Plant flavonoid apigenin inactivates Akt to trigger apoptosis in human prostate cancer: an in vitro and in vivo study

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Abbreviations: DMSO, dimethyl sulfoxide; GSK3, glycogen synthase kinase-3; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PARP, poly-ADP-ribose-polymerase; PDK, phosphoinositide-dependent kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a negative regulator of the PI3K–Akt-signaling pathway (5). The N-terminal pleckstrin homology domain in the N-terminal region of Akt interacts with PIP3 at the plasma membrane resulting in the recruitment of Akt to the plasma membrane. Recruitment to the membrane results in a conformation change in Akt that exposes two crucial amino acids: threonine 308 (Thr308) in the kinase domain and serine 473 (Ser473) in the hydrophobic motif domain of Akt1. These amino acids need to be phosphorylated for the activation of Akt (6). Thr308 is phosphorylated by constitutively active phosphoinositide-dependent kinase (PDK1), which is also recruited to the plasma membrane by PIP3s through its N-terminal pleckstrin homology domain, thereby stabilizing the activation loop, whereas phosphorylation of the Ser473 in the hydrophobic C-terminal domain by PDK2 is necessary for full activation because it stabilizes the active conformation state. Several different potential PDK2s have been proposed, including Akt itself, the mammalian target of rapamycin rictor complex, integrin-linked kinase 1, protein kinase CβII and the members of the atypical PI3K-related protein kinase family (3–6).

Once activated, Akt transduces signals from growth factors and oncogenes to downstream targets that control tumor-associated crucial cellular processes including cell growth, cell cycle progression, survival, migration, tissue invasion and angiogenesis (7,8). Aberrant activation of the Akt pathway and hence these tumor-associated pathways have been detected in a number of human malignancies (9). A number of studies have demonstrated PKB/Akt gene amplifications in human cancers such as PKBβ/Ark1 amplification in gastric carcinoma (10) and PKBβ/Akt2 amplification in ovarian, pancreatic, gastric and breast tumors (11,12). Messenger RNA overexpression and selective activation of PKBβ/Akt3 protein by growth factors has also been documented in hormone-independent breast and prostate cancer cell lines (13–15). Furthermore, ectopic expression of constitutively activated Akt and even wild-type Akt2 results in oncogenic transformation of cells and tumor formation in transgenic mice (16,17). Akt activation has been shown to promote tumor invasion and prostate cancer progression in autochthonous transgenic adenocarcinoma of the mouse prostate (18). Frequent deregulation of the PI3K–Akt pathway in cancer has prompted significant interest in blocking this pathway to prevent and/or treat cancer (19–21). Specific inhibition of the activation of Akt by small molecules may be a valid approach to prevent and/or treat human malignancies.

Apigenin (4’, 5, 7-trihydroxyflavone), a naturally occurring plant flavone that is abundantly present in common fruits and vegetables, has been shown to possess cancer preventive and therapeutic properties (22–26). It has low toxicity, is non-mutagenic and has shown selective effects in inhibiting cell growth and inducing apoptosis in cancer cells without affecting normal cells (27). Our laboratory has conducted extensive research to investigate the mechanisms underlying the anticarcinogenic effects of apigenin in several human prostate cancer cell lines and in the transgenic adenocarcinoma of the mouse prostate model (28–30). We previously demonstrated that apigenin impairs cell cycle machinery through modulation of mitogen-activated protein kinase and Akt and loss of cyclin D1 associated with dephosphorylation of the retinoblastoma (31). However, the mechanisms of apigenin-induced decreased cell survival in human prostate cancer cells have not been fully elucidated. We undertook studies of...
human prostate cancer PC-3 cells as well as studies of prostate cancer xenografts in athymic nude mice to investigate the effects of apigenin on Akt inactivation. Our findings provide experimental evidence indicating that apigenin-induced decreased cell survival and apoptosis in PC-3 cells are mediated by inactivation of Akt, leading to BAD dephosphorylation and activation of caspase-9.

