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Role of Bax in quercetin-induced apoptosis in human prostate cancer cells

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ABSTRACT

The aim of this study was to investigate the effect of quercetin, a flavonoid, on the apoptotic pathway in a human prostate cell line (LNCaP). We observed that treatment of cells for 24 h with quercetin-induced cell death in a dose-dependent manner. A sustained inhibition of the major survival signal, Akt, occurred in quercetin-treated cells. Treatment of LNCaP cells with an apoptosis inducing concentration of quercetin (100 μM) resulted in a rapid decrease in the inhibitory Ser(^{473}) phosphorylation of Akt leading to inhibition of its kinase activity. Quercetin treatment (100 μM) also caused a decrease in Ser(^{136}) phosphorylation of Bad, which is a downstream target of Akt. Protein interaction assay revealed that during treatment with quercetin, Bcl-xL dissociated from Bax and then associated with Bad. Our results also show that quercetin decreases the Bcl-xL:Bax ratio and increases translocation and multimerization of Bax to the mitochondrial membrane. The translocation is accompanied by cytochrome *c* release, and procaspases-3, -8 and -9 cleavage and increased poly(ADP-ribose) polymerase (PARP) cleavage. Similar results were observed in human colon cancer HCT116Bax$^{+/+}$ cell line, but not HCT116Bax$^{-/-}$ cell line. Interestingly, at similar concentrations (100 μM), quercetin treatment did not affect the viability or rate of apoptosis in normal human prostate epithelial cell line (PrEC) and rat prostate epithelial cell line (YPEN-1). Our results indicate that the apoptotic processes caused by quercetin are mediated by the dissociation of Bax from Bcl-xL and the activation of caspase families in human prostate cancer cells.

1. Introduction

Prostate cancer has become the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among men in the United States [1,2]. Since prostate cancer has a long latency often requiring more than 10 years to develop into a symptomatic and clinically significant disease, if we can block the multi-step process involving progression from small, low-grade lesions to large, high-grade and metastatic carcinomas, we can reduce the incidence of advanced prostate cancer. One such approach is the development of chemoprevention agents. In this study, we will focus on one such promising agent, quercetin, which is a constituent of many fruits and vegetables (e.g., onions and apples).

The natural product quercetin (3,5,7,3′,4′-pentahydroxy-flavone), which is orally bioavailable, is a flavonoid found in

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; PI3K, phosphatidylinositol-3 kinase; TNF, tumor necrosis factor.

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many fruits and vegetables [3]. Through epidemiological studies and preliminary data, quercetin has been found to inhibit the onset/growth of prostate cancer. It was noted that there is a 27% risk reduction for prostate cancer for those who consume at least 26 mg of quercetin a day [4]. Quercetin and its metabolites are potent antioxidants, which have oxygen radical scavenging properties and inhibit xanthine oxidase and lipid peroxidation *in vitro* [5–7]. Previous research has also shown that quercetin has anti-tumor, anti-inflammatory, anti-allergic, and anti-viral activities [8–12]. Our previous studies showed that quercetin enhances TRAIL-induced apoptosis in human prostate cancer DU145 cells via Akt dephosphorylation [13]. However, knowledge of the molecular mechanisms of quercetin-induced apoptosis was rudimentary and remained to be delineated. This study was undertaken to evaluate the induction of apoptosis caused by treatment with quercetin of LNCaP, a human prostate cancer cell line, and the probable apoptotic molecular mechanisms.

Apoptosis, which is also known as programmed cell death, plays a crucial role in the maintenance of cell homeostasis. Apoptosis may occur via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. In the extrinsic pathway, tumor necrosis factor (TNF) family (Fas/APO-1 ligand, TNF, TRAIL) proteins bind to the death receptors and these interactions result in conformational changes that expose a binding surface for Fas-associated death domain (FADD), an adaptor protein [14,15]. The apoptosis initiator procaspase-8 is recruited through the adaptor FADD [16]. Caspase-8 can directly activate downstream effector caspases including procaspase-3, -6, and -7 [17]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome *c* release [18]. Cytochrome *c* in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9 [19]. Caspase-9 cleaves and activates procaspase-3 [20]. Previous studies have shown that mitochondria play a central role in cell death in response to DNA damage, and mediate the interaction(s) of various cytoplasmic organelles, including the endoplasmic reticulum, Golgi apparatus, and lysosomes. The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins, a group of antiapoptotic and proapoptotic proteins that regulate the passage of small molecules, such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor, which activates caspase cascades, through the mitochondrial transition pore. The activation of caspases is counteracted by anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-xL), because these Bcl-2 family proteins heterodimerize with pro-apoptotic members of the Bcl-2 family (Bax, Bak) and interfere with release of cytochrome *c* by pore-forming proteins (Bid, Bik) [21]. Accordingly, we turned our attention to the proapoptotic Bcl-2 family member Bad, a key downstream effector of Akt [22], and related Bcl-2 family members. It has been demonstrated that in the absence of activated Akt, Bad forms heterodimers with Bcl-xL, an antiapoptotic protein that prevents the release of cytochrome *c* from mitochondria [21,23,24]. This complex formation abrogates the antiapoptotic function of Bcl-xL [25–26], thereby facilitating apoptotic death via a cytochrome *c*-dependent pathway. Conversely, when Akt is activated, Bad becomes phosphorylated and translocated into the cytoplasm through binding with the phosphoserine-binding protein 14-3-3 [27,28]. The sequestration of Bad from mitochondria frees Bcl-xL to facilitate antiapoptotic signaling. As a consequence, the dynamic interaction between Bcl-xL and Bad represents a critical determinant of cell fate downstream of the phosphatidylinositol-3 kinase (PI3K)/Akt cascade, and may represent an alternative mechanism for cancer cells to evade apoptosis.

