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Modulation of Phosphatidylinositol-3-Kinase/Protein Kinase B- and Mitogen-Activated Protein Kinase-Pathways by Tea Polyphenols in Human Prostate Cancer Cells

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Abstract We have earlier shown that oral infusion of a polyphenolic fraction isolated from green tea, at a human achievable dose (equivalent to six cups of green tea per day), significantly inhibits prostate cancer (PCA) development and metastasis in *t*ransgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model that closely mimics progressive form of human prostatic disease (Gupta et al. [2001]: Proc. Natl. Acad. Sci. U.S.A. 98:10350–10355.). A complete understanding of the mechanism(s) and molecular targets of PCA chemopreventive effects of tea polyphenols may be useful in developing novel approaches for its prevention. In this study, we employed two distinct human PCA cell lines viz. DU145 (androgen-unresponsive prostate carcinoma cells) and LNCaP (androgen-responsive prostate carcinoma cells) and, employing immunoblot analysis, we evaluated the effect of epigallocatechin-3-gallate (EGCG), the major polyphenol present in green tea and theaflavins (TF), the major polyphenol present in black tea on phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) and mitogen-activated protein kinase (MAPK) pathways. Both EGCG and TF treatment were found to (i) decrease the levels of PI3K and phospho-Akt and (ii) increase Erk1/2 in both DU145 and LNCaP cells. Our data showing the inhibition of the constitutive levels of PI3K and the phosphorylation of Akt could be important because the treatment approaches should be aimed at the inhibition of the constitutive levels of PI3K and Akt. Our data also suggest that Erk1/2 could be involved in the anti-cancer effects of EGCG and TF. Taken together, our study, for the first time demonstrated the modulation of the constitutive activation of PI3K/Akt and Erk1/2 pathways by EGCG as well as TF. We suggest that detailed studies in appropriate tumor model system are needed to establish the relevance of the cell culture work to in vivo models. J. Cell. Biochem. 91: 232–242, 2004.

Key words: prostate cancer; PI3K/PKB; MAPK; EGCG; TF; polyphenols

Prostate cancer (PCA) is one of the most frequently diagnosed malignancies and the second leading cause of cancer related deaths in American men. According to estimates of the American Cancer Society, in the year 2003, an estimated 2,20,900 men in the United States will be diagnosed with PCA and approximately 28,900 PCA related deaths are expected [Jemal et al., 2003]. In recent years, "*Chemoprevention*" via naturally occurring agents present in human diet and beverages has shown promise for the management of several cancers [Trevisanato and Kim, 2000]. Indeed, PCA represents an excellent candidate disease for chemoprevention because of its particularly long latency period, and because it is typically diagnosed in elderly men. Thus, even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could

This paper from the laboratory of Hasan Mukhtar is dedicated to the memory of Ed Bresnick, who in addition to being my mentor was my scientific father. Ed has made many contributions to the field of cancer research, toxicology, and pharmacology. Many of his postdoctoral fellows and graduate students, through a meticulous training provided by Ed, are full time faculty members at many institutions worldwide. Ed: much of what I am today is a result of your superb mentoring. I will miss you sorely as long as I live.

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result in substantial reduction in the incidence of the clinically detectable disease. For a variety of reasons, naturally occurring dietary substances for chemoprevention are preferred [Ames, 1983; Block, 1993].

We have recently shown that oral infusion of a polyphenolic fraction isolated from green tea, at a human achievable dose (equivalent to six cups of green tea per day), significantly inhibits PCA development and metastasis in *tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) model that closely mimics progressive form of human prostatic disease [Gupta et al., 2001]. Our in vitro studies have shown that (−)-epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea causes cell cycle arrest and apoptosis of androgen-responsive human prostate carcinoma LNCaP as well as androgen-unresponsive human prostate carcinoma DU145 cells [Ahmad et al., 1997; Gupta et al., 2000]. The polyphenolic antioxidants present in black tea have also been suggested to possess chemopreventive potential against PCA. A complete understanding of the mechanism(s) and molecular targets of PCA chemopreventive effects of tea polyphenols may be useful in developing novel approaches for its prevention and treatment.

This study was designed to assess the involvement of PI3K/PKB- and MAPK-signaling pathways, which are regarded to play a critical role in cellular proliferation, cell cycle regulation, and apoptosis. Defects in these signaling pathways may result in the development of cancer. PI3K catalyses the formation of the 3′-phosphoinositides, phosphatidylinositol 3,4-diphosphate, and phosphatidylinositol 3,4,5-triphosphate. Increase in 3′-phosphoinositides leads to membrane translocation of downstream effectors such as the serine/threonine protein kinase Akt resulting in increased cellular proliferation and protection from apoptosis [Van de Sande et al., 2002]. Similarly, the MAPK family such as extracellular signal regulated protein kinase (Erk) 1 and 2 are shown to be constitutively active in human PCA, and possibly play a causative role in the progression of this malignancy from an androgen-sensitive phenotype to an advanced and androgen-insensitive metastatic disease.