Materials and methods

Cell lines and treatments

Androgen-refractory human prostate cancer PC-3 and DU145 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in RPMI 1640 supplemented with 5% fetal bovine serum and 1% penicillin–streptomycin. Monolayer cultures of PC-3 and DU145 cells were maintained at 37°C and 5% CO₂ in a humid environment. At 60% confluence, PC-3 cells were treated either with 40 μM of apigenin (Sigma, St. Louis, MO, Cat#A13415) for various time intervals or with different concentration of apigenin for 24 h. The cells were treated with varying concentrations of apigenin dissolved in dimethyl sulfoxide (DMSO), which was provided to the control group within permissible concentrations.

Proliferation assay

The effect of apigenin on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay. Briefly, the PC-3 and DU145 cells were plated at 1 × 10⁴ cells per well in 96-well microtiter plates and allowed to attach overnight. After 16–18 h, cells were treated with different concentration of apigenin or DMSO (control). To analyze the effect of caspase inhibitors, cells were treated with 20 μM of caspase-9 inhibitor, Z-LEHD-FMK (R&D, Minneapolis, MN, Cat#FMK008) and general caspase inhibitor, Z-VAD-FMK (Calbiochem, Calbiochem/EMD Biochemicals, Gibbstown, NJ, Cat#627610) for 2 h followed by 40 μM of apigenin treatment. Each treatment was repeated in at least six wells. Cell viability was determined after incubating the treated cells for 24 h at 37°C in a humidified incubator. Working MTT solution (1 mg/ml) was prepared in complete media from the stock solution of 5 mg MTT dissolved per ml of phosphate-buffered saline (pH 7.4). Following 24 h of treatment, 50 μl of working MTT solution was added to each well and incubated for 2 h, after which the plate was centrifuged at 2800 rpm for 5 min at 4°C. The MTT solution was carefully removed from the wells by aspiration followed by the addition of 0.1 ml DMSO. The plates were shaken for 10 min in dark and the absorbance was read on a microplate reader at the wavelength of 540 nm.

Immunoprecipitation

Total cell lysate (200 μg) from control- and apigenin-treated PC-3 cells were blocked with 40 μl Protein A/G PLUS–agarose (Santa Cruz Biotechnology, Santa Cruz, CA, Cat#SC-2003) beads for 2 h at 4°C followed by centrifugation at 13 000 r.p.m. for 10 min. Supernatants were incubated with 5 μl antibody 14-3-3-β antibody overnight at 4°C. Thirty microliter of Protein A/G–agarose beads was then added to each sample, and the incubation was continued for an additional 2 h at 4°C followed by centrifugation at 13 000 r.p.m. for 10 min. Supernatant was aspirated from each sample and the immunoprecipitates were washed four times with lysis buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting with anti-BAD or anti-14-3-3-β antibody.

Immunoblotting

The cell lysate was cleared by centrifugation at 13 000 r.p.m. for 15 min at 4°C, and the protein concentration was measured in the supernatant by Bio-Rad protein assay using the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). A total of 20–40 μg of supernatant proteins was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4–20% Tris–glycine gel and then transferred onto the nitrocellulose membrane either for 2 h or overnight. The blots were blocked using 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 and probed using primary antibodies followed by secondary antibodies. Primary antibodies against pAkt (Ser473) (Cat#9275), BAD (Cat#2922), p-BAD Ser136 (Cat#9295) caspase-3 (Cat#9662), caspase-9 (Cat#5502), GSK-3β (Cat#9332), p-GSK-3βSer21/9 (Cat#9331), IGF-IRs (Cat#3022), p-IGF-IR Tyr1131 (Insulin Receptor, Tyr1136, Cat#3021), cleaved PARPAsp214 (Cat#9545) from Cell Signaling Technology (Beverly, MA) and Akt1/2 (Cat#8312) and p-Akt Ser473 (Cat#8795-R) from Santa Cruz, CA in blocking buffer either at room temperature for 1 h or overnight at 4°C. The membrane was then incubated with appropriate secondary antibody and the immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham Life Sciences, GE Healthcare, Pittsburgh, PA). For equal protein loading, the membrane was stripped and reprobed with anti-β-actin (Calbiochem, Cat#P01) or anti-α-tubulin antibodies (Santa Cruz, Cat#SC-8035). Bands were determined with densitometric analysis and the fold change was calculated as the protein level of apigenin-treated cells relative to the control-treated cells and thereafter normalizing the resulting protein levels with control β-actin or α-tubulin.

