Chapter III

Modulation of phosphatidylinositol-3-kinase/protein kinase B- and mitogen-activated protein kinase-pathways by tea polyphenols in human prostate cancer cells
Chapter 3

Modulation of phosphatidylinositol-3-kinase/protein kinase B- and mitogen-activated protein kinase-pathways by tea polyphenols in human prostate cancer cells

Studies described in this chapter have compared the effect of epigallocatechin-3-gallate (EGCG), the major polyphenol present in green tea with that of theaflavins (TF), the major polyphenol present in black tea on phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB) and mitogen-activated protein kinase (MAPK) signaling pathways. A detailed description of the study is as follows:

3.1 Introduction and Rationale:

Recent studies have shown that oral infusion of a polyphenolic fraction isolated from tea leaves, at a human achievable dose (equivalent to six cups of green tea per day), significantly inhibits CaP development and metastasis in transgenic adenocarcinoma of mouse prostate (TRAMP) model that closely mimics progressive form of human prostatic disease (Gupta et al., 2001). In vitro studies have shown that EGCG, the major polyphenolic constituent of green tea and also present in black 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). Similarly, the polyphenolic antioxidants present in black tea have also been suggested to possess chemopreventive potential against CaP (Klein & Fisher, 2002 and the references therein). A complete understanding of the mechanism(s) and molecular targets of CaP chemopreventive effects of tea polyphenols may be useful in developing novel approaches for its prevention and treatment. The studies described in this chapter were designed to assess the involvement of phosphatidylinositol-3-kinase (PI3K; also known as protein kinase B, PKB) and mitogen activated protein kinase (MAPK)-signaling pathways, during the prostate cancer chemopreventive effects of tea polyphenols. Both PI3K- and MAPK- pathways are regarded to play critical roles in cellular
proliferation, cell cycle regulation and apoptosis. Defects in these signaling pathways have been associated with the development of CaP.

PI3K catalyzes 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 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 CaP, 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.

The serine/threonine kinase Akt/PKB has been a focus of intense research. It seems that Akt/PKB lies in the crossroads of multiple cellular signaling pathways and acts as a transducer of many functions initiated by growth factor receptor that activate PI3K. Akt/PKB is particularly important in mediating several metabolic actions and cell survival. Akt/PKB is the direct target of PI3K and overexpression of constitutively activated PI3K can activate Akt/PKB (Klippel et al., 1997). Because Akt/PKB activity is dependent on PI3K, any mechanism that activates PI3K can theoretically lead to stimulation of Akt/PKB activity. MAP Kinase cascades are among the most thoroughly studied of signal transduction systems, and have been shown to participate in a diverse array of cellular programs including cell differentiation, cell movement, cell division and cell death.

The data presented in this chapter demonstrated that both EGCG and TF result in significant inhibition of the constitutive activation of PI3K and phosphorylation of Akt/PKB in human prostate carcinoma LNCaP and DU145 cells; suggesting 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 CaP cells.
3.2 Materials and Methods

Chemicals

Purified preparations of epigallocatechin-3-gallate (EGCG) and theaflavins (TF) (>98% pure) were 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/PI3Kα and anti-Akt1/PI3Kα). 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 units/ml)-streptomycin (100 μg/ml) at 37° C in a humidified 5% CO₂ 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 hours in complete cell medium. Cells that were used as controls were treated with the vehicle (PBS) only for similar times.

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, 150mM NaCl, 1mM EGTA, 1mM EDTA, 20mM NaF, 100mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1mM PMSF (pH 7.4)] with freshly added protease inhibitor cocktail (Protease...
Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA) over ice for 30 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14000 X g for 25 minutes 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, Rockford, IL) as per the manufacturer’s protocol. The kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium. The purple colored reaction product of this assay is formed by chelation of the two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 552 nm that is nearly linear with the increasing protein concentration. The kit consists of two components A and B, reagent A consists of sodium carbonate, sodium bicarbonate, bicinchininic acid and sodium tartarate in 0.1 M sodium hydroxide and the reagent B contains 4% cupric sulfate. For a working reagent (WR) the kit components are mixed in the ratio 50:1 (A: B) and a diluted 0.1 ml of unknown protein sample is added to 2 ml of this WR and mixed well. This mixture is incubated for 30 min at 37° C, cooled, and read at 562 nm against a control devoid of protein.

For Western blot analysis, 30-50 µg protein was aliquoted in a 1.5 ml eppendorf tube and equal amount of 2x loading dye (62 mM 4x Stacking gel buffer, 5% β-mercaptoethanol, 3%SDS, 20% Glycerol, 0.1% Bromophenol blue and sterile distilled water) was added, the samples were mixed and boiled at 90° C for 5 minutes and centrifuged for 5 seconds to collect the total protein in the bottom of the tube. The proteins were resolved over 8-12% Tris-Glycine gels by SDS poly acrylamide gels electrophoresis at 110-120 volts for about 90 min (or until the gel has run off unneeded proteins according to colored protein ladder loaded in first lane) using 1x running buffer (Tris-HCl, Glycine and SDS) and transferred onto a nitrocellulose membrane sandwiching the gel and membrane (Sponge - filter paper - gel - membrane - filter paper – sponge) using 1x transfer
buffer (containing Tris-HCl, Glycine and Methanol) at a fixed voltage (25 volts) for 90 minutes.