In the present study, employing LNCaP and DU145 PCA cells, we show that EGCG and theaflavins (TF) result in significant inhibition of the constitutive activation of PI3K and phosphorylation of Akt/PKB. Our data suggests that the inhibition of the constitutive activation of Akt and modulation in MAPK-pathway may be important events by which EGCG and TF inhibit the proliferation of human PCA cells.

MATERIALS AND METHODS

Reagents

A purified preparation of EGCG and TF (>98% pure) was a kind gift from Dr. Yukihiko Hara of Mitsui Norin Co. Ltd. (Shizuoka, Japan). The antibodies used in this study were obtained from Cell Signaling Technology, Beverly, MA (anti-phospho–Erk1/2 p42/44); and Upstate Technology, Lake Placid, NY (anti-phosphatidylinositol-3-kinase, anti-phospho-Akt1/PKBα and anti-Akt1/PKBα). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology (Rockford, IL). Novex pre-cast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA).

Cell Culture

The androgen-unresponsive (DU145) and androgen-responsive (LNCaP) cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in minimum essential medium (MEM) and RPMI1640 (Mediatech, Herndon, VA), respectively, supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

Treatment of Cells

EGCG and TF dissolved in PBS (stock solution of 50 mM, pH 7.4) were employed for the treatment of cells. For dose- and time-dependent studies, the cells (70–80% confluent) were treated with EGCG (10, 20, and 40 μg/ml) or TF (10, 25, and 50 μg/ml) for 12 and 24 h in complete cell medium. Cells that were used as controls were incubated with the vehicle only.

Preparation of Cell Lysates and Western Blot Analysis

Following EGCG and TF treatments, the cells were harvested at 12 and 24 h post-treatment and then washed with cold PBS (10 mM, pH 7.4). The cells were then incubated in ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM

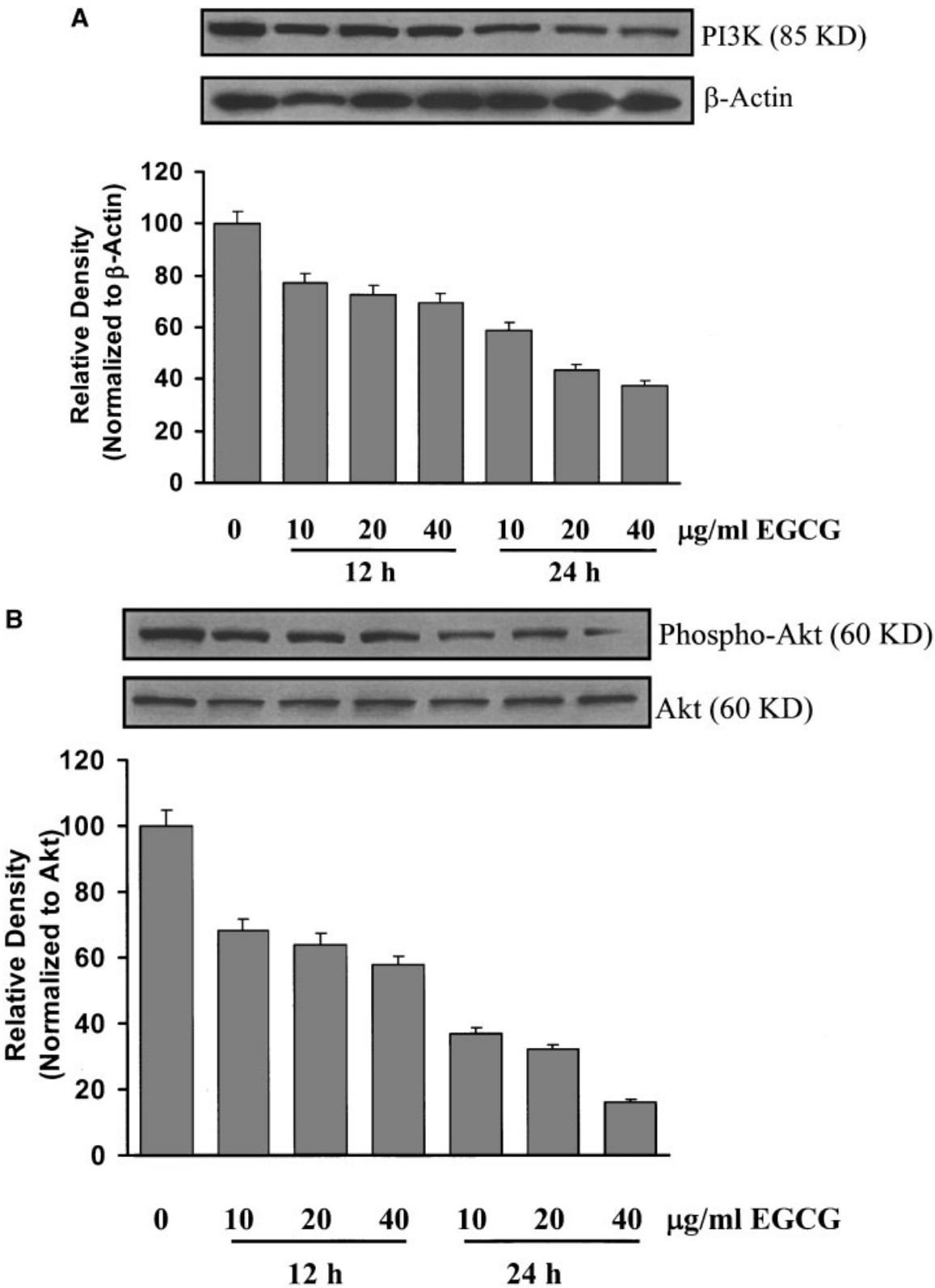

Fig. 1. Effect of EGCG on PI3K and Akt proteins in DU145 cells. **A**: Immunoblot analysis and densitometry of PI3K proteins. **B**: Immunoblot analysis and densitometry of Akt proteins. Following treatments of the cells with EGCG (as specified), the cell lysates were prepared and 30 μg protein was subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminiscent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin in case of PI3K and Akt in case of p-Akt. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT^{TM} software (Silk Scientific, Inc., Orem, UT). The immunoblots shown here are representative of three independent experiments with similar results. The densitometry data represent means ± standard errors from three immunoblots and are shown as relative density of protein bands normalized to β-actin or Akt.

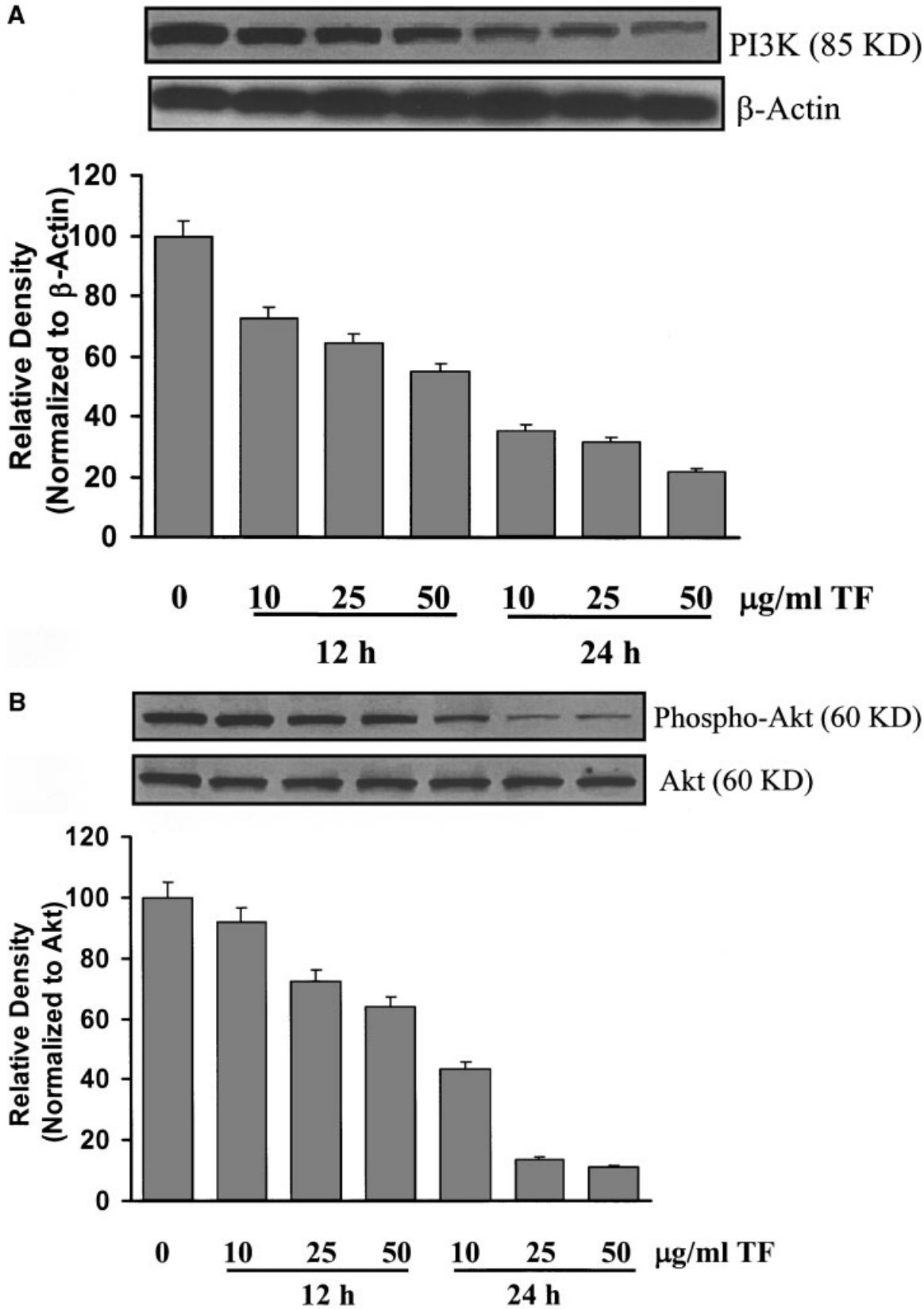
A
PI3K (85 KD)
β-Actin
Relative Density (Normalized to β-Actin)
120
100
80
60
40
20
0
0 10 25 50 10 25 50 μg/ml TF
12 h
24 h
B
Phospho-Akt (60 KD)
Akt (60 KD)
Relative Density (Normalized to Akt)
120
100
80
60
40
20
0
0 10 25 50 10 25 50 μg/ml TF
12 h
24 h