Transient transfection

Briefly, DU145 cells were plated in 100 mm plates and allowed to attach overnight. Approximately 60% confluent cells were transiently transfected with 8 μg of either pLNCX vector containing Akt expression plasmid (kindly provided by Dr William Sellers, Harvard Medical School, Boston, MA) or empty vector using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA). For MTT assay, DU145 cells were plated at the density of 1 × 10⁴ cells per well of 96-well microtiter plates, allowed to attach overnight and received 0.13 μg of Akt-expressing construct or empty vector per well. After 5 h, the medium was replaced with fresh complete medium, and the cells were incubated overnight at 37°C in a humidified incubator. Cells were treated with different concentrations of apigenin or DMSO (control) after 16–18 h of transfection and incubated at 37°C for additional 24 h. Then the cells were either processed for cell survival assay or immunoblotting as described before.

M-30 apoptosis by enzyme-linked immunosorbent assay

Apoptosis was assessed by M30-Apoptose™ ELISA kit (Alexis Biochemicals, San Diego, CA) according to the manufacturer’s protocol and color development was read at 450 nm against the blank and values were plotted against standards provided and expressed as units per liter.

Tumor xenograft studies

PC-3 tumors were grown subcutaneously in athymic nude mice. Approximately 1 million PC-3 cells suspended in 0.05 ml of medium and mixed with 0.05 ml of Matrigel were subcutaneously injected into the left and right flank of each mouse to initiate tumor growth. The animals were equally divided into three groups. The first group received only 0.2 ml of vehicle material by gavage daily and served as a control group. The second and third groups of animals received 20 and 50 μg/mouse/day doses of apigenin in vehicle, respectively, for 8 weeks. These doses are comparable with the daily consumption of flavonoid in humans as reported in previously published studies (29,30). Apigenin intake was started 2 weeks after cell inoculation and was continued for 8 weeks. Animals were monitored daily, and their body weights were recorded weekly throughout the studies. Once the tumors started growing, their sizes were measured twice weekly in two dimensions with calipers. At the termination of the experiment, tumors were excised and weighed to record wet tumor weight. A portion of the tumors from control and treated animals was used for preparation of tumor lysate used in further experiments.

Statistical analysis

Changes in tumor volume and body weight during the course of the experiments were visualized by scatter plot. Differences in tumor volume (cubic millimeters) at the endpoint and body weight were determined for each treatment group among the three treatment groups. The first group received only 0.2 ml of vehicle material by gavage daily and served as a control group. The second and third groups of animals received 20 and 50 μg/mouse/day doses of apigenin in vehicle, respectively, for 8 weeks. These doses are comparable with the daily consumption of flavonoid in humans as reported in previously published studies (29,30). Apigenin intake was started 2 weeks after cell inoculation and was continued for 8 weeks. Animals were monitored daily, and their body weights were recorded weekly throughout the studies. Once the tumors started growing, their sizes were measured twice weekly in two dimensions with calipers. At the termination of the experiment, tumors were excised and weighed to record wet tumor weight. A portion of the tumors from control and treated animals was used for preparation of tumor lysate used in further experiments.