In this study, we have shown that quercetin inhibits the PI3K/Akt pathway, suppresses phosphorylation of Bad, and subsequently alters interaction between Bcl-xL and Bax in human prostate carcinoma LNCaP cells. Our results suggest that an increase in the pro-apoptotic activity of Bax by dissociation from Bcl-xL leads to cytochrome *c* release, activation of caspases and consequently apoptotic death.

2. Materials and methods

2.1. Reagents and antibodies

Quercetin (>99% pure) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Galangin and chrysin were purchased from Calbiochem (San Diego, CA, USA). Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-caspase-8, anti-phospho-Ser^{473} Akt, anti-Akt, anti-phospho Bad-Ser^{136}, anti-Bad, anti-Bax, and anti-Bcl-xL antibodies were from Cell Signaling (Beverly, MA, USA). Monoclonal antibodies were purchased from each of following companies: anti-caspase-9 antibody from Upstate Biotechnology (Lake Placid, NY, USA), anti-PARP antibody from Biomol Research Laboratory (Plymouth Meeting, PA, USA), anti-cytochrome *c* from PharMingen (San Diego, CA, USA) and anti-actin antibody from ICN (Costa Mesa, CA, USA).

2.2. Cell lines

Human prostate carcinoma LNCaP cells obtained from ATCC (Manassas, VA, USA) were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 26 mM sodium bicarbonate for monolayer cell culture.

Primary cultures of normal human prostate epithelial cells (PrEC) were purchased from Cambrex Bio Science Walkersville (Cambrex Corporation; East Rutherford, NJ, USA), cultivated in PrEBM™ (Prostate Epithelial Cell Basal Medium; Cambrex) and supplemented with PrEGM SingleQuots® (bovine pituitary extract, hydrocortisone, hEGF, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin; Cambrex). Cells were maintained in accordance with manufacturer's instructions (Clonetics™ Prostate Epithelial Cell System). Rat prostate endothelial cells (YPEN-1) were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine at 233.6 mg/ml, penicillin–streptomycin at 72 mg/ml, and amphotericin B at 0.25 mg/ml, and were adjusted to pH 7.4–7.6 with $NaHCO_3$ in an atmosphere of 5% CO_2. The human colon cancer cell line HCT116 and its Bax-deficient derivative, Bax Knock Out cells (Bax KO), were kindly provided by Dr. Lin Zhang (University of Pittsburgh, Pittsburgh, PA, USA). These cell lines were cultured

in McCoy's 5A medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum and antibiotics. The dishes containing cells were kept in a 37 °C humidified incubator with 5% CO_2.

2.3. Treatment of cells

Quercetin, galangin, and chrysin (dissolved in DMSO) were used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies, LNCaP cells and PrEC cells were treated with quercetin at 10, 30, 50, 100 μM final concentrations for various times in complete cell medium. HCT116 (containing $Bax^{+/+}$) and HCT116 (containing $Bax^{-/-}$) cells were treated with quercetin at 100 μM final concentration for 24 h in complete cell medium. Control cells were treated with vehicle alone. A stock solution was prepared in DMSO.

2.4. Measurement of cell viability

One or 2 days prior to the experiment, cells were plated into 60-mm dishes at a density of 1×10^5 cells/plate in 5 ml 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 solution (PBS). 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.

2.5. Annexin V binding

Phosphatidylserine externalization, a marker of early apoptotic events, was detected by binding of FITC conjugated Annexin V, whereas counterstaining with propidium iodide (PI) allowed for the detection of cells with permeable cell membrane. Cells were treated with quercetin and harvested by trypsinization, washed with serum-free medium, and suspended in PBS at the density 1×10^6 cells/ml. Aliquots of 1×10^6 cells were suspended in binding buffer (500 μl, Annexin V-FITC Staining Kit, PharMingen). This cell suspension (100 μl) was stained with mouse anti-human Annexin V antibody (mIgG type, 5 μl) and PI (500 μg/ml, 10 μl) for 15 min in the dark. The immunostaining was terminated by addition of binding buffer and cells were immediately analyzed by flow cytometry. The negative control included staining with mIgG only. Typically, 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for Annexin V and PI, respectively.

2.6. Bax oligomerization

To detect the formation of Bax multimeric complexes, aliquots of isolated mitochondrial fractions and cytosolic fractions were cross-linked with 1 mM dithiobis (succinimidyl propionate) (Pierce, Rockford, Illinois, USA) at 37 °C for 30 min. DMSO alone was used as the control. The cross-linked samples were then centrifuged at 13,000 rpm for 15 min at 4 °C. After the supernatant was removed, the pellet was washed once with homogenization buffer (sucrose 0.25 M, HEPES pH 7.4 10 mM, EGTA 1 mM) and lysed with 2 × native sample buffer (125 mM Tris–HCl, pH 6.8, 40% glycerol, 0.02% bromophenol blue). Samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions followed by immunoblotting for Bax.

2.7. Measurement of cytochrome c release

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

2.8. Immunoblot analysis

Cells were lysed with 1 × 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, USA). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli [29] using a Hoefer gel apparatus. 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, USA). Quantitation of X-ray film was carried out by scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) using area integration. To ensure equal protein loading, each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

2.9. Interaction between Bcl-xL and Bad or Bax

To examine the interaction between Bcl-xL and Bad/Bax, LNCaP cells were treated with vehicle or 100 μM quercetin 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 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 12,000 × *g* for 15 min and the supernatant was collected. Bax, 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. Immunoprecipitates were washed with lysis

buffer and subsequently subjected to SDS-PAGE on 12% gels followed by immunoblotting using anti-Bax, Bad, and Bcl-xL antibodies.

