ARTICLE

Magnolol Induces Apoptosis Via Inhibiting the EGFR/PI3K/Akt Signaling Pathway in Human Prostate Cancer Cells

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ABSTRACT

We observed that treatment of prostate cancer cells for 24 h with magnolol, a phenolic component extracted from the root and stem bark of the oriental herb *Magnolia officinalis*, induced apoptotic cell death in a dose- and time-dependent manner. A sustained inhibition of the major survival signal, Akt, occurred in magnolol-treated cells. Treatment of PC-3 cells with an apoptosis-inducing concentration of magnolol (60 μM) resulted in a rapid decrease in the level of phosphorylated Akt leading to inhibition of its kinase activity. Magnolol treatment (60 μM) also caused a decrease in Ser^{136} phosphorylation of Bad (a proapoptotic protein), which is a downstream target of Akt. Protein interaction assay revealed that Bcl-xL, an anti-apoptotic protein, was associated with Bad during treatment with magnolol. We also observed that during treatment with magnolol, translocation of Bax to the mitochondrial membrane occurred and the translocation was accompanied by cytochrome *c* release, and cleavage of procaspase-8, -9, -3, and poly(ADP-ribose) polymerase (PARP). Similar results were observed in human colon cancer $HCT116Bax^{+/-}$ cell line, but not $HCT116Bax^{-/-}$ cell line. Interestingly, at similar concentrations (60 μM), magnolol treatment did not affect the viability of normal human prostate epithelial cell (PrEC) line. We also observed that apoptotic cell death by magnolol was associated with significant inhibition of pEGFR, pPI3K, and pAkt. These results suggest that one of the mechanisms of the apoptotic activity of magnolol involves its effect on epidermal growth factor receptor (EGFR)-mediated signaling transduction pathways. J. Cell. Biochem. 106: 1113–1122, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: MAGNOLOL; APOPTOSIS; EGFR; PI3K/Akt PATHWAY; Bax; Bad

Prostate cancer is the sixth most commonly diagnosed cancer in the world. It is also the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among men in the United States. The American Cancer Society estimated that approximately 232,090 new cases were diagnosed in the United States in the year 2005 [Jemal et al., 2005]. Treatment options are limited and are associated with significant morbidity and mortality. In China, Korea, and Japan, Complementary and Alternative Medicine (CAM) therapies such as traditional Chinese medicine have been an option to cancer patients for centuries, whereas in Europe and North America, CAM has been growing in popularity only in the past few decades. In this study, we identified magnolol as a potential agent for CAM and investigated its biological mechanism as an anti-neoplastic agent for prostate cancer patients.

Magnolol is a phenolic component isolated from the root and bark of *Magnolia officinalis*. It has been used in traditional Chinese and Japanese medicine for the treatment of various ailments due to its muscle relaxant, antioxidative, anti-atherosclerosis, anti-inflammatory, anti-gastric ulcer, anti-allergic, anti-bacterial, anti-thrombotic, and steroidogenesis-stimulating activities [Teng et al., 1988; Wang et al., 2000; Li, 2002; Huang et al., 2004; Chen et al., 2005a,b]. Recently, attention has been paid to its anti-cancer action. Previous studies have revealed that magnolol also inhibits proliferation and induces apoptosis in several cancer cell lines

including colon cancer, HepG2 hepatoma, leukemia, fibrosarcoma, melanoma, squamous carcinoma, and thyroid carcinoma cells [Lin et al., 2001, 2002; Ikeda and Nagase, 2002; Yang et al., 2003; Zhong et al., 2003; Huang et al., 2007]. Moreover, in animals inoculated subcutaneously with B16-BL6 melanoma cells, magnolol effectively inhibits invasion and metastasis [Nagase et al., 2001; Ikeda and Nagase, 2002].

Several researchers have shown that magnolol inhibits proliferation of cancer cells at low concentrations and induces apoptosis at high concentrations [Yang et al., 2003]. Magnolol arrests the cells at the G_0/G_1 phase of the cell cycle. Lin et al. [2002] reported that the magnolol-induced cell cycle arrest occurs when the cyclin-CDK system is inhibited, just as p21 protein expression is augmented. Several researchers also reported that magnolol increases translocation of cytochrome c from mitochondria to cytosol, activates caspases, and subsequently leads to apoptotic death [Lin et al., 2001; Yang et al., 2003; Huang et al., 2007]. However, recent studies claim that magnolol-induced cell death occurs via autophagy but not apoptosis [Li et al., 2007]. This discrepancy has led us to examine the mechanism of magnolol-induced cell death. In this study, we clearly demonstrate that magnolol induces apoptotic death. Our studies also suggest that magnolol-induced apoptotic death is mediated through blockade of the epidermal growth factor receptor (EGFR)-PI3K–Akt pathway and subsequent activation of the Bad–Bax–cyctochrome c-caspase pathway.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Magnolol (>99% pure) was purchased from Calbiochem (San Diego, CA). Rabbit polyclonal anti-caspase-3, anti-PI3K, and anti-phospho-PI3K antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EGFR, anti-phospho-EGFR^{Y845}, anti-phospho-EGFR^{Y1068}, anti-phospho-EGFR^{Y1086}, anti-PDK-1, anti-phospho-PDK-1, anti-caspase-8, anti-phospho-Ser^{473} Akt, anti-Akt, anti-phospho Bad-Ser^{136}, anti-Bad, anti-Bax, anti-Bcl-xL, and anti-COX-IV antibody were from Cell Signaling (Beverly, MA). Monoclonal antibodies were purchased from each of the following companies: anti-caspase-9 antibody from Upstate Biotechnology (Lake Placid, NY), anti-poly(ADP-ribose) polymerase (anti-PARP) antibody from Biomol Research Laboratory (Plymouth Meeting, PA), anti-cytochrome c antibody from PharMingen (San Diego, CA), and anti-actin antibody from ICN (Costa Mesa, CA).

CELL CULTURES

Human prostate cancer PC-3, DU-145, and LNCaPcells, and rat prostate endothelial YPEN-1 cells obtained from ATCC (Manassas, VA) were cultured in either DMEM or RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), and 26 mM sodium bicarbonate for monolayer cell culture. Immortalized nonmalignant human breast epithelial MCF-10A cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml EGF, 0.1 μg/ml cholera enterotoxin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L L-glutamine, and 0.5 μg/ml Fungizone. Primary cultures of normal human prostate epithelial cells (PrECs) were purchased from Cambrex Bio Science Walkersville (Cambrex Corporation, East Rutherford, NJ), cultivated in PrEBM^{TM} (Prostate Epithelial Cell Basal Medium; Cambrex Corporation) and supplemented with PrEGM SingleQuots$^{®}$. Cells were maintained in accordance with manufacturer's instructions (Clonetics^{TM} Prostate Epithelial Cell System). The human colon cancer cell line HCT116 and its Bax-deficient derivative, Bax Knock Out cells (Bax KO), were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). These cell lines were cultured in McCoy's 5A medium (Gibco-BRL) containing 10% fetal bovine serum and antibiotics. The dishes containing cells were kept in a 37°C humidified incubator with 5% CO_2.