Results

Apigenin inhibits Akt Ser473 phosphorylation and downstream targets

Our previous studies have indicated that treatment of PC-3 cells with 40 μM of apigenin decreased Ser473 phosphorylation of Akt significantly in a time-dependent manner (31). We reproduced these data and found that 40 μM of apigenin inhibited Ser473 phosphorylation drastically after 3 h of treatment (Figure 1). Phosphorylation of Ser473 and Thr380 activates Akt to phosphorylate its targets through its kinase activity to promote survival and inhibit apoptosis (4,6). We further continued the study to examine whether downstream targets of Akt are also affected by the decreased phosphorylation of Akt. We observed that phosphorylation of glycogen synthase kinase-3 (GSK3β), which is a well-recognized direct target of Akt (32), was dramatically decreased upon apigenin treatment confirming the inhibition of Akt kinase activity by apigenin treatment (Figure 1). Apigenin inhibited the Akt Ser473 phosphorylation in a dose-dependent manner with prominent effect at 20 μM concentration for 24 h (Figure 2). Phosphorylation of Akt at Ser473 was reduced by 20, 59 and 62% upon 10, 20 and 40 μM apigenin treatment, respectively. Consistently, phosphorylation of BAD and GSK3β was decreased significantly as
a result of decreased phosphorylation and kinase activity of Akt. BAD phosphorylation was diminished by 42% at 10 μM, 51% at 20 μM and 55% at 40 μM apigenin treatment, whereas phosphorylation of GSK3β was reduced by 13, 40 and 47% upon 10, 20 and 40 μM apigenin treatment, respectively. Likewise, exposure to apigenin decreased GSK3β protein expression; however, the level of BAD was found to be elevated even after 5 μM apigenin treatment to PC-3 cells (Figure 2).

**Fig. 1.** Immunoblotting for phospho-Ser(473)-Akt, Akt, p-GSK-3β and α-Tubulin for the indicated time periods. The blots were stripped and reprobed with anti-α-tubulin antibody to normalize the protein loading. Bands were quantitated by densitometric analysis. Fold change represents the protein level of the apigenin-treated cells relative to the control cells treated with vehicle and the resulting protein levels were then normalized to the α-tubulin protein.

**Fig. 2.** Immunoblotting for phospho-Ser(473)-Akt, Akt, phospho-BAD, BAD, p-GSK-3β and GSK-3β using lysates from PC-3 cells treated with either vehicle (DMSO, lanes 1, 2, 4, 6 and 8) or 40 μM apigenin (lanes 3, 5, 7 and 9) for the indicated time periods. The blots were stripped and reprobed with anti-α-tubulin antibody to normalize the protein loading. Bands were quantitated by densitometric analysis. Fold change represents the protein level of the apigenin-treated cells relative to the control cells treated with vehicle and the resulting protein levels were then normalized to the α-tubulin protein.

**Apigenin causes downregulation of IGF-IR protein and its phosphorylation**

To explore whether phosphorylation of Akt by apigenin treatment was impaired as a result of upstream events, we examined the effect of apigenin on insulin like growth factor receptor (IGF-IR) expression and its autophosphorylation. Receptor tyrosine kinases such as insulin-like growth factor receptor or other growth factor receptors are auto- phosphorylated and induced upon ligand binding and activate the PI3K-signaling pathway that recruits Akt to the plasma membrane, resulting in its phosphorylation and activation (4,33). In our studies, apigenin reduced the IGF-IR protein expression level by 20, 35, 53 and 52% upon 5, 10, 20 and 40 μM apigenin treatment, respectively. The IGF-IR phosphorylation was downregulated by 10% at 5 μM, 38% at 10 μM, 66% at 20 μM and 74% at 40 μM apigenin treatment (Figure 3A).