Fig. 2

PMSF (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA) over ice for 30 min. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21½-G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000*g* for 25 min at 4°C and the supernatant (total cell lysate) was collected, aliquoted, and stored at −70°C. The protein content in the lysates was measured by BCA protein assay kit (Pierce Biotechnology) as per the manufacturer's protocol.

For Western blot analysis, 30–50 μg protein was resolved over 8–12% Tris-glycine polyacrylamide–SDS gels and transferred onto a nitrocellulose membrane. The non-specific sites were blocked by incubating the blot with 5% non-fat dry milk in buffer (containing 10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 1 h at room temperature or overnight at 4°C. The blot was washed with wash buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 2×10 min and then incubated overnight with appropriate primary antibody specific for the protein to be assessed. The antibodies were used at dilutions specified by the manufacturer. The blot was washed for 2×10 min, 2×5 min and then incubated with the corresponding secondary antibody HRP conjugate (Amersham Life Science, Inc., Arlington Height, IL) at 1:2,000 dilutions for 1 h at room temperature. The blot was washed for 2×10 min, 4×5 min, and the protein was detected by chemiluminiscence using ECL kit (Amersham Life Science, Inc., Arlington Height, IL) and autoradiography with XAR-5 film (Amersham Life Science, Inc.). Each blot was stripped and reprobed with β-actin to assess any problems with protein loading. The data are presented as the relative density of protein bands normalized to β-actin. In the case of immunoblot analysis of p-Akt the data was normalized to whole-Akt.

RESULTS AND DISCUSSION

Catechins and TF are strong antioxidant polyphenols abundantly present in green tea and black tea, respectively [Ahmad et al., 1997; Gupta et al., 1999, 2000; Yang, 1999; Mukhtar and Ahmad, 2000]. EGCG is the most abundant polyphenol present in green tea and is regarded to be responsible for most of the biological activities of green tea. Similarly, TF is the major constituent of black tea. [Ahmad et al., 1998; Yang, 1999; Gupta et al., 2000; Mukhtar and Ahmad, 2000]. Both EGCG and TF have been shown to possess anti-proliferative effects in in vitro cell culture and in in vivo animal studies [Yang and Wang, 1993; Mukhtar et al., 1994; Yang et al., 1998; Gupta et al., 1999, 2000; Yang, 1999; Mukhtar and Ahmad, 2000]. Studies from our laboratory have shown that EGCG treatment of PCA cells DU145 and LNCaP resulted in an induction of apoptosis and cell cycle dysregulation [Ahmad et al., 1997; Gupta et al., 2000]. These findings were verified by Paschka et al. [1998]; Chung et al. [2001]. Recently, Klein and Fischer [2002] suggested that black tea polyphenols inhibit insulin like growth factor (IGF)-1 mediated activation of Akt pathway and suggested that this may be involved in the anti-proliferative effects of black tea. However, understanding of the exact mechanism of the biological effects of tea polyphenols is far from complete.

Because of the critical role of PI3K/Akt and MAPK pathways in the development of cancer including PCA cells [Davies et al., 1999; Graff et al., 2000; Chen et al., 2001; Chaudhary and Hruska, 2003], in this study, we examined the role of PI3K/Akt and MAPK in EGCG- and TF-mediated inhibition of cell growth and induction of apoptosis. We employed two distinct human PCA cell lines viz. LNCaP (androgen-responsive prostate carcinoma cells) and DU145 (androgen-unresponsive prostate

Fig. 2. (*Overleaf*) Effect of TF on PI3K and Akt proteins in DU145 cells. **A**: Immunoblot analysis and densitometry of PI3K proteins. **B**: Immunoblot analysis and densitometry of Akt proteins. Following treatments of the cells with TF (as specified), the cell lysates were prepared and 30 μg protein was subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminiscent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin in case of PI3K and Akt in case of p-Akt. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ software (Silk Scientific, Inc.). The immunoblots shown here are representative of three independent experiments with similar results. The densitometry data represent means ± standard errors from three immunoblots, and are shown as relative density of protein bands normalized to β-actin or Akt.