DRUG TREATMENT

Magnolol (dissolved in dimethyl sulfoxide (DMSO)) was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies, PC-3, DU-145, LNCaP, and PrECs were treated with magnolol at 10–100 μM final concentrations for 24 h in complete cell medium. HCT116 Bax$^{+/-}$ and HCT116 Bax$^{-/-}$ cells were treated with magnolol at 20, 40, and 80 μM final concentration for 24 h in complete cell medium. Control cells were treated with vehicle alone. A stock solution was prepared in DMSO.

DETERMINATION OF CELL VIABILITY

One or two days prior to the experiment, cells were plated into 60-mm dishes at a density of 1×10^5 cells/plate in 5 ml of tissue culture medium in triplicate. For trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline (PBS) solution. The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined under a light microscope. At least 300 cells were counted for each survival determination. For colony formation assay, cells were trypsinized, counted, and plated at appropriate dilutions. After 1–3 weeks of incubation at 37°C, colonies were stained and counted.

DAPI STAINING AND DNA FRAGMENTATION

Morphological changes in the nuclear chromatin in cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI). Cells were grown on glass coverslips at a density of 2×10^5 cells/well. Cells were treated with 60 μM magnolol for 8 h. Cells were washed twice with PBS and fixed by incubation in 70% ethanol for 30 min. Following washes with PBS, cells were incubated in 1 mg/ml DAPI solution for 30 min in the dark. The detection of nuclear DNA fragmentation was performed as described previously [Saeki et al., 2002] with some modifications. Briefly, the cell pellets were resuspended in lysis buffer (10 mM Tris–HCl, pH 8.0; 25 mM EDTA; 0.5% SDS, 100 mM NaCl, and 200 μg/ml proteinase K) and incubated at 55°C for 2 h. The cell lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and then centrifuged for 15 min. The supernatant was incubated with RNase A (0.2 μg/ml) at 37°C. After 1 h, the DNA was extracted with phenol and precipitated with one-tenth of the volume of 3 M sodium acetate and 3 volumes

of 100% ethanol. The DNA samples were separated by horizontal electrophoresis on 1.8% agarose gels, stained with EtBr, and visualized under UV light.

IMMUNOCYTOCHEMISTRY OF Bax

For immunocytochemical analysis, 1×10^5 cells were plated on glass slides 1 day before the experiment. Cells were treated with 60 μM magnolol for 8 h. Following treatment, cells were washed three times with 0.5% BSA in PBS, followed by fixation in 2% paraformaldehyde for 15 min. The fixed cells were permeablized with 0.05% Triton-X, washed and blocked with 2% BSA in PBS for 45 min to eliminate nonspecific binding of secondary Ab. Cells were treated with polyclonal rabbit anti-human Bax (1:100 in 0.5% BSA; Santa Cruz Biotechnology) and incubated for 1 h in moist chamber, then washed and incubated with secondary Ab. The cells were washed and incubated for 45 min with a FITC-conjugated donkey anti-rabbit antibody (1:500 in 0.5% BSA; Santa Cruz Biotechnology). Mitochondria were stained with 300 nM MitoTracker (Invitrogen). Slides were mounted on a medium with DAPI (Vector Laboratories) in order to trace cell nuclei. Cells were visualized in 0.4-μm sections using an inverted Olympus Fluoview 1000 laser scanning confocal microscope under an Olympus x 60 oil immersion objective. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

MEASUREMENT OF CYTOCHROME c RELEASE

To determine the release of cytochrome c from mitochondria, subconfluent PC-3 cells growing in 100 mm dishes were used. These cells were treated with magnolol (60 μM) for 24 h. Using Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA), mitochondria and cytosol fractions were prepared from treated cells using instructions and reagents included in the kit.

IMMUNOBLOT ANALYSIS

Cells were lysed with $1\times$ Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromophenol blue) and boiled for 7 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with $1\times$ lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–polyacrylamide gels. Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS–Tween-20 (0.1%, v/v) for 1 h. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) at 4°C overnight. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL; Amersham, Arlington Heights, IL). To ensure equal protein loading, each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

INTERACTION BETWEEN Bcl–xL AND Bad

To examine the interaction between Bcl-xL and Bad, cells were treated with vehicle or 60 μM magnolol for 24 h. After treatment, cells were scraped, washed in PBS, washed in lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin) and resuspended in 400 μl of lysis buffer. After 1 h incubation on ice, cells were lysed by forcing them through a 27-gauge needle 5–10 times. The lysate was centrifuged at 12,000g for 15 min and the supernatant was collected. Bad and Bcl-xL proteins were immunoprecipitated from whole-cell lysates using specific antibodies (4 μg) after incubation overnight at 4°C, followed by the addition of protein A/G-plus agarose beads (50 μl; Santa Cruz Biotechnology), and incubation was continued for 2 h. Immuno-precipitates were washed with lysis buffer and subsequently subjected to SDS–PAGE on 12% gels followed by immunoblotting using anti-Bad or anti-Bcl-xL antibody.

STATISTICAL ANALYSIS

Statistical analysis was carried out using Graphpad InStat 3 software (GraphPad Software, Inc., San Diego, CA). Results were considered statistically significant at $P < 0.05$.

RESULTS

MAGNOLOL INDUCES CYTOTOXICITY IN HUMAN PROSTATE CANCER CELLS

To investigate morphological changes, human prostate carcinoma PC-3 cells were treated with 0–100 μM magnolol for 24 h and then observed under a light microscope and photographed. Observations made under the microscope showed that, after magnolol treatment, the cell number decreased in a dose-dependent manner and, more interestingly, the shape of cells changed in comparison to control cells (Fig. 1A). Apoptotic cell death, which is associated with typical morphological features like cell shrinkage and cytoplasmic membrane blebbing, was observed. Unlike magnolol, DMSO caused little or no cytotoxicity. We further examined the effect of various concentrations of magnolol on cell viability in human prostate cancer cells (PC-3, DU-145, and LNCaP) and noncancer cells (PrEC, MCF-10A, and PrEC) with various concentrations of magnolol. Data from trypan blue exclusion assay show that magnolol treatment resulted in a dose-dependent decrease in the viability of cancer cells (Fig. 1B). Unlike cancer cells, noncancer cells were resistant to magnolol-induced cytotoxicity. Similar results were obtained by colony formation assay (Fig. 1C). Magnolol treatment for 24 h caused dramatic cell mortality of prostate cancer cell lines LNCaP, PC-3, and DU-145 with an estimated 50% of cell death (IC50) value of 53, 60, and 70 μM, respectively.