**Apigenin exposure reduced interaction between BAD and 14-3-3β**

Phosphorylation of BAD is a key regulator in promoting either cell survival or apoptosis. In its dephosphorylated state, BAD is trans- located to the outer mitochondrial membrane, where it binds to anti-apoptotic Bcl-2 family proteins such as Bcl-2 or Bcl-XL, and releases proapoptotic protein Bax to perform its apoptotic function (34). However, growth factor-mediated phosphorylation of BAD promotes its binding to 14-3-3β protein, which results in the sequestration of protein complex to the cytosol, leading to cell survival (35,36). To ascertain the effect of apigenin on the interaction of BAD and 14-3-3β proteins, PC-3 cells were treated with 20 μM apigenin for 24 h, cell lysates were prepared and immunoprecipitation was performed using antibody against 14-3-3β protein. Immunoprecipitated complexes were then subjected to immunoblotting using anti-BAD antibody (Figure 3B). Same blot was reprobed for 14-3-3β (lower panel) using immunoprecipitates from PC-3 cell lysate proteins (200 μl) from control- and apigenin-treated cells were used to ascertain whether apigenin increased cell death through apoptosis.

**Apigenin decreased viability of human prostate cancer cells by inducing apoptosis**

As shown in Figure 4A, survival of PC-3 cells was reduced significantly after treatment with 20 and 40 μM apigenin for 24 h. Since the viability of cancer cells was decreased upon apigenin exposure, to ascertain whether apigenin increased cell death through apoptosis,
activation of caspases was analyzed. Caspases are present as inactive zymogens. During apoptosis, upon receipt of a death stimulus, initiator caspases (caspase-9 and caspase-8) are activated and cleaved and their products in turn activate and cleave effector caspases (caspase-3 and caspase-7) (37). Treatment of PC-3 cells with increasing concentrations of apigenin elevated the protein expression of cleaved caspase-9 (Figure 4B, upper panel). Likewise, caspase-3 becomes activated, as evident by an increase in caspase-3 protein expression after apigenin treatment (Figure 4B, lower panel).

To confirm the role of caspases in cell death, the viability of PC-3 and DU145 cells was evaluated in the presence of apigenin and caspase inhibitors. As evident in Figure 4C, apigenin treatment to PC-3 cells reduced the cell survival; however, this effect was attenuated when apigenin was given in the presence of a caspase-9-specific inhibitor, zLEHD-fmk. A general caspase inhibitor, zVAD-fmk, impaired the effect of apigenin as well, although more effectively than zLEHD-fmk (Figure 4C). DU145 adenocarcinoma cells also exhibit responsiveness to apigenin as cell survival was reduced markedly upon 40 μM apigenin treatment for 24 h (Figure 4D). Both zLEHD-fmk and zVAD-fmk weakened the apigenin effect in the DU145 cell line, confirming the role of caspases in apigenin-induced cell death (Figure 4D).

Overexpression of Akt in DU145 cells confers resistance to apigenin treatment

DU145 cells were transfected either with empty vector (Figure 5A, lanes 1–2) or vector containing Akt expression plasmid (Figure 5A, lanes 3–4). It is apparent from Figure 5A, that phosphorylation of Akt is ~4-fold higher after transfecting Akt-expressing plasmid in DU145 cells as compared with transfection with empty vector (Figure 5A, compare lane 3 with lane 1). As observed in PC-3 cells, 20 μM apigenin treatment for 24 h reduced the Akt Ser473 phosphorylation markedly in DU145 cells transfected with Akt expression plasmid (Figure 5A, compare lane 4 with lane 3). However, overexpression of Akt in DU145 cells confers resistance against apigenin-induced cell death (Figure 5B). Apigenin exposure reduced the survival of DU145 cells transfected with empty vector, whereas Akt-transfected DU145 cells remain unaffected upon apigenin exposure (Figure 5B). To further support these data, cleavage of poly-ADP-ribose-polymerase (PARP) was detected in DU145 cells transfected with empty vector or Akt-expressing vector upon apigenin treatment (Figure 5C). The 85 kDa fragment resulting from PARP cleavage by active caspase-3 is considered to be a hallmark of apoptosis (38). The content of 85 kDa-cleaved PARP was found to be increased drastically after exposing DU145 cells transfected with empty vector to increasing concentration of apigenin (Figure 5C, lanes 2–4) as compared with control-treated cells (Figure 5C, lane 1); however, Akt-overexpressing DU145 cells were unresponsive to increasing concentration of apigenin treatment (Figure 5C, lanes 6–8). These data are consistent with the cell viability data (Figure 5B), which showed that survival of Akt-overexpressing DU145 cells was unaffected after apigenin treatment and confirmed that cell death was reduced due to lack of apoptosis (Figure 5C).