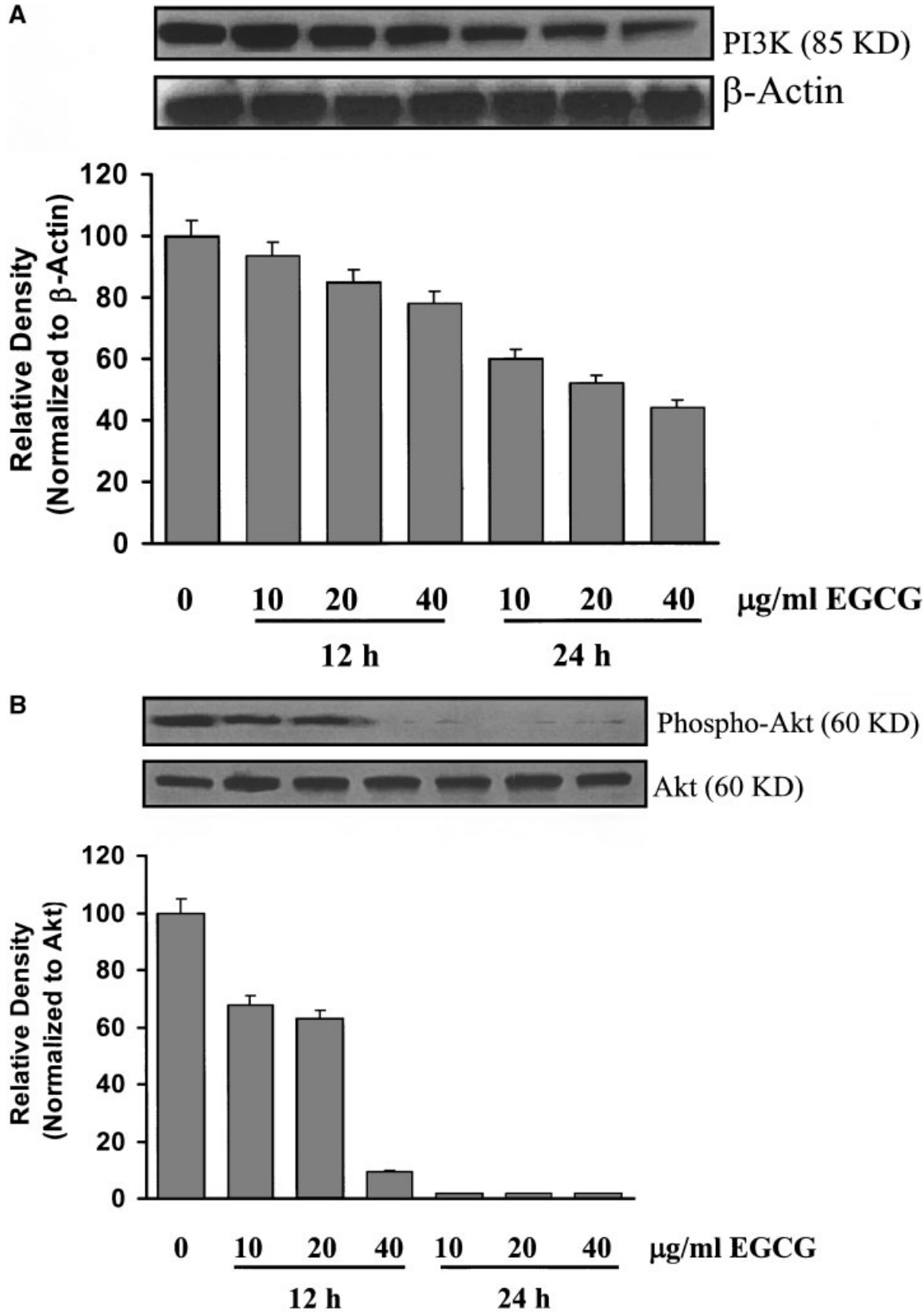

Fig. 3. Effect of EGCG on PI3K and Akt proteins in LNCaP cells. **A**: Immunoblot analysis and densitometry of PI3K proteins. **B**: Immunoblot analysis and densitometry of Akt proteins. Following treatments of the cells with EGCG (as specified), the cell lysates were prepared and 30 μg protein was subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminiscent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin and Akt for PI3K and p-Akt, respectively. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT^{TM} software (Silk Scientific, Inc.). The immunoblots shown here are representative of three independent experiments with similar results. The densitometry data represent means ± standard errors from three immunoblots, and are shown as relative density of protein bands normalized to β-actin or Akt.