MAGNOLOL INDUCES APOPTOTIC DEATH

To confirm whether magnolol induces apoptosis, we further examined magnolol-induced cytotoxicity. PC-3 cells were treated with 60 μM magnolol for 8 h and analyzed with DAPI staining. DAPI staining of cells treated with magnolol showed the presence of many cells with condensed nuclei, a morphological change that is associated with apoptosis (Fig. 2A). Similar results were observed by DNA fragmentation (Fig. 2B). DNA ladder formation (DNA banding characteristic of late apoptosis) occurred during treatment with 60 μM magnolol. Additional studies were designed to examine
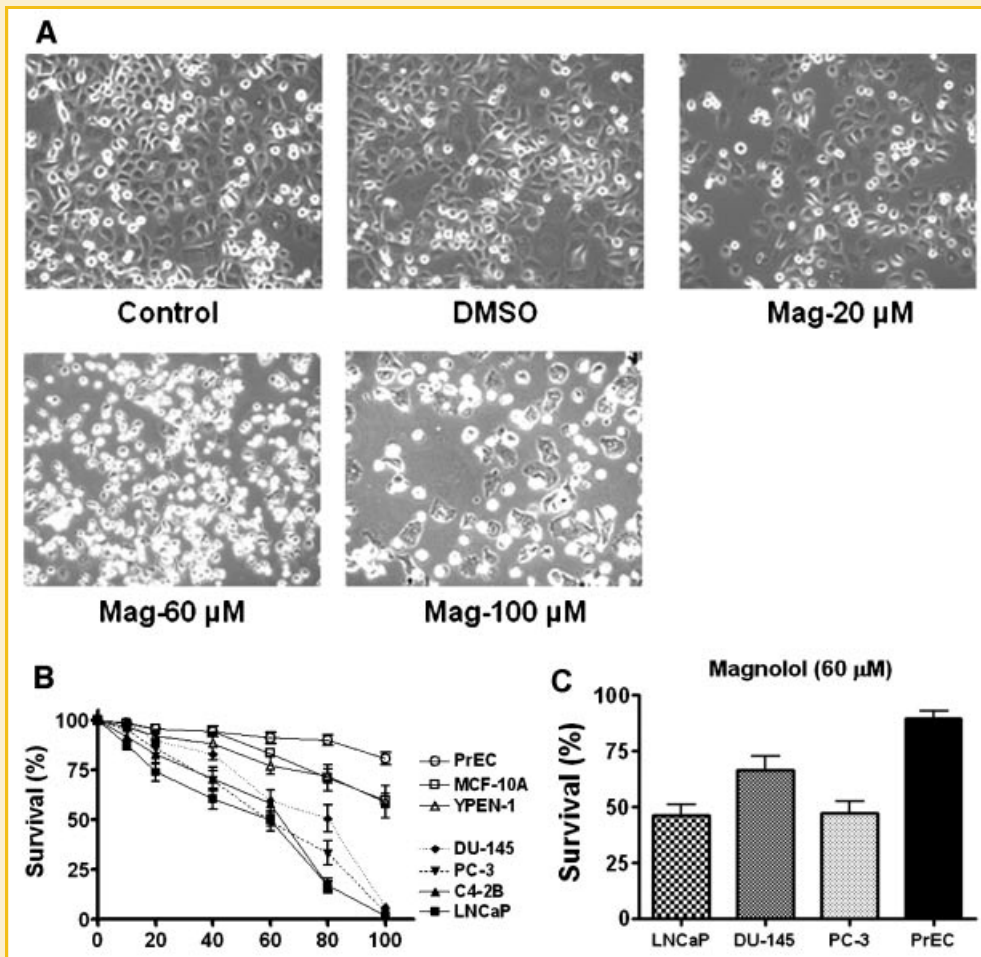
Fig. 1. Magnolol induces cytotoxicity in human prostate cancer cells. A: Phase-contrast photograph of PC-3 cells: untreated control, treated with 0.1% DMSO, or various concentrations of magnolol (20–100 μM) for 24 h. B: PC-3, DU-145, LNCaP, MCF-10A, YPEN-1, and PrECs were treated with various concentrations (10–100 μM) of magnolol for 24 h. The cytotoxic effects of magnolol on cells were determined using the trypan blue dye exclusion assay as described in the Materials and Methods Section. Error bars represent SEM from triplicate experiments. C: PC-3, DU-145, LNCaP, and PrECs were treated with 60 μM of magnolol for 24 h. The cytotoxic effects of magnolol on cells were determined using the colony formation assay as described in the Materials and Methods Section. Error bars represent SEM from triplicate experiments.

whether magnolol induces apoptotic death by measuring PARP cleavage, the hallmark feature of apoptosis. Previous studies show that PARP (116 kDa) is cleaved yielding a characteristic 85 kDa fragment during apoptotic death [Lee et al., 2004]. Figure 2C shows that the cleavage of PARP was observed during treatment with magnolol in a dose-dependent manner. Figure 2C also demonstrates that magnolol activated caspases in a dose-dependent manner. Western blot analysis shows that procaspase-8 (55/57 kDa) was cleaved to the intermediate forms (41 and 43 kDa) by treatment with magnolol. Magnolol induced proteolytic processing of procaspase-9 (48 kDa) into its active form (34 kDa). Western blot analysis also shows that magnolol induced caspase-3 activation. Procaspase-3 (32 kDa), the precursor form of caspase-3, was cleaved to active form (17 kDa) by treatment with magnolol. Figure 2D shows that magnolol-induced PARP cleavage was observed even at 40 and 60 μM in human prostate carcinoma LNCaP cells and human prostate adenocarcinoma DU-145 cells, respectively. However, PARP cleavage occurred at 100 μM magnolol in normal human

PrECs. These results suggest that magnolol effectively induces apoptotic death in prostate cancer cells but not in normal prostate cells.

