer (21,22). Loss of PTEN in cancer cells such as PC-3 cells (23) leads to constitutive activation of the PI3K/Akt signal transduction pathway (21).

The combination effect of PEITC and curcumin may thus be explained by their dual roles with positive impact on cell death in prostate cancer: the inhibition of NF-κB activity and the inhibition of the EGFR activity. Intervention in these signaling pathways by these molecules can culminate in the execution of the apoptotic cell death program, as demonstrated by measuring apoptotic biomarkers such as PARP and caspase 3. Thus, the ultimate effect of inhibition of NF-κB and EGFR activation by PEITC and curcumin is multifactorial, a result which could explain in part their pro-apoptotic properties and could potentially be exploited for prostate cancer prevention. The exact chemical or biochemical mechanisms by which PEITC and curcumin exhibit their additive effects could be related to the combination of their signaling mechanisms, including direct inhibition of IKKs by PEITC (9); direct inhibition of tyrosine kinases by curcumin (24); direct inhibition of JNK phosphatases leading to sustained JNK activation (25); inhibition of histone deacetylases (HDACs) (26) and/or other as yet to be discovered signaling molecules.

In summary, we have demonstrated that PEITC in combination with curcumin inhibits Akt and NF-κB cell survival signal mechanisms by blocking of EGFR activation in PC-3 cells. Our results suggest that PEITC in combination with curcumin could induce programmed cell death by inhibiting the EGFR/Akt/NF-κB signaling pathways (Figure 5), which are critical for maintaining cell survival. These mechanisms may be exploited for the prevention and/or treatment of human prostate cancer.

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References


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