Apigenin intake inhibits growth of PC-3 xenografts in athymic nude mice

Apigenin has been shown to be effective in cell culture, inactivating Akt Ser473 phosphorylation and downstream targets viz. GSK and BAD in PC-3 cells; therefore, we extended our study to determine whether these events occur in vivo using a xenograft mouse model. We designed a protocol that simulates a therapy regimen, wherein apigenin was provided at 20 and 50 μg/mouse/day through gavage, beginning 2 weeks after cell inoculation and continuing for 8 weeks. In this experimental protocol, intake of apigenin inhibited the growth of tumor xenograft at both doses of apigenin. As shown in Figure 6A and B, tumor volume was inhibited by 32 and 51% (P < 0.005 and 0.0001) and the wet weight of tumor was decreased by 28 and 40% (P < 0.001), respectively, at the termination of the experiment. Furthermore, apigenin intake by these mice did not seem to induce any adverse effects as judged by monitoring body weight gain, dietary intake and prostate weight (data not shown).

Fig. 4. (A) Viability of PC-3 cells was assessed by MTT assay after treating the cells with 20 and 40 μM apigenin for 24 h as compared with the vehicle-treated cells. (B) PC-3 cells were exposed to vehicle alone (DMSO, lane 1) or 5, 10, 20 and 40 μM apigenin (lanes 2, 3, 4 and 5, respectively) for 24 h, cell lysates were prepared and immunoblotted for procaspase-9, cleaved caspase-9 and procaspase-3. Fold change was calculated as described in Figure 1. (C) Effect of caspase inhibitors zLEHD-fmk and zVAD-fmk was assessed on apigenin-induced cell death in PC-3 cells and (D) DU145 cells. Cells were treated with 20 μM caspase inhibitors for 2 h, followed by 40 μM apigenin treatment for 24 h. Viability of cells was determined by MTT assay. Data are mean ± SE and **P < 0.001, significantly different from untreated, treated cells.
Apigenin intake causes apoptosis in PC-3 tumors through inactivation of Akt-signaling pathway

Earlier results in cell culture demonstrated that apigenin induces decreased cell survival and apoptosis in PC-3 cells; therefore, we evaluated the effects of apigenin intake on the induction of apoptosis in tumor xenografts. As shown in Figure 6C, peroral intake of apigenin at doses of 20 and 50 μg/mouse/day resulted in a marked induction of apoptosis in PC-3 tumor xenografts as shown by M-30 reactivity measured by enzyme-linked immunosorbent assay. Compared with vehicle-treated control, 1.68- and 2.86-fold increases (P 0.0001) in the induction of apoptosis were observed in PC-3 tumors at 20 and 50 μg/day apigenin treatment. Furthermore, consistent with the findings in cell culture, apigenin administration to nude mice resulted in a dose-dependent decrease in the expression of p-Akt Ser473, p-GSK3β, p-BAD Ser136, with concomitant increases in cleaved caspase-9 and -3, compared with mice receiving vehicle treatment. These results suggest that Akt inactivation and dephosphorylation of BAD causes induction of apoptosis in PC-3 tumor xenografts (Figure 6D).