MAGNOLOL INHIBITS PI3K/Akt ACTIVITY

It is well known that phosphorylation (activation) of Akt is associated with protection of cells from apoptosis [Nicholson and Anderson, 2002]. We postulated that magnolol inhibits Akt activity and consequently leads to apoptosis. To examine whether magnolol inhibits Akt activity by dephosphorylating Akt, PC-3 cells were treated with 60 μM magnolol for various periods (2–24 h) and the level of Akt was measured. Figure 3 shows that Akt was rapidly dephosphorylated within 2 h of magnolol treatment without changing the Akt protein level. Since PI3K and PDK1 are involved in the activation of Akt, we examined whether dephosphorylation of Akt during magnolol treatment is mediated through inactivation (dephosphorylation) of PI3K and PDK1. Figure 3 shows that magnolol dephosphorylated Akt upstream proteins PI3K and PDK1.
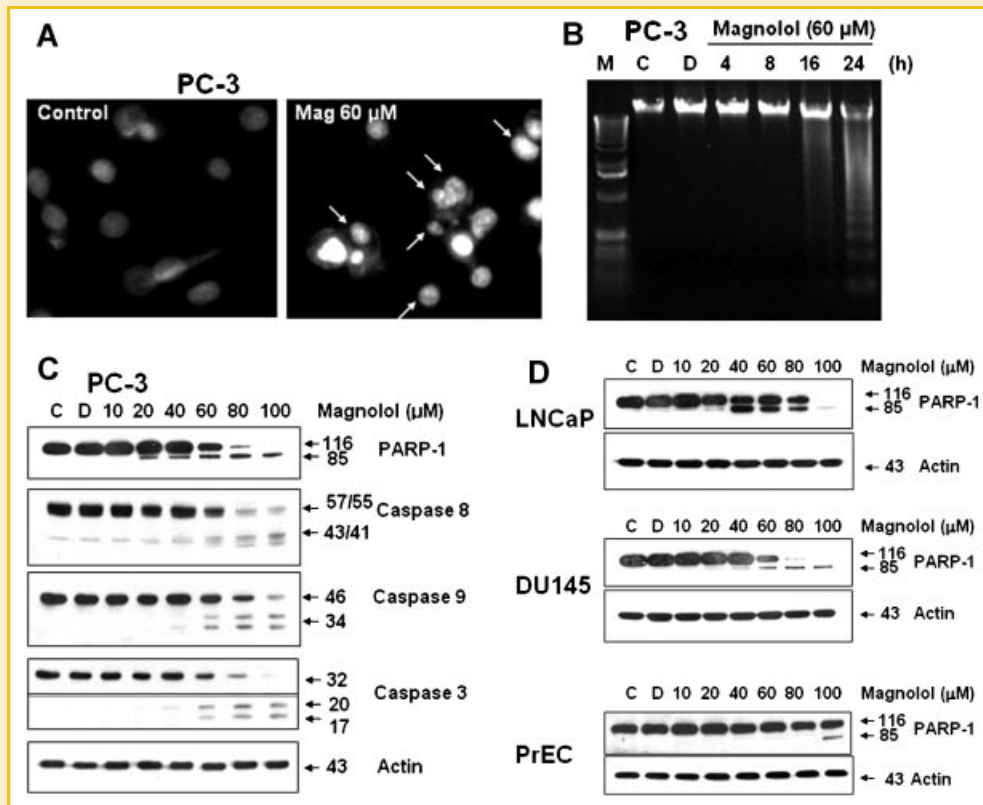
Fig. 2. Magnolol induces apoptotic death in PC–3 cells. A: Cells were treated with either 0.1% DMSO or 60 μM magnolol for 8 h. After treatment, cells were stained with DAPI (1 μg/ml), and morphological features were analyzed with a fluorescence microscope. B: Cells were treated with either 0.1% DMSO (D, lane 3) for 24 h or various times (2–24 h, lanes 4–8) with 60 μM magnolol. DNA isolated from cells was fractionated by electrophoresis. Oligonucleosomal length DNA fragments were visualized by staining gels with ethidium bromide. Lane 1, DNA ladder. C, untreated control. C,D: Prostate cancer cells (LNCaP, PC–3, DU–145) or normal human prostate epithelial cells (PrECs) were treated with either 0.1% DMSO (D, lane 2) or various concentrations (10–100 μM) of magnolol for 24 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted. Actin was used to confirm the equal amount of protein loaded in each lane. C, untreated control.

These results suggest that magnolol inhibits Akt activity by inhibiting the PI3K–PDK1 signaling pathways.

MAGNOLOL ALTERS INTERACTION BETWEEN Bcl-xL AND Bad AND LEADS TO CYTOCHROME *c* RELEASE

The proapoptotic BH3-only Bcl-2 family member Bad plays an important role in regulating apoptosis in cells [Yang et al., 1995]. In the presence of survival signals, Bad is phosphorylated and sequestered in the cytoplasm bound to 14-3-3 proteins; thus, the proapoptotic activity of Bad is repressed by phosphorylation [Zha et al., 1996]. Phosphorylation of Bad occurs on a MAPK-dependent site, serine 112, and also on serine 136, a site phosphorylated directly by Akt [Datta et al., 1997]. Phosphorylation of either serine 112 or serine 136 is sufficient to render Bad inactive [Zha et al., 1996]. Since magnolol inactivates Akt, we therefore examined whether treatment with magnolol lead to the dephosphorylation of Bad at serine 136, which would promote apoptosis. Figure 4A shows that the level of phosphorylated Bad was decreased during treatment with 60 μM magnolol. As a next step, we investigated whether dephosphorylation of Bad alters interaction between Bad and Bcl-xL during treatment with magnolol. The interaction between Bad and Bcl-xL was examined by immunoprecipitation with anti-Bcl-xL antibody. Figure 4B shows an increase in interaction between Bad

and Bcl-xL during treatment with magnolol; densitometric analysis illustrates that magnolol increased the Bcl-xL:Bad ratio to 2.2. These results suggest that dephosphorylated Bad dissociates from 14-3-3 and interacts with pro-survival Bcl-2 family members, such as Bcl-xL. We previously reported that association of Bad with Bcl-xL results in dissociation of Bax from Bcl-xL [Lee et al., 2008a]. We also reported that oligomerization of Bax occurs when Bax is dissociated from Bcl-xL [Lee et al., 2008a]. The oligomerized Bax integrates in the outer mitochondria membrane, where it triggers cytochrome *c* release [Costantini et al., 2000]. Integration of Bax on mitochondria was indeed detected by confocal microscopy during treatment with magnolol (Fig. 5A). Figure 5B clearly shows that cytochrome *c* release occurred during treatment with magnolol.