2.10. Immuocytochemistry of Bax

For immunocytochemical analysis, 1×10^5 cells were plated on glass slide 1 day before experiment. Cells were treated with 100 μM quercetin 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 permablized with 0.05% Triton-X, washed and blocked with 2% BSA in PBS for 45 min to eliminate non-specific binding of secondary Ab. Cells were treated with polyclonal rabbit anti-human Bax (1:100 in 0.5% BSA; Santa Cruz Biotechnology) and incubated 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 in a medium with 4′, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) in order to trace cell nuclei. Cells were visualized in 0.4-μm sections using an inverted Olympus Fluoview 1000 laser scaning confocal microscope under an Olympus 60× oil immersion objective. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

2.11. Statistical analysis

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

3. Results

3.1. Quercetin induces cytotoxicity in human prostate cancer LNCaP cells

To investigate the effect of quercetin on viability, human prostate cancer LNCaP cells were treated with quercetin. The effects of quercetin on the viability of LNCaP cells were studied using trypan blue exclusion assay. As shown in Fig. 1A and B, our data clearly show that quercetin treatment resulted in a dose and time-dependent decrease in the viability of LNCaP cells. Unlike quercetin, DMSO caused little or no cytotoxicity. Fig. 1C shows morphological changes such as cell surface blebbing and formation of apoptotic bodies during treatment with quercetin.

3.2. Quercetin-induced apoptosis is mediated through activation of caspases in LNCaP cells

Activation of both extrinsic and intrinsic caspase pathways has been well established to be the major mechanism of apoptotic cell death in most cellular systems [30]. First of all, to examine whether quercetin-induced cell death is associated with apoptosis, human prostate cancer LNCaP cells were treated with 100 μM quercetin for 24 h and then analyzed with flow cytometric assay. Data from cytometric assay show that quercetin induced apoptosis (panel c in Fig. 2A). Based on our findings showing that quercetin causes apoptotic death of LNCaP cells, we assessed whether quercetin activates caspase pathways. As poly(ADP-ribose) polymerase (PARP) cleavage is a hallmark of caspase activation, we investigated whether the apoptosis machinery was activated by quercetin treatment, using an anti-PARP antibody. Treatment of cells with

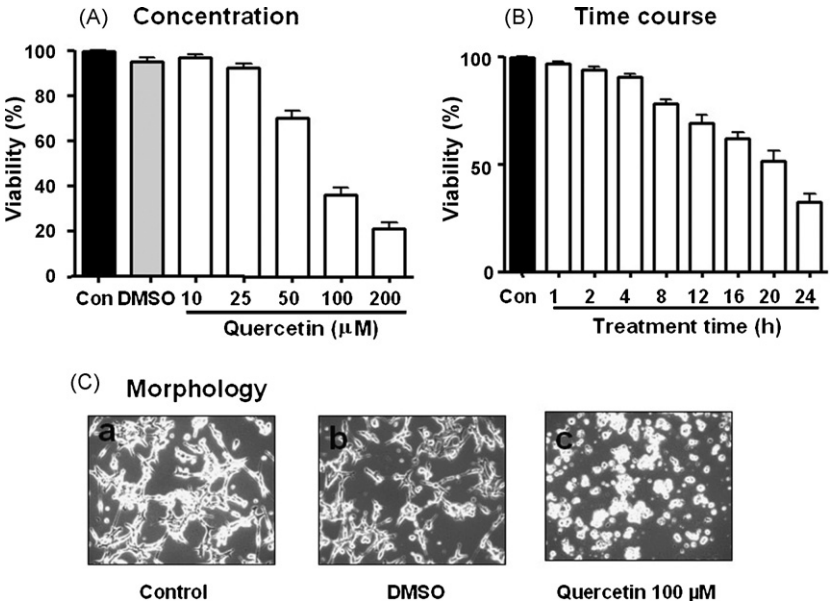

Fig. 1 – Quercetin induces cytotoxicity in human prostate cancer LNCaP cells. (A) Cells were treated with various concentrations (10–200 μM) of quercetin for 24 h. (B) Cells were treated with 100 μM quercetin for various times (1–24 h). The cytotoxic effect of quercetin on LNCaP cells was determined using the trypan blue dye exclusion assay as described in Section 2. Error bars represent standard error of the mean (S.E.M.) from three separate experiments. (C) Phase contrast photograph of LNCaP cells untreated control (a), or treated with 0.1% DMSO (b) or 100 μM quercetin (c) for 24 h.