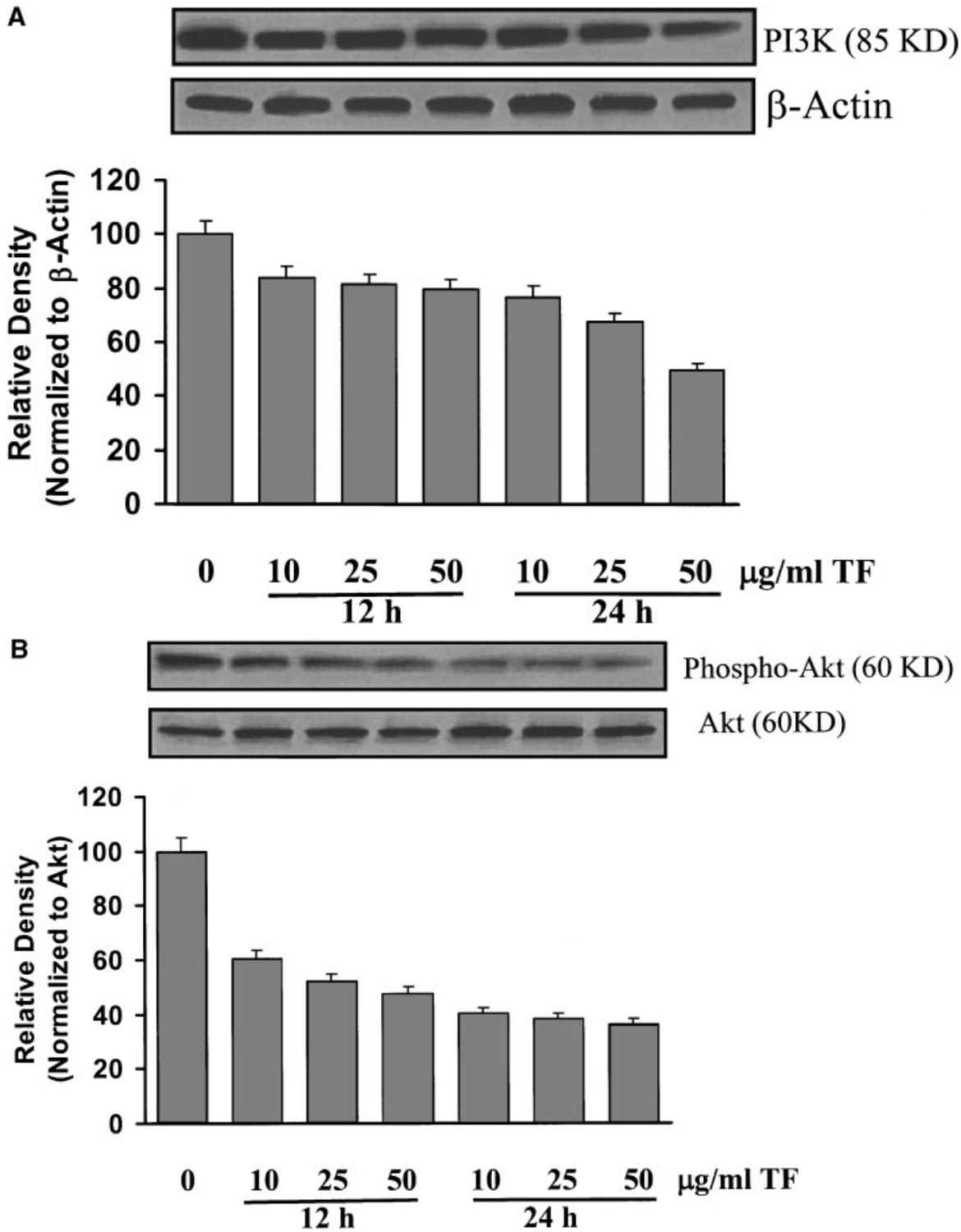

Fig. 4. Effect of TF on PI3K and Akt proteins in LNCaP cells. **A**: Immunoblot analysis and densitometry of PI3K proteins. **B**: Immunoblot analysis and densitometry of Akt proteins. Following treatments of the cells with TF (as specified), the cell lysates were prepared and 30 μg protein was subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminiscent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin and Akt for PI3K and p-Akt, respectively. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ software (Silk Scientific, Inc.). The immunoblots shown here are representative of three independent experiments with similar results. The densitometry data represent means ± standard errors from three immunoblots, and are shown as relative density of protein bands normalized to β-actin or Akt.