ROLE OF Bax IN MAGNOLOL–INDUCED APOPTOSIS IN HCT116 HUMAN COLON CANCER CELLS

It is well known that Bax is a cytosolic protein that undergoes a conformational change during apoptosis and migrates to the mitochondria, an event essential for the release of cytochrome *c*. In order to verify the importance of Bax in magnolol-induced cell death, we used HCT116Bax$^{+/-}$ and HCT116Bax$^{-/-}$ cells to compare the apoptotic death induced by magnolol. These cells were treated with various concentrations (20–60 μM) of magnolol for 24 h,
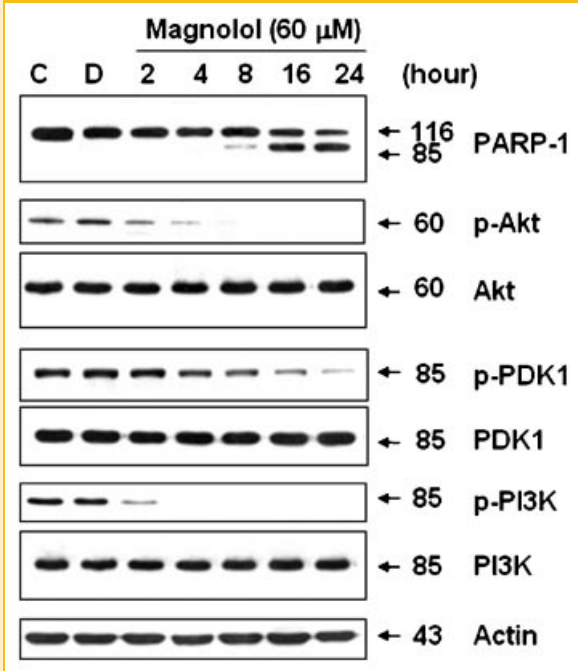
Fig. 3. Effect of magnolol on the PI3K–PDK–Akt pathway in PC-3 cells. Cells were treated with 0.1% DMSO for 24 h or 60 μM magnolol for various times (2–24 h). Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti–PARP-1, anti–phospho–PI3K, anti–PI3K, anti–phospho–PDK, anti–PDK, anti–phospho–Akt, and anti–Akt antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

occurred in Bax$^{+/-}$ cells, but not in Bax$^{-/-}$ cells, during treatment with 40 μM magnolol (Fig. 5D). These results confirm that Bax plays an important role in magnolol-induced apoptotic death.

MAGNOLOL INHIBITS THE EGFR–PI3K–Akt SIGNAL TRANSDUCTION PATHWAY

It is well known that the PI3K pathway is regulated by a variety of growth factors (i.e., EGF), and the activation of the PI3K–Akt signaling pathway is strongly implicated in the regulation and survival or protection of cells [Nicholson and Anderson, 2002]. We further examined whether magnolol inhibits growth factor-associated signals. The first task was to examine the effect of EGF on the EGFR-associated signal transduction pathway. As shown in Figure 6A, phosphorylation of EGFR, PI3K, and Akt occurred during treatment with EGF (100 ng/ml). Phosphorylation at three residues (Y845, Y1068, and Y1086) of EGFR was detected at 5 min after EGF administration and gradually decreased with time. PI3K (p85), a functional subunit of PI3K, was activated within 5 min of EGF administration and gradually decreased by 30 min. One of the downstream targets of EGFR–PI3K is Akt, which was gradually phosphorylated during EGF treatment. The second task was to examine the effect of magnolol on EGF-induced activation of the EGFR–PI3K–Akt signal transduction pathway. As shown in Figure 6B, magnolol inhibited EGF-induced phosphorylation (Y845, Y1068, and Y1086) of EGFR as well as that of PI3K and Akt. We next examined whether the effect of magnolol on EGFR differs from that on other growth factor receptors. Figure 6C shows that insulin, like EGF, stimulated phosphorylation of Akt. However, unlike its inhibitory effect on the EGFR–PI3K–Akt signal transduction pathway, magnolol did not inhibit insulin-induced Akt phosphorylation. Interestingly, insulin partially protected the cells from magnolol-induced cytotoxicity (Fig. 6D). These results suggest that magnolol-induced cytotoxicity is mediated through several pathways.

and cell death was analyzed both by trypan blue exclusion assay and Western blotting. The trypan blue exclusion data suggest that HCT116Bax$^{+/-}$ cells were more sensitive to magnolol than HCT116Bax$^{-/-}$ cells (Fig. 5C). Also, we observed that PARP cleavage
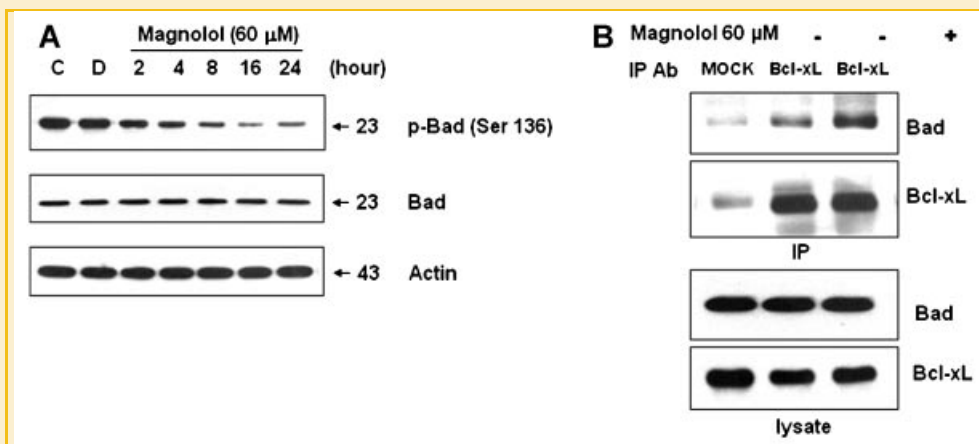