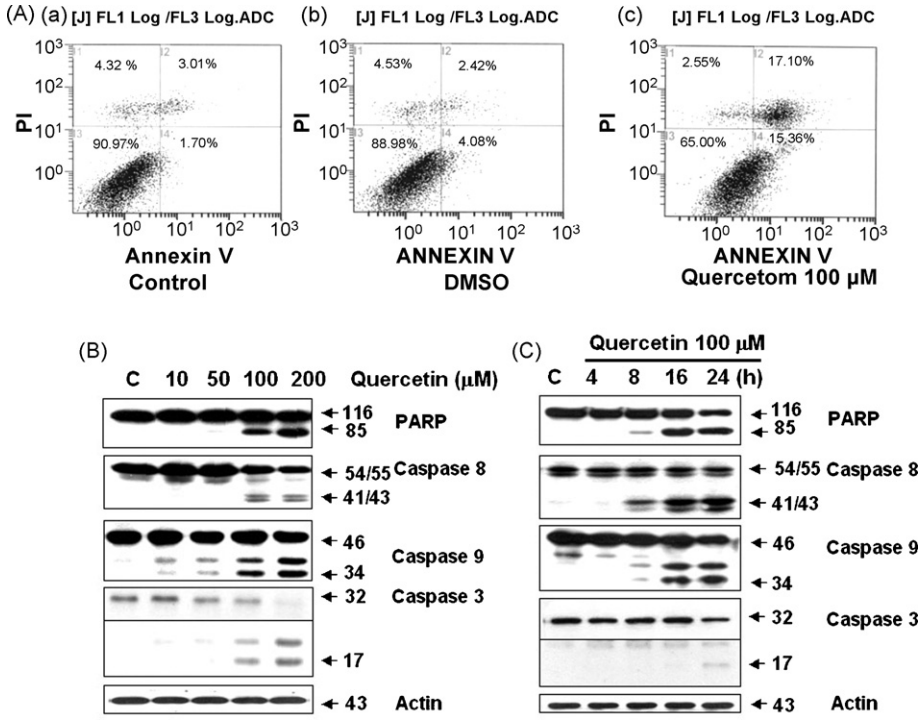

Fig. 2 – Quercetin induces apoptotic death in LNCaP cells. (A) Cells were treated with either 0.1% DMSO or 100 μM quercetin for 24 h. (a–c) After treatment, cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI). Apoptosis was detected by the flow cytometric assay. Cells were treated with various concentrations of quercetin for 24 h (B) or 100 μM quercetin for various times (C). Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. Actin was used to confirm the equal amount of proteins loaded in each lane.

quercetin did result in caspase-8 activation (cleavage); also, it caused a strong dose- and time-dependent cleavage of caspase-9, caspase-3, and PARP (Fig. 2B and C). Because we observed prominent apoptotic cell death and its associated cleavage of caspases and PARP at 100 μM quercetin dose at 24 h of treatment, we selected this quercetin dose level to study the time kinetics of molecular events involved in quercetin-induced apoptosis. As shown in Fig. 2C, quercetin treatment of LNCaP cells at this dose resulted in caspase-9 and its downstream caspase-3 cleavage as early as 8 h with an increase at later time points, although prominent effect was evidenced at 24 h. We further examined whether low concentrations of quercetin also induce cytotoxicity when cells are treated with quercetin for more than 1 day. Fig. 3 clearly shows that cytotoxicity was observed by treatment with a physiologically relevant concentration (10 μM) of quercetin for 2 days. As mentioned previously, PARP cleavage suggests that apoptotic death occurred by treatment with 10 μM quercetin.

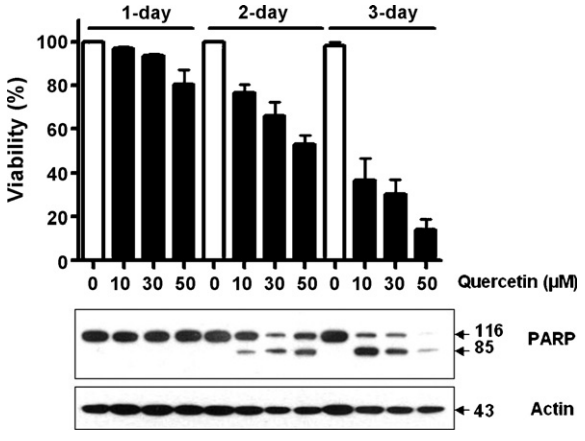

Fig. 3 – Low concentrations of quercetin also induce cytotoxicity. LNCaP cells were treated with various concentrations (10–50 μM) for various times (1–3 days). The cytotoxic effect of quercetin on LNCaP cells was determined as described in Fig. 1. Immunoblot analysis was performed as described in Fig. 2.

3.3. *Quercetin inhibits Akt activity*

Phosphorylation (activation) of Akt is associated with protection of cells from apoptosis [31]. We postulated that quercetin inhibits Akt activity and consequently leads to apoptosis. In our previous research, to analyze whether inhibition of Akt phosphorylation is related to quercetin-induced apoptosis, we measured total and phosphorylated levels of this protein treated with ascending amounts of quercetin, and reported that concentrations of quercetin above 200 μM inhibited Akt by decreasing the level of phosphorylated active Akt [13]. In this current study, we treated LNCaP cells with 100 μM quercetin for various times. We observed that Akt was dephosphorylated within 1 h of quercetin treatment without

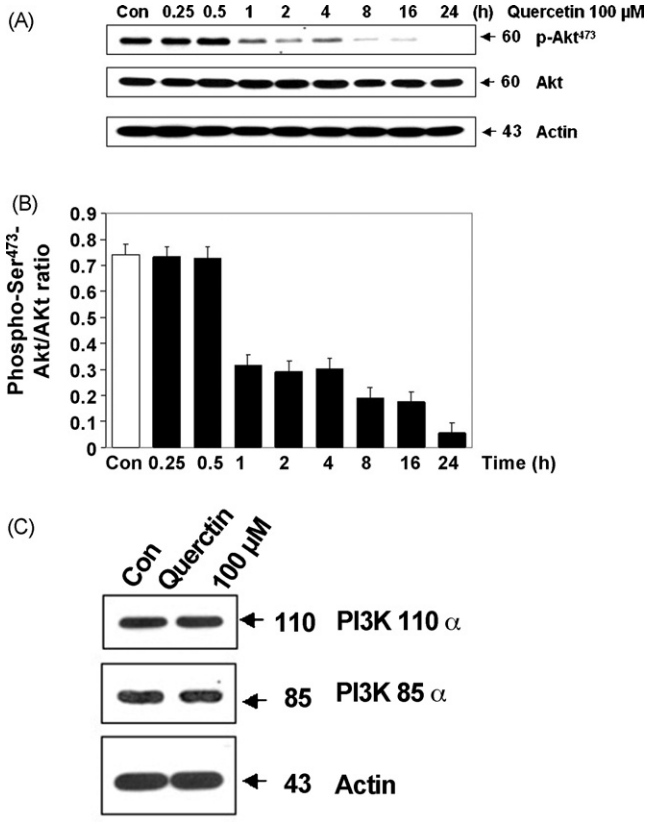

Fig. 4 – Effect of quercetin on the levels of phospho-Akt, Akt, and PI3K proteins in LNCaP cells. (A) Cells were treated with 100 μM quercetin for various times (0.25–24 h). Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt (S^{473}), or anti-Akt antibody. Actin was used to confirm the equal amount of proteins loaded in each lane. (B) Quantitative measurement of Akt dephosphorylation. The immunoblots in Fig. 4A and other experiments were analyzed with a densitometer. The ratio of the intensity of phosphor-Akt to the intensity of Akt is plotted as a function of treatment times. (C) Cells were treated with 100 μM quercetin for 24 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PI3K (110α) or anti-PI3K (85α) antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