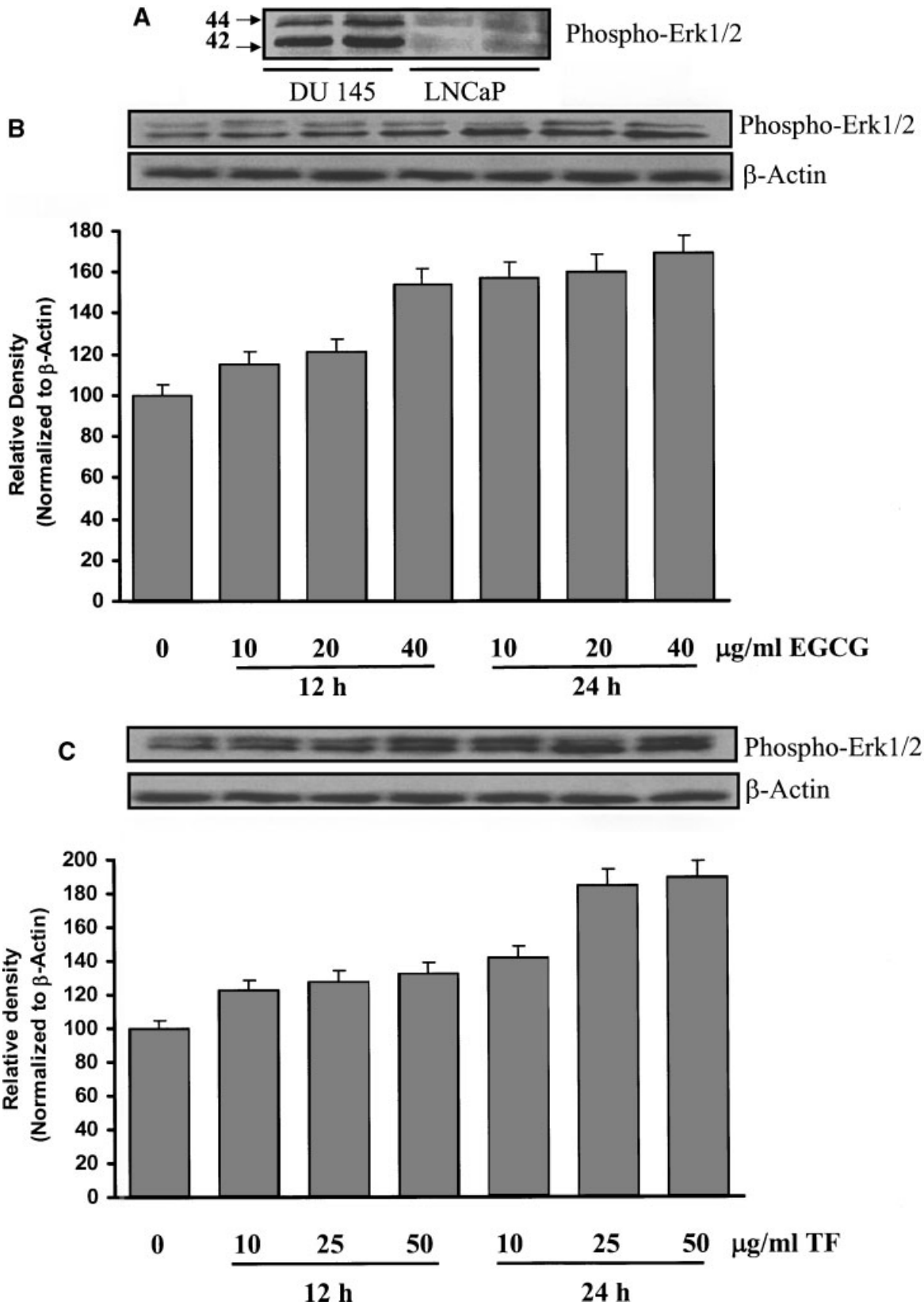
A
44
42
Phospho-Erk1/2
DU 145
LNCaP
B
Phospho-Erk1/2
β-Actin
Relative Density
(Normalized to β-Actin)
180
160
140
120
100
80
60
40
20
0
0
10
20
40
10
20
40
μg/ml EGCG
12 h
24 h
C
Phospho-Erk1/2
β-Actin
Relative density
(Normalized to β-Actin)
200
180
160
140
120
100
80
60
40
20
0
0
10
25
50
10
25
50
μg/ml TF
12 h
24 h

Fig. 5

carcinoma cells). The choice of these two distinct cell lines is based on the fact that PCA, as occurs in humans, is either androgen-responsive (great majority at presentation of the disease) or androgen-unresponsive (often late in the disease process). Thus, the strategies should be aimed at the elimination of both these cell types via mechanism-based approaches.

In the first set of experiments, we evaluated the effect of EGCG and TF on the constitutive levels of protein expression of PI3K, phospho-Akt, and MAPK in lysates of androgen-unresponsive prostate adenocarcinoma DU145 cells. The cells treated with EGCG showed marked decrease in the levels of PI3K and phospho-Akt at 12 and 24 h post-treatment. (Fig. 1A,B). Although EGCG inhibited the expression of PI3K at all doses and time periods, the maximum inhibition (~60% over control) was observed at 24 h with 40 μg EGCG. The inhibition in p-Akt levels was much more pronounced (~80% over control) at the same dose of EGCG. Next, we evaluated the effect of TF treatment on the protein expression of PI3K and phospho-Akt in DU145 cells. As shown in Figure 2A,B, TF-treatment to the cells resulted in a noticeable inhibition of PI3K and Akt proteins at 12 and 24 h post-treatment. Although TF was able to decrease the expression of PI3K and Akt proteins in dose dependent manner the maximum inhibition was observed at 50 μg/ml dose (24 h) in both the proteins (Fig. 2). An interesting observation from our experiment was that TF was more effective in inhibiting PI3K as compared to EGCG.