Fig. 4. Dephosphorylation of Bad and association of Bcl–xL with Bad after magnolol treatment in PC-3 cells. A: Cells were treated with 0.1% DMSO for 24 h or 60 μM magnolol for various times (2–24 h). Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti–phospho–Bad (S136) or anti–Bad antibody. Actin was used to confirm the equal amount of proteins loaded in each lane. B: Cells were treated with magnolol (60 μM) for 24 h. Cell lysates were immunoprecipitated with anti–Bcl–xL antibody or mock antibody (IgG) and then immunoblotted with anti–Bcl–xL or anti–Bad antibody (upper panels). The presence of Bad or Bcl–xL in the lysates was verified by immunoblotting (lower panels).
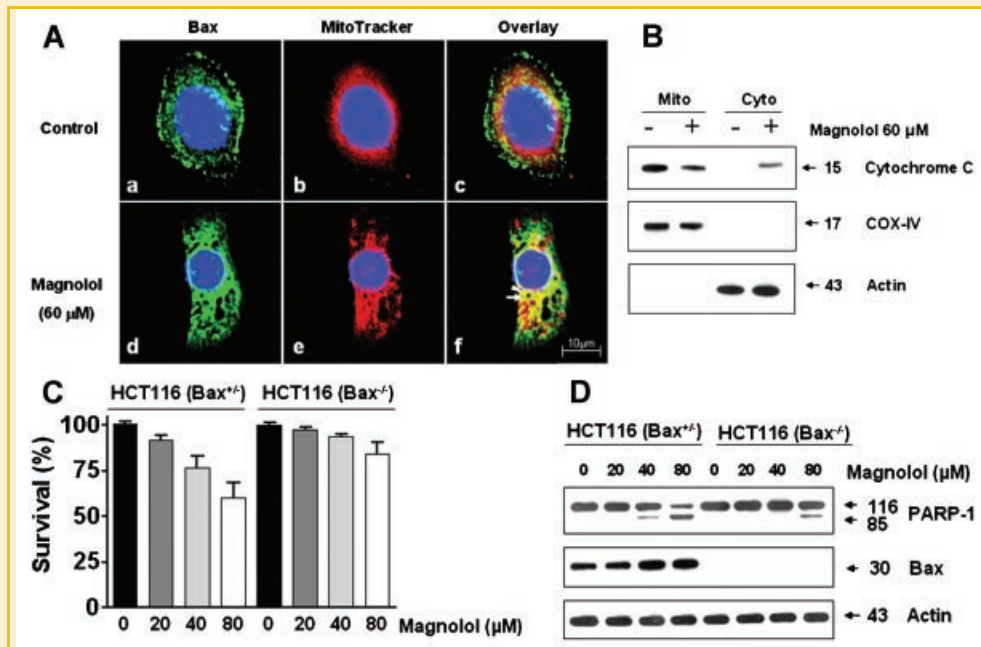
Fig. 5. Magnolol treatment increases Bax translocation and cytochrome *c* release from mitochondria to the cytosol in PC-3 cells. A: Localization of Bax after magnolol treatment was examined by confocal microscope. Cells were untreated (panels a–c) or treated (panels d–f) with 60 μM magnolol for 16 h. Bax was stained green with anti-Bax antibody (panels a and d). Mitochondria were stained red with MitoTracker (panels b and e). Nuclei were stained blue with DAPI (panels a–f). Panel c is an overlay of panels a and b. Panel f is an overlay of panels d and e. Co-localization of Bax and mitochondria is shown in yellow (arrows). B: Cytochrome *c* (15 kDa) release into cytosol was determined by immunoblotting for cytochrome *c* in the mitochondrial and cytosolic fraction after 24 h of 60 μM magnolol treatment. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-cytochrome *c* antibody. We used COX-IV as a mitochondrial marker and actin as a cytosolic marker. C,D: HCT 116 (Bax$^{+/-}$) and HCT 116 (Bax$^{-/-}$) cells were treated with various concentrations (20–80 μM) of magnolol for 24 h. Cell viability was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (SEM) from three separate experiments. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-PARP-1 or anti-Bax antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

DISCUSSION

In this study, we examined the cytotoxic mechanism of one potential CAM agent, magnolol, which has been used with other phenolic compounds in Chinese medicine for thousands of years and reflects a long and safe record of usage. We observed the apoptosis induction activity of magnolol in human prostate cancer PC-3, LNCaP, and DU-145 cells and human colon cancer HCT116 cells. We observed that magnolol-induced apoptosis is mediated by inhibition of the EGFR–PI3K–Akt signal transduction pathway and subsequent activation of the Bad–Bax–cytochrome *c*-caspase pathway. These observations are consistent with previous studies showing that magnolol induces cytochrome *c* release, which causes apoptosis through activating caspases [Lin et al., 2001; Yang et al., 2003; Huang et al., 2007]. However, in several recent reports, magnolol has been shown to increase the activity of ERK and to prevent cells from apoptosis [Ou et al., 2007; Jin et al., 2008; Lee et al., 2008b]. This discrepancy may be due that the effects of magnolol are cell type-dependent. Nonetheless, an important observation of our studies is that noncancer cells (normal human PrECs, immortalized nonmalignant human mammary epithelial MCF-10A cells, and rat prostate epithelial YPEN cells) are resistant to magnolol toxicity (Fig. 1B). These results provide a molecular basis for the development of phenolic compounds as anti-cancer agents to achieve better management of human prostate cancer.

During this study, we employed three different prostate cancer cell lines: LNCaP, DU-145, and PC-3. LNCaP cells contain wild type p53 and RB. These cells, which are androgen-dependent cells, are poorly invasive and tumorigenic. Unlike LNCaP cells, DU-145 cells are androgen-independent and express mutated p53 and RB. They are tumorigenic in nude mice. PC-3 cells lack p53 expression, but contain wild-type RB. They are androgen-independent and tumorigenic in nude mice. Our studies with these various human prostate cancer cells reveal that magnolol-induced cytotoxicity can be generalized regardless of invasiveness, androgen-dependency, p53 and RB status.

The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins, a group of anti-apoptotic and proapoptotic proteins that regulate the passage of small molecules such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor, which activate caspase cascades through the mitochondrial transition pore. Anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-xL) counteract the activation of caspases because these Bcl-2 family proteins heterodimerize with proapoptotic members of the Bcl-2 family (Bax, Bak) and interfere with release of cytochrome *c* by pore-forming proteins (Bid, Bik) [Gross et al., 1999]. To examine the involvement of the mitochondrial pathway in magnolol-induced apoptotic death, we turned our attention to the proapoptotic Bcl-2 family member Bad, a key downstream effector of Akt [Cantley, 2002] and related Bcl-2 family members during treatment with
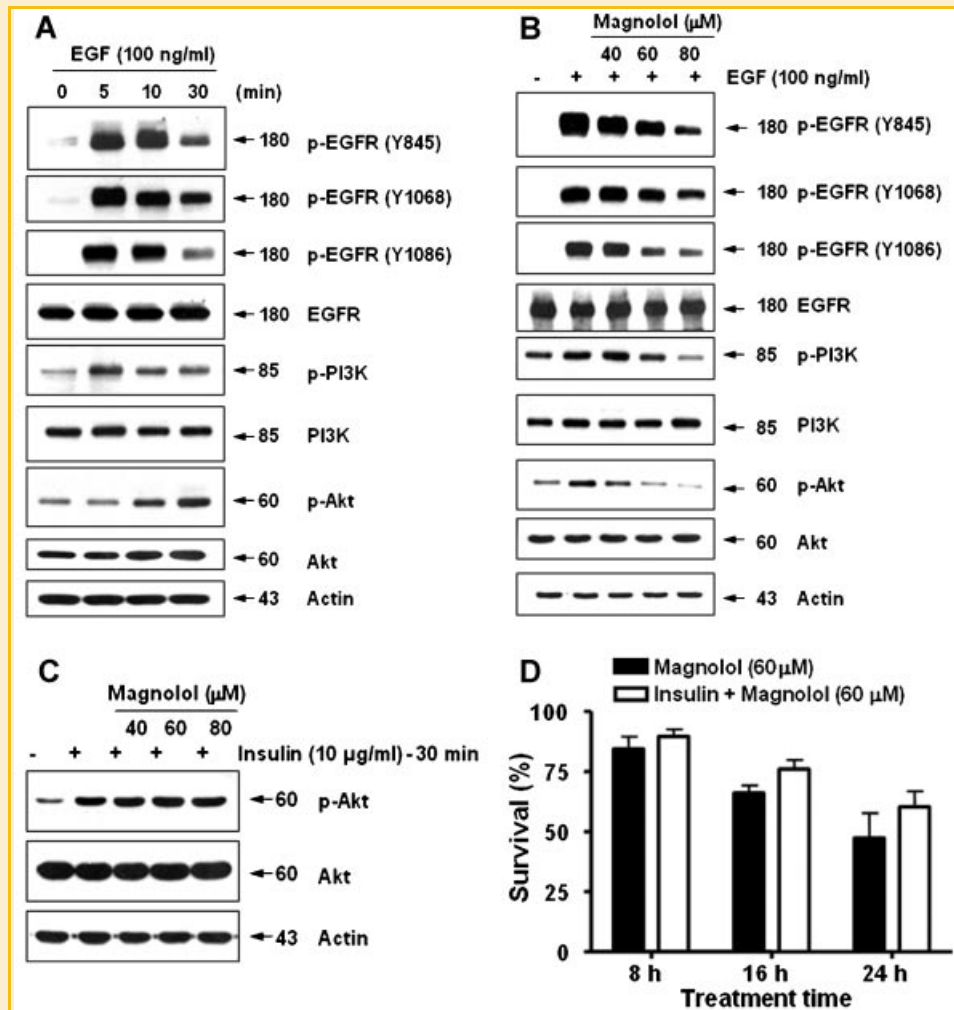
Fig. 6. Inhibition of EGFR signaling by magnolol in PC–3 cells. A: PC–3 cells were incubated with EGF (100 ng/ml) for the indicated times. Representative EGFR signaling proteins (p–EGFR, p–PI3K, and p–Akt) were detected by immunoblotting using five different types of phosphor–specific antibodies, which recognize specific phosphorylation sites on tyrosine residues. Anti–EGFR, anti–PI3K (p85), and anti–Akt antibodies were used to determine the total expression levels of each protein. Actin was used to ensure equal protein loading. B: Cells were pretreated with magnolol (40–80 μM) for 1 h prior to treatment with EGF (100 ng/ml) for 10 min. Activated EGFR signaling proteins were detected by immunoblotting analysis as described above. Actin was used to ensure equal protein loading. C: Cells were pretreated with magnolol (40–80 μM) for 1 h prior to treatment with insulin (100 μg/ml) for 30 min. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti–phospho–Akt or anti–Akt antibody. Actin was used to confirm the equal amount of proteins loaded in each lane. D: Cells were pretreated with or without insulin (100 μg/ml) for 30 min prior to treatment with 60 μM magnolol for various times (8–24 h). The cytotoxic effects of magnolol on cells were determined using the trypan blue dye exclusion assay as described in the Materials and Methods Section. Error bars represent SEM from triplicate experiments.