changing the intracellular Akt level in LNCaP cells (Fig. 4A). To quantify the level of dephosphorylation during treatment with 100 μM quercetin for various times (0.25–24 h), the immunoblots were analyzed with a densitometer. Separate experiments were normalized by taking the ratios of the intensity of the relative amounts of the phosphorylated Akt to that of Akt (Fig. 4B). The level of phosphorylated Akt was decreased as a function of treatment time. It is well known that the PI-3-kinase (PI3K) pathway is regulated by a variety of growth factors, and the activation of the PI3K-Akt signaling pathway is strongly implicated in the regulation and survival or protection of cells [31]. Thus, we also examined the effect of quercetin on the levels of the p85 regulatory subunit and the p110 catalytic subunit of PI-3-kinase. The expression of both subunits was not altered by quercetin treatment (Fig. 4C). Our results indicate that apoptosis by quercetin is coupled with PI3K-Akt pathway activity without changes in the level of PI3K.

3.4. *Quercetin alters interaction between Bcl-xL and Bad/Bax and leads to cytochrome c release*

The proapoptotic BH3-only Bcl-2 family member Bad plays an important role in regulating apoptosis in cells [32]. 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 [27]. Phosphorylation of Bad occurs on a MAPK-dependent site, serine 112 [33], and also on serine 136, a site phosphorylated directly by Akt [34]. Phosphorylation of either serine 112 or serine 136 is sufficient to render Bad inactive [27]. We therefore examined whether treatment with quercetin leads to the dephosphorylation of Bad at serine 136, which would promote apoptosis. Levels of phosphorylated Bad were examined after 8 h quercetin treatment, at which time Bad phosphorylation was reduced (Fig. 5A). Quercetin treatment of prostate cancer cells caused dephosphorylation of Bad at serine 136 residues (Fig. 5A). Therefore, it is possible that activation of Bad may play an important role in mediating quercetin-induced apoptosis in prostate cancer cells. To test this possibility, we examined how quercetin-induced inacti-

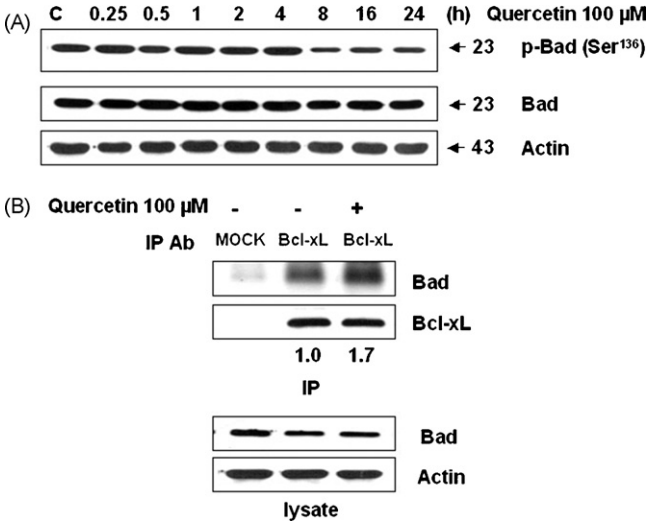

Fig. 5 – Dephosphorylation of Bad and dissociation of Bcl-xL from Bad after quercetin treatment in LNCaP cells. (A) Cells were treated for various times with 100 μM quercetin, and then harvested. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-phospho-Bad (S^{136}) or anti-Bad antibody. Actin was used to confirm the equal amount of proteins loaded in each lane. (B) Cells were treated with quercetin (100 μM) for 24 h. Cell lysates were immunoprecipitated with anti-Bad antibody or mock antibody (IgG) and then immunoblotted with anti-Bcl-xL or anti-Bad antibody (upper panels). The presence of Bad in the lysates was verified by immunoblotting (lower panel).

vation of Akt leads to caspase activation and consequently results in apoptotic death. The first task investigated was whether dephosphorylation of Bad alters interaction between Bad and Bcl-xL during treatment with quercetin. The interaction between Bad and Bcl-xL was examined by immunoprecipitation with anti-Bcl-xL antibody. Fig. 5B shows an increase in interaction between Bad and Bcl-xL during treatment with quercetin; densitometric analysis illustrates that quercetin increased the Bcl-xL:Bad ratio to 1.7-fold. These results suggest that dephosphorylated Bad dissociates from 14-3-3 and interacts with pro-survival Bcl-2 family members, such as Bcl-xL. Association of Bad with Bcl-xL may result in dissociation of Bax from Bcl-xL. Indeed, Fig. 6A shows that Bax dissociated from Bcl-xL during treatment with quercetin; densitometric analysis illustrates that quercetin decreased the Bcl-xL:Bax ratio to 0.4-fold. We also observed that the apoptosis induced by quercetin is dependent upon the presence of Bax. After treatment with quercetin, Bax multimerized in the mitochondria (Fig. 6B). We used actin for a cytosolic marker and COX IV for a mitochondrial marker as fractional markers and loading controls. Previous studies reported that the pro-apoptotic molecule Bax when dissociated from Bcl-xL may activate the Bax-dependent mitochondrial apoptotic pathway through its oligomerization [35]. The oligomerized Bax integrates in the outer mitochondria membrane, where it triggers cytochrome c release [36]. Oligomerization of Bax on mitochondria was indeed detected