The non-specific sites were blocked by incubating the blot with 5% non-fat dry milk in buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) for 1 hour 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) twice for 10 minutes each 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 2x10 minutes, 2x5 minutes and then incubated with the corresponding secondary antibody HRP conjugate (Amersham Life Science Inc., Arlington Height, IL) at 1: 2000 dilutions for 1 hour at room temperature. The blot was washed for 2x10 minutes, 4x5 minutes 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., Arlington Height, IL). Each blot was stripped and reprobed with β-Actin to confirm 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.

3.3 Results and Discussion

Catechins and Theaflavins are strong antioxidant polyphenols abundantly present in green tea and black tea respectively (Mukhtar and Ahmad, 2000; Yang, 1999; Ahmad et al., 1997; Gupta et al., 1999; Gupta et al., 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. (Mukhtar and Ahmad, 2000; Yang, 1999; Gupta et al., 2000) Both EGCG and TF have been shown to possess antiproliferative effects in in vitro cell culture and in in vivo animal studies (Mukhtar and Ahmad, 2000; Yang, 1999; Gupta et al., 1999; Gupta et al., 2000; Yang et al., 1998; Mukhtar et al., 1994; Yang and Wang, 1993). Studies have shown that EGCG treatment of prostate cancer 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 Chung et al., (2001). Recently, Klein et al., (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 antiproliferative 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 prostate cancer cells (Chaudhary and Hruska, 2003; Chen et al., 2001; Davies et al., 1999; Graff et al., 2000), 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. The study in this chapter employed two distinct human CaP cell lines viz. LNCaP (androgen-responsive prostate carcinoma cells) and DU145 (androgen-unresponsive prostate carcinoma cells). The choice of these two distinct cell lines is based on the fact that CaP, 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, the study 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 hours post-treatment (Figure 1 and 2).

Although EGCG inhibited the expression of PI3K at all doses and time periods, the maximum inhibition (~60% over control) was observed at 24 hrs with 40 μg EGCG. The inhibition in p-Akt levels was much more pronounced (~80% over control) at the same dose of EGCG.

Next, the study evaluated the effect of TF treatment on the protein expression of PI3K and phospho-Akt in DU145 cells. As shown in figure 3 and 4, TF-treatment to the cells resulted in a noticeable inhibition of PI3K and Akt.
proteins at 12 and 24 hours 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 doses (24 hours) in both the proteins. An interesting observation from our experiment was that TF was more effective in inhibiting PI3K as compared to EGCG.

Next, the effect of EGCG and TF on the protein levels of PI3K and phospho-Akt in LNCaP cells was evaluated. As shown in figures 5, 6, 7 and 8, both the polyphenols significantly inhibited PI3K as well as phosphorylated form of Akt at 24 hours post treatment. However the inhibition in PI3K proteins was only moderate at 12 hours post treatment. TF treatment to LNCaP cells resulted in only a slight decrease in PI3K levels at 12 hours post treatment.

Klein et al. (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 theaflavins inhibit UVB-induced PI3K activation. However, none of these studies evaluated the effect of tea polyphenols on the constitutive activation of these proteins.

The MAPKs 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.
Figure 1: Effect of EGCG on PI3K protein in DU145 cells. 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 chemiluminescent detection. Equal loading was confirmed by stripping the immunoblot and re-probing it for β-actin. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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.
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Figure 2: Effect of EGCG on Akt proteins in DU145 cells. 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 Akt. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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 Akt.
Constitutive levels of Erk1/2 is significantly up regulated in human prostate cancer cell lines as well as human prostate cancer tissues (Guo et al., 2000). Data in figure 9A 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 hours post treatment (Fig 9B and 10). Studies have shown that Erk1/2 is up regulated in cancer 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 (Malik et al., 2002; Zimmermann and Moelling, 1989) 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.

Taken together, this study, demonstrated the modulation of PI3K/Akt and Erk1/2 pathways by EGCG as well as TF.
Figure 3: Effect of TF on PI3K protein in DU145 cells. 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. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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.
Figure 4: Effect of TF on Akt proteins in DU145 cells. 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 Akt. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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 Akt.
Figure 5: Effect of EGCG on PI3K protein in LNCaP cells. 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. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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.
Figure 6: Effect of EGCG on p-Akt protein in LNCaP cells. 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 Akt. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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 Akt.
Figure 7: Effect of TF on PI3K protein in LNCaP cells. 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. The quantification of protein was performed by densitometric analysis using UN-SCAN-IT™ 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.
Figure 8: Effect of TF on p-Akt protein in LNCaP cells. 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 Akt. The quantification of protein was performed by densitometric analysis using UNSCAN-IT™ 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 Akt.
Figure 9: (A) Constitutive expression of Erk1/2 in DU145 and LNCaP cells, Effect of EGCG on protein levels of Erk1/2. Following treatments of the cells with EGCG (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 independent experiments with similar results. The quantification of protein levels was performed by densitometric analysis using UN-SCAN-IT™ software (Silk Science Inc., Orem, UT). The densitometry data represent means ± standard errors from the immunoblots, and are shown as relative density of protein bands (relative background) normalized to β-Actin.
Figure 10: Effect of TF on protein levels of Erk1/2. Following treatments of cells with TF (as specified), the cell lysates were prepared and 50 µg protein subjected to Tris-glycine gel electrophoresis followed by immunoblot analysis and chemiluminescent 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 Scientif Inc., Orem, UT). The densitometry data represent means ± standard errors from the immunoblots, and are shown as relative density of protein bands (relative background) normalized to β-actin.
3.4 References


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