magnolol. It has been demonstrated that in the absence of activated Akt, Bad forms heterodimers with Bcl-xL, an anti-apoptotic protein that inhibits the release of cytochrome *c* from mitochondria [Gross et al., 1999]. This complex formation abrogates the anti-apoptotic function of Bcl-xL [Green and Reed, 1998], thereby facilitating apoptotic death via a cytochrome *c*-dependent pathway. Conversely, when Akt is phosphorylated (activated), Bad becomes phosphorylated and translocated into the cytoplasm through binding with the phosphoserine-binding protein 14-3-3 [Zha et al., 1996]. The sequestration of Bad from mitochondria frees Bcl-xL to facilitate anti-apoptotic signaling. As a consequence, the dynamic interaction between Bcl-xL and Bad represents a critical determinant of cell fate downstream of the PI3K/Akt cascade. Our studies clearly demon-strate that Bad is associated with Bcl-xL, Bax translocates from the cytosol to the mitochondrial membrane, and leakage of cytochrome *c* occurs during treatment with magnolol (Figs. 4B and 5A,B).

A number of studies have demonstrated that the protein kinase Akt (protein kinase B) plays a major role in the survival of cells under a variety of conditions [Datta et al., 1999]. Akt is an important regulator of cell survival and cell proliferation, which 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 [Datta et al., 1999; Hill and Hemmings, 2002]. Akt blocks the induction of apoptosis by a large number of stimuli; these include DNA damage, growth factor withdrawal, and ultraviolet irradiation

as well as anti-Fas antibody and transforming growth factor β-mediated apoptosis. Akt promotes cell survival by inhibiting apoptosis, and its phosphorylation has been considered a critical factor in the aggressiveness of cancer. Magnolol was found to induce inactivation of Akt by decreasing the level of phosphorylated Akt in a concentration-dependent manner, contributing to the promotion of apoptosis [Huang et al., 2007; Fig. 6]. Although the precise anti-apoptotic effects of Akt are still unclear, Akt directly phosphorylates and inactivates procaspase-9 and blocks caspase-9-mediated apoptosis [Cardone et al., 1998]. Alternatively, inhibition of Akt promotes phosphorylation of the proapoptotic Bad, which favors the apoptotic process [Nakanishi et al., 2005; Fig. 4A].

There is increasing evidence that the malignant behavior of some tumors is sustained by deregulated activation of several growth factor receptors [Yarden and Ullrich, 1988; Glenney 1992]. Interference with the activation of growth factor receptor and/or with intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anti-cancer therapies [Gibbs, 2000]. A large body of experimental evidence has accumulated for a key role for EGFR activation in a variety of human epithelial cancers [Salomon et al., 1995; Ciardiello et al., 2001]. EGFR-driven intracellular signaling controls not only cancer cell proliferation but also several processes that are important for tumor progression, including apoptosis, invasion, angiogenesis, and metastasis [Woodburn, 1999]. 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 [Chott et al., 1999; Huang et al., 2002]. Thus, inhibition of EGFR activity is promising for successful treatment of prostate cancer. The present study demonstrates that magnolol can effectively inhibit the EGFR–PI3K–Akt survival pathway.

The results of the present study show that magnolol caused minimal toxicity in normal PrEC human PrECs. At the present time, we can only speculate how normal cells in comparison to cancer cells are resistant to magnolol-induced cytotoxicity. Magnolol may bind to the tyrosine kinase domain of EGFR, alter the structural configuration, and inhibit kinase activity. Previous studies have shown that reduced amounts of antioxidant enzymes, especially MnSOD, are found in a variety of cancer cells [Oberley and Buettner, 1979]; also, lowered amounts of CuZnSOD have been found in many, but not all, tumors. These differential amounts of antioxidant enzymes cause different redox states in cells. Although we are far from understanding how normal cell are resistant to magnolol, it is possible that the binding affinity of magnolol to EGFR is dependent upon the intracellular redox states. We believe that this model will provide a framework for future studies.

REFERENCES

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. Science 296: 1655–1657.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282:1318–1321.

Chen JS, Chen YL, Greenberg AS, Chen YJ, Wang SM. 2005a. Magnolol stimulates lipolysis in lipid-laden RAW 264.7 macrophages. Cell Biochem 94:1028–1037.