In our next set of experiments, we evaluated the effect of EGCG and TF on the protein levels of PI3K and phospho-Akt in androgen-responsive LNCaP cells. As shown in Figures 3 and 4, both the polyphenols significantly inhibited PI3K as well as phosphorylated form of Akt at 24 h post-treatment. However, the inhibition in PI3K proteins was only moderate at 12 h post-treatment. TF treatment to LNCaP cells resulted in only a slight decrease in PI3K levels at 12 h post-treatment (Fig. 4A). However, treatment of the cells with TF significantly inhibited Akt proteins at all the doses used (Fig. 4B). Klein and Fischer [2002] have shown that black tea polyphenols inhibit IGF-I induced signaling through Akt in DU145 cells. Nomura et al. [2001] have observed that pretreatment with EGCG and TB inhibit UVB-induced PI3K activation. However, none of these studies evaluated the effect of tea polyphenols on the constitutive activation of these proteins in human cancer cells. Our data showing the inhibition of the constitutive levels of PI3K and the phosphorylation of Akt could be important because the treatment approaches should be aimed at the inhibition of the constitutive levels of PI3K and Akt.

The MAPKs, the proline-directed kinases, which are critical for cellular proliferation as the transcription of many early genes is mediated via the sequential activation of MAPK. Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified and include the extracellular regulated kinase (ERK), c-jun N-terminal kinases (JNK), and p38 (also known as CSBP, RK, Mkp2). MAPK proteins play a critical role in cell growth, differentiation, and apoptosis. Among the different members of MAPK protein family, ERK sub-group is the best studied. Disruption of Erk has been linked to the induction of oncogenesis. Constitutive expression of Erk1/2 is significantly up regulated in human PCA cell lines as well as human PCA tissues [Guo et al., 2000]. As shown in Figure 5A, our data demonstrated that in DU145 cells the expression of Erk1/2 was significantly higher while its basal expression in LNCaP cells was negligible. The striking observation of this study was that the treatment of DU145 cells with either EGCG or TF resulted in an up regulation of the phosphorylation of Erk1/2 proteins that was more evident at 24 h post-treatment (Fig. 5B,C). Earlier studies have shown that Erk1/2 is up regulated in cancer

Fig. 5. (*Overleaf*) Effect of EGCG and TF on Erk1/2 protein in human prostate cancer cells. **A**: Constitutive expression of Erk1/2 in DU145 and LNCaP cells, (**B**) effect of EGCG on protein levels of Erk1/2, and (**C**) effect of TF on protein levels of Erk1/2. Following treatments of the cells with EGCG or TF (as specified), the cell lysates were prepared and 50 μg protein was subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminiscent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin. The immunoblots shown here are representative of four independent experiments with similar results. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ software (Silk Scientific, Inc.). The densitometry data represent means ± standard errors from three immunoblots, and are shown as relative density of protein bands (relative to background) normalized to β-actin.

cells treated with green tea polyphenols [Chen et al., 2000; Opare et al., 2001]. Opare et al. [2001] have shown that growth inhibitory effect of green tea extract and EGC involves a cellular thiol-dependent activation of MAPK in Ehrlich ascites tumor cells. Chen et al. [2000] have shown the activation of antioxidant response element (ARE), MAPKs, and Caspases by tea polyphenols in certain cancer cells. Recent studies [Zimmermann and Moelling, 1989; Malik et al., 2002] have provided explanation for the observed upregulation of Erk1/2 by tea polyphenols. These studies suggested that phospho-Akt inactivates Raf by a direct phosphorylation on Ser259, resulting in inhibition of the Raf-MEK-Erk signaling pathway. However, further detailed studies are required to establish a cause-and-effect relationship between Akt inhibition and subsequent MAPK-activation by EGCG and TF. We also evaluated the effect of EGCG and TF on the protein levels of p38 and JNK, other members of MAPK family; and our results (data not shown) showed no significant change in the expression of p38 with either EGCG or TF, whereas JNK constitutive expression was not observed in either cell line suggesting that only Erk1/2 could be involved in the anti-cancer effects of EGCG and TF.

Taken together, our study, for the first time, demonstrated the modulation of the constitutive activation of PI3K/Akt and Erk1/2 pathways by EGCG as well as TF. This study suggested the probable basis of the anti-proliferative effects of tea polyphenols TF and EGCG in PCA cells. However, detailed in vivo studies in appropriate model system are needed to establish the relevance of the cell culture work to in vivo models.

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