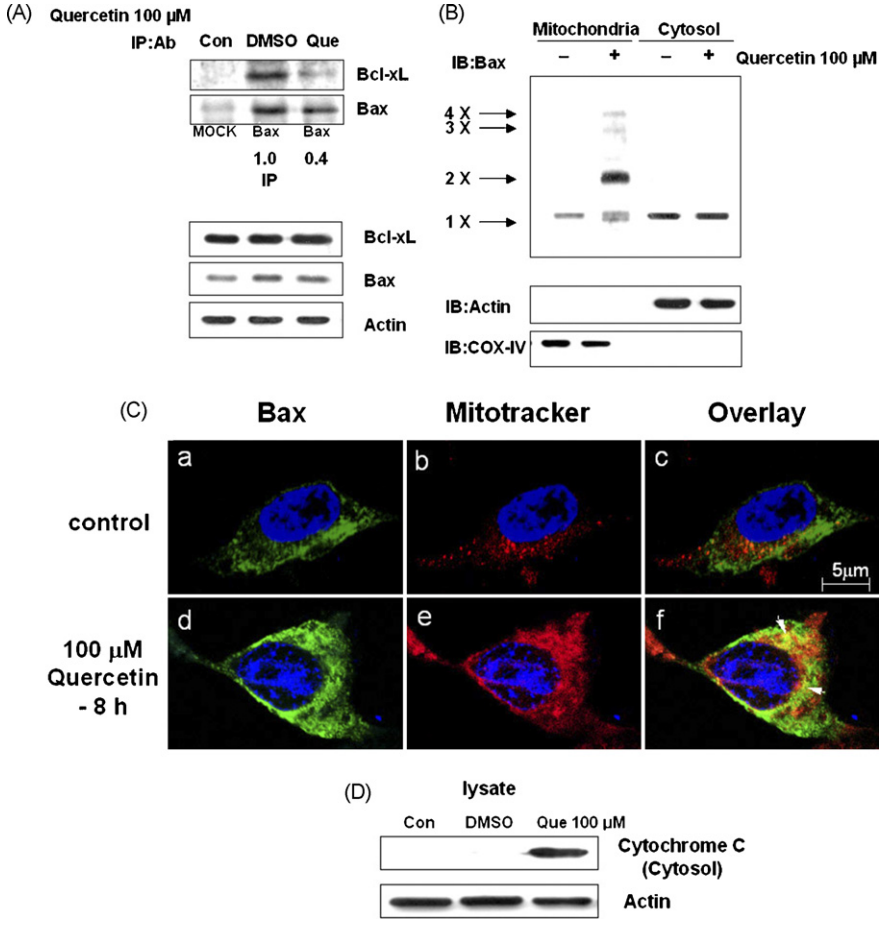

Fig. 6 – Quercetin treatment increases Bax oligomerization and cytochrome c release from mitochondria to the cytosol in LNCaP cells. Cells were treated with quercetin (100 μM) for 24 h. (A) Cell lysates were immunoprecipitated with anti-Bax antibody or mock antibody (IgG) and immunoblotted with anti-Bcl-xL or anti-Bax antibody (upper panels). The presence of Bcl-xL and Bax in the lysates was verified by immunoblotting (lower panels). (B) Mitochondrial and cytosolic fractions were obtained from cell lysates. Each subcellular fraction was cross-linked with 1 mM dithiobis (succinimidyl propionate) and subjected to immunoblotting with anti-Bax antibody. Bax monomer (1×) and multimers (2×, 3×, and 4×) are indicated. We used actin as a cytosolic marker and COX IV as a mitochondrial marker. (C) Localization of Bax after quercetin treatment was examined by confocal microscope. Cells were untreated (panels a–c) or treated (panels d–f) with 100 μM quercetin for 8 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 overlay of panels (a) and (b). Panels (f) is overlay of panels (d) and (e). Co-localization of Bax and mitochondria is shown as yellow (arrows). (D) Cytochrome c (15 kDa) release into cytosol was determined by immunoblotting for cytochrome c in the cytosolic fraction. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-cytochrome c antibody. Actin was used to confirm the equal amount of proteins loaded in each lane. DMSO (0.1%) and untreated control cells were used as sham and control group, respectively.

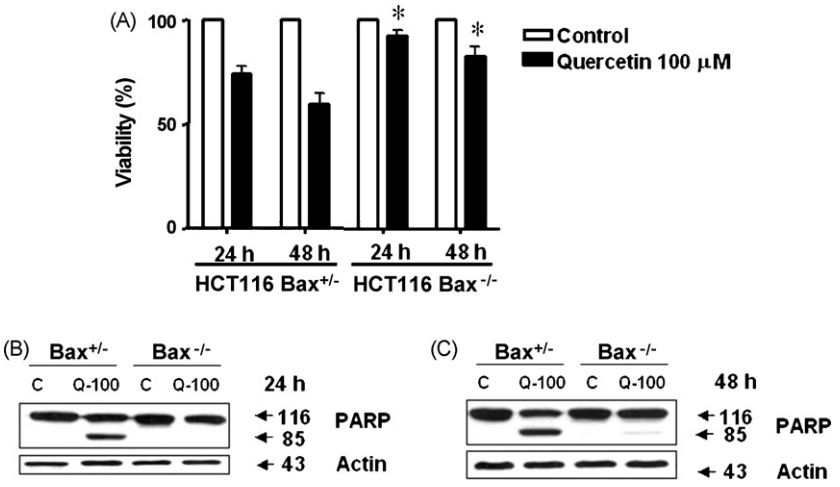

Fig. 7 – Role of Bax in quercetin-induced apoptosis in HCT 116 cells. HCT 116 ($Bax^{+/-}$) cells and HCT 116 ($Bax^{-/-}$) cells were treated with 100 μM quercetin for 24 or 48 h. (A) Cell viability was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (S.E.M.) from three separate experiments. Asterisk (*) represents statistically significantly difference between HCT 116 ($Bax^{+/-}$) cells and HCT 116 ($Bax^{-/-}$) cells at $p < 0.05$. (B and C) Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