Chen YC, Chang MF, Chen Y, Wang SM. 2005b. Signaling pathways of magnolol-induced adrenal steroidogenesis. FEBS Lett 579:4337–4343.

Chott A, Sun Z, Morganstern D, Pan J, Li T, Susani M, Mosberger I, Upton MP, Bubley GJ, Balk SP. 1999. Tyrosine kinases expressed in vivo by human prostate cancer bone marrow metastases and loss of the type 1 insulin-like growth factor receptor. Am J Pathol 155:1271–1279.

Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, De Placido S, Bianco AR, Tortora G. 2001. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth tyrosine kinase inhibitor. Clin Cancer Res 7:1459–1465.

Costantini P, Jacotot E, Decaudin D, Kroemer G. 2000. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 92:1042–1053.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. 1997. Akt phosphorylation of Bad couples survival signals to the cell-intrinsic death machinery. Cell 91:231–241.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: A play in three Akts. Genes Dev 13:2905–2927.

Gibbs JB. 2000. Anticancer drugs targets: Growth factors and growth factors signaling. J Clin Invest 105:9–13.

Glenney JR. 1992. Tyrosine-phosphorylated proteins: Mediators of signal transduction from the tyrosine kinases. Biochim Biophys Acta 1134:113–127.

Green DR, Reed JC. 1998. Mitochondria and apoptosis. Science 281:1309–1312.

Gross A, McDonnell JM, Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899–1911.

Hill MM, Hemmings BA. 2002. Inhibition of protein kinase B/Akt implications for cancer therapy. Pharmacol Ther 93:243–251.

Huang SM, Li J, Harari PM. 2002. Molecular inhibition of angiogenesis and metastatic potential in human squamous cell carcinomas after epidermal growth factor receptor blockade. Mol Cancer Ther 1:507–514.

Huang SH, Shen WJ, Yeo HL, Wang SM. 2004. Signaling pathway of magnolol-stimulated lipolysis in sterol ester-loaded 3T3-L1 preadipocytes. J Cell Biochem 91:1021–1029.

Huang SH, Chen Y, Tung PY, Wu JC, Chen KH, Wu JM, Wang SM. 2007. Mechanisms for the magnolol-induced cell death of CGTH W-2 thyroid carcinoma cells. J Cell Biochem 101:1011–1022.

Ikeda K, Nagase H. 2002. Magnolol has the ability to induce apoptosis in tumor cells. Biol Pharm Bull 25:1546–1549.

Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. 2005. Cancer stastistics. CA Cancer J Clin 55:10–30.

Jin YC, Kim KJ, Kim YM, Ha YM, Kim HJ, Yun UJ, Bae KH, Kim YS, Kang SS, Seo HG, Lee JH, Chang KC. 2008. Anti-apoptotic effect of magnolol in myocardial ischemia and reperfusion injury requires extracellular signal-regulated kinase1/2 pathways in rat in vivo. Exp Biol Med 233:1280–1288.

Lee YJ, Song JJ, Kim JH, Kim HR, Song YK. 2004. Low extracellular pH augments TRAIL-induced apoptotic death through the mitochondria-mediated caspase signal transduction pathway. Exp Cell 293:129–143.

Lee DH, Kim C, Zhang L, Lee YJ. 2008a. Role of p53, PUMA, and Bax in wogonin-induced apoptosis in human cancer cells. Biochem Pharmacol 75:2020–2033.

Lee SJ, Cho YH, Park K, Kim EJ, Jung KH, Park SS, Kim WJ, Moon SK. 2008b. Magnolol elicits activation of the extracellular signal-regulated kinase pathway by inducing p27KIP1-mediated G2/M-phase cell cycle arrest in human urinary bladder cancer 5637 cells. Biochem Pharmacol 75:2289–2300.

Li TSC. 2002. Chinese and related North American herbs: Phytopharmacology and therapeutic values. Boca Raton, FL: CRC Press. p. 5.

Li HB, Yi X, Gao JM, Ying XX, Guan HQ, Li JC. 2007. Magnolol-induced H460 cells death via autophagy but not apoptosis. Arch Pharm Res 30:1566–1574.

Lin SY, Chang YT, Liu JD, Yu CH, Ho YS, Lee YH, Lee WS. 2001. Molecular mechanisms of apoptosis induced by magnolol in colon and liver cancer cells. Mol Carcinog 32:73–83.

Lin SY, Lau JD, Chang HC, Yeh SD, Lin CH, Lee WS. 2002. Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis. J Cell Biochem 84:532–544.

Nagase H, Ikeda K, Sakai Y. 2001. Inhibitory effect of magnolol and honokiol from Magnolia obovata on human fibrosarcoma HT-1080 invasiveness in vitro. Planta Med 67:705–708.

Nakanishi K, Sakamoto M, Yamasaki S, Todo S, Hirohashi S. 2005. Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis in hepatocellular carcinoma. Cancer 103:307–312.

Nicholson K, Anderson N. 2002. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 14:381–395.

Oberley LW, Buettner GR. 1979. Role of superoxide dismutase in cancer: A review. Cancer Res 39:1141–1149.

Ou HC, Chou FP, Sheu WH, Hsu SL, Lee WJ. 2007. Protective effects of magnolol against oxidized LDL-induced apoptosis in endothelial cells. Arch Toxicol 81:421–432.

Saeki K, Kobayashi N, Inazawa Y, Zhang H, Nishitoh H, Ichijo H, Saeki K, Isemura M, You A. 2002. Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): A distinct pathway from those of chemically induced and receptor-mediated apoptosis. Biochem J 368:705–720.

Salomon DS, Brandt R, Ciardielllo F, Normanno N. 1995. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 19:183–232.

Teng CM, Chen CC, Ko FN, Lee LG, Huang TF, Chen YP, Hsu HY. 1988. Two anti-platelet agents from Magnolia officinalis. Thromb Res 50:757–765.

Wang SM, Lee LJ, Huang YT, Chen JJ, Chen YL. 2000. Magnolol stimulates steroidogenesis in rat adrenal cells. Br J Pharmacol 131:1172–1178.

Woodburn JR. 1999. The epidermal growth factor receptor and its inhibition in cancer therapy. Pharmacol Ther 82:241–250.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. 1995. Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces Bax and promotes cell death. Cell 80:285–291.

Yang SE, Hsieh MT, Tsai TH, Hsu SL. 2003. Effector mechanism of magnolol-induced apoptosis in human squamous carcinoma CH27 cells. Br J Pharmacol 138:193–201.

Yarden Y, Ullrich A. 1988. Growth factor receptor tyrosine kinases. Annu Rev Biochem 57:443–478.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist Bad in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619–628.

Zhong WB, Wang CY, Ho KJ, Lu FJ, Chang TC, Lee WS. 2003. Magnolol induces apoptosis in human leukemia cells via cytochrome c release and caspase activation. Anticancer Drugs 14:211–217.