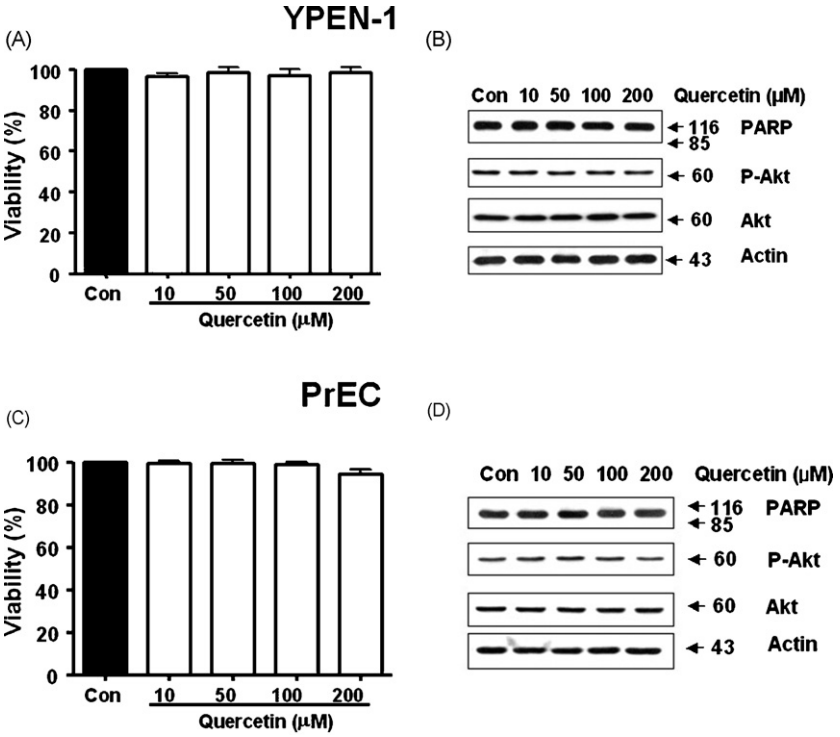

Fig. 8 – Effect of quercetin on cell viability, PARP and Akt activity in rat endothelial cells (YPEN-1) and human prostate epithelial cells (PrEC). PrEC and YPEN-1 cells were treated with various concentrations (10–200 μM) of quercetin for 24 h. (A and C) Cell viability was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (S.E.M.) from three separate experiments. (B and D) Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP, anti-phospho-Akt (S^{473}), or anti-Akt antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

by confocal microscopy (Fig. 6C). Fig. 6D clearly shows that cytochrome *c* release occurred during treatment with quercetin.

3.5. Role of Bax in quercetin-induced apoptosis in HCT116 human colon cancer cells

Bax and Bak are two important multidomain proapoptotic proteins essential for the mitochondrial release of cytochrome c. 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 examine the importance of Bax in quercetin-induced cell death we used HCT116Bax$^{+/-}$ and HCT116Bax$^{-/-}$ cells to compare the apoptotic death induced by quercetin. These cells were treated with 100 μM of quercetin for 24 and 48 h, 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 quercetin than HCT116Bax$^{-/-}$ cells (Fig. 7A). Also, we observed that PARP cleavage occurred in Bax$^{+/-}$ cells, but not in Bax$^{-/-}$ cells, during treatment with 100 μM quercetin (Fig. 7B and C). These results confirm that Bax plays an important role in quercetin-induced apoptotic death.

3.6. Quercetin neither induces cytotoxicity nor inhibits Akt in normal prostate cells

We further examined whether quercetin also induces cytotoxicity and inhibits Akt in normal human prostate epithelial PrEC and normal rat prostate endothelial YPEN-1 cells. Cells were exposed to various concentrations (10–200 μM) of quercetin for 24 h. While our studies show that quercetin treatment for 24 h caused dramatic cell mortality of LNCaP cells with an estimated 50% of cell death (IC_{50}) value of 75 μM (Fig. 1A), quercetin treatment did not affect the viability and did not lead to apoptosis in both normal cell lines (Fig. 8). We also observed that quercetin did not alter the phosphorylation of Akt in both normal cell lines (Fig. 8).

3.7. Quercetin analogues also induce apoptosis and inhibit Akt activity

As the biological activities of chemicals are dependent on their individual structure, we have investigated effects of quercetin, galangin and chrysin, three structurally related flavonoids, on cell viability. Additional studies were designed to examine whether quercetin analogues such as galangin and chrysin induce apoptotic death and inactivate (dephosphorylate) Akt. Similar to quercetin, galangin and chrysin induced cytotoxicity in LNCaP cells (Fig. 9A). PARP (116 kDa) was cleaved yielding a characteristic 85-kDa fragment during treatment with galangin or chrysin. Akt was also inactivated in the presence of galangin or chrysin (Fig. 9B).

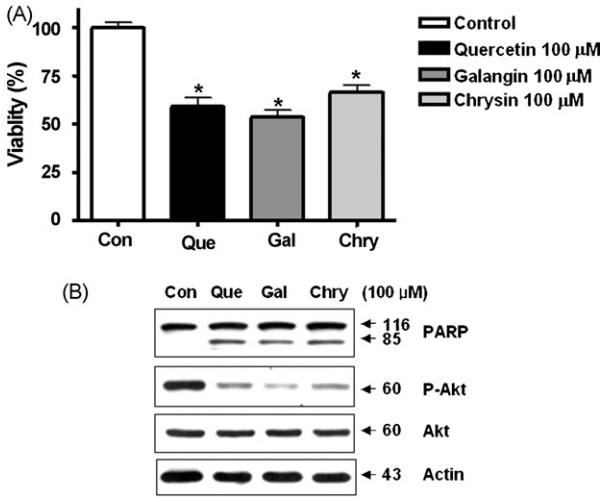

Fig. 9 – Comparison between quercetin and its analogues of cell viability and Akt activity in LNCaP cells. Cells were treated with 100 μM quercetin, galangin, or chrysin for 24 h. (A) Cell viability was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean (S.E.M.) from three separate experiments. Asterisk (*) represents statistically significantly difference between control and drug-treated cells at $p < 0.05$. (B) Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-PARP, anti-phospho-Akt (S^{473}), or anti-Akt antibody. Actin was used to confirm the equal amount of proteins loaded in each lane.

4. Discussion

Epidemiological studies have demonstrated the relation between diet and cancer and the potential of dietary components, in particular, polyphenols, as antiproliferative agents [3,37–39]. Chemoprevention can be achieved through prevention in carcinogenesis, inhibition or delay of cancer progression, or suppression of cancer recurrence. We believe that induction of apoptosis and inhibition of proliferation could be an important preventive approach. In this context, a continuous daily intake of polyphenols like quercetin might result in steady-state plasmatic concentrations, even to levels that might provoke an apoptotic cell death (10–50 μM), which has been observed after the intake of dietary supplements [40]. Our study showed that quercetin modulates apoptosis induction in LNCaP and HCT116 cells; that is, it stimulates proteolytic activities of caspase-3 and -9, alters the interaction between some of the Bcl-2 family of proteins, and dephosphorylates Akt and Bad.

Quercetin concentrations that significantly decrease LNCaP cell viability have similar effects on other cancer cell cultures, such as hepatoma [41], human colon [42], lung [43], leukemia [44], breast [45], and murine hepatoma [46]. These observations suggest that quercetin can be used as an anticancer agent.

Our studies clearly demonstrate that Bax is dissociated from Bcl-xL during treatment with quercetin (Fig. 6A). Our data clearly reveal that dissociated Bax forms oligomers (Fig. 6B). An increase in Bax oligomerization leads to mitochondria-mediated caspase activation (Fig. 6D): mitochondria dysfunction promoted by Bax translation leads to the leakage of cytochrome c from mitochondria [36]; cytochrome c binds to Apaf-1, which then permits recruitment of procaspase-9;

caspase-9 cleaves and activates executioner caspases including procaspase-3. Obviously, we need to clarify if and how Bax leads to caspase activation in the near future. Several researchers have reported the activation of the mitochondrial pathway, and therefore caspase-9, by quercetin in leukemia [47] and colon cancer cells [42]. In colon cancer cells, caspase-9 was also cleaved by other polyphenols such as resveratrol [48].

Akt promotes cell survival by inhibiting apoptosis, and its phosphorylation has been considered a critical factor in the aggressiveness of cancer. Quercetin induced inactivation of Akt by decreasing the level of phosphorylated Akt in a concentration-dependent manner, contributing to the promotion of apoptosis. Although the precise anti-apoptotic effects of Akt are still unclear, Akt directly phosphorylates and inactives procaspase-9 and blocks caspase-9-mediated apoptosis [49]. Alternatively, inhibition of Akt promotes phosphorylation of the proapoptotic Bad, which favors the apoptotic process [50].

In contrast to the marked effects of quercetin on Akt phosphorylation, quercetin did not affect PI3K protein levels. This observation has been previously demonstrated in studies of human breast [45], lung [43] and hepatoma [41] cancer cells. Quercetin and other flavonoids are reported to be PI3K inhibitors, reducing enzymatic PI3K activity without changing either p85 or p110 subunit levels [51–52], which agrees with the lack of effect of quercetin on PI3K levels observed in our study (Fig. 4C). Therefore, an inhibition of PI3K activity by quercetin might be taking place, which might explain the observed decrease of Akt phosphorylation, its downstream target. PI3K activity will be investigated further in future studies.

As our results have shown in Figs. 5 and 6, interaction between Bcl-xL and Bad and interaction between Bcl-xL and Bax could be regulated by quercetin differently, suggesting that the balance in the interaction of these proteins might be involved in the control of the apoptotic process. Although quercetin increased the Bad:Bcl-xL ratio, quercetin decreased the Bax:Bcl-xL ratio; thus, regulation of Bcl-xL protein levels seems to be, at least in part, caspase-dependent in LNCaP cells, which agrees with previous results in human hepatoma treated with quercetin [41]. Quercetin also increased Bax translocation from the cytosol to the mitochondrial membrane, an event that promotes apoptotic death [53]. This accords with the increased levels of total Bax found in other studies of fetal hepatocytes [53] as well as in studies of human lung [43], prostate [54], and liver [55] cancer cells.

Conceivably, enhanced Bcl-xL expression in advanced prostate cancer cells may antagonize the sensitizing effect of PI3K/Akt inhibition to apoptosis. Thus, modulation of the interaction of Bax, Bcl-xL, and Bad as demonstrated by the mechanism of quercetin-induced apoptosis may represent an important strategy to optimize the efficacy of chemotherapeutic agents; this modulation is currently under investigation in this laboratory.

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