Discussion

The PI3K–Akt pathway has been shown to be activated in a wide range of human cancers (9). Upregulation of PI3K–Akt signaling through mutations in the PTEN gene and constitutive activation of growth factor receptors leads to evasion of apoptosis in tumor cells. Mutation and/or loss of function in the negative regulator PTEN has been observed in advanced stage human prostate cancer and in xenograft models (39,40). We have recently demonstrated the potential role of PI3K–Akt in prostate cancer progression in cell culture and in autochthonous transgenic adenocarcinoma of the mouse prostate (18). In nearly 50% of prostate cancers, the PI3K–Akt survival pathway has been shown to constitutively upregulated because of loss of function and/or mutation of tumor suppressor PTEN, which functions as a negative regulator of PI3K through its lipid phosphatase activity (5,39,40). Therefore, agents that inactivate the PI3K–Akt-signaling pathway are potentially effective in the prevention and/or treatment of prostate cancer. Our studies demonstrate that apigenin reproducibly inhibits Akt phosphorylation in a dose- and time-dependent manner in human prostate cancer cells.

Activation of cells by growth factors or cytokines leads to recruitment of PI3K to the plasma membrane, where it catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate in the D3 position to generate PIP3. The accumulation of PIP3 creates a docking site for Akt at the plasma membrane with conformational change in the Akt that exposes two crucial amino acids, threonine 308 in the kinase domain and serine 473 in the hydrophobic motif domain of Akt1, critical for cell survival (6–9). Our studies demonstrate that apigenin treatment causes a rapid decrease in activating phosphorylations of Akt at Ser473 and Thr308 in PC-3 cells. The apigenin-mediated suppression of Akt phosphorylation in PC-3 cells is coupled by inhibition of GSK-3β and BAD phosphorylation, essential downstream targets of the Akt-signaling pathway. Similar observations have been demonstrated where apigenin treatment to human prostate cancer PC-3M cells caused inhibition of hypoxia-inducible factor-1α and vascular endothelial growth factor via PI3K–Akt–GSK pathway (41). Furthermore, relative resistance of DU145 cells transfected with constitutively active Akt against apigenin-induced apoptosis compared with vector-transfected controls supports the concept that Akt inactivation contributes to cell death caused by apigenin. Additional studies are required in other tumor types to determine whether the association between apigenin-mediated Akt inactivation and cell death is only observable in prostate cancer.

Activated Akt can phosphorylate several apoptosis-regulating proteins, including the proapoptotic Bcl-2 family protein, BAD (3–6). BAD promotes cell death by interacting with anti-apoptotic Bcl-2 members such as Bcl-xL, which allows the multidomain proapoptotic Bcl-2 family members Bax and Bak to aggregate and cause release of apoptogenic molecules (e.g. cytochrome c) from mitochondria to the cytosol, culminating into caspase activation and cell death (42). BAD is phosphorylated at Ser136 by Akt and also at Ser112 and Ser155 by other kinases, reducing its ability to interact with Bcl-xL (43). Phosphorylation of BAD promotes its interaction with 14-3-3 proteins, which sequesters BAD in the cytosol (44). Our study indicates that apigenin-mediated inactivation of Akt in prostate cancer cells is...
associated with reduced phosphorylation of BAD at Ser136. We also observed that apigenin treatment reduces interaction between BAD and 14-3-3 that impairs cell survival and triggers apoptosis in prostate cancer cells.

The phosphorylation of caspase family member caspase-9 (Mch-6/ICE-LAP6) at Ser196 by Akt has been reported as another mechanism of Akt-mediated cell survival (45). Caspase-9 may activate caspase-3, initiating the apoptotic cascade that leads to the cleavage and inactivation of key cellular proteins such as PARP (38,46). In the present study, we have demonstrated the role of caspase-9 and -3 in apigenin-induced apoptosis in human prostate cancer PC-3 and DU145 cells. The apigenin-induced cleavage of caspase-9 and -3 and decreased cell survival are significantly blocked on pretreatment with both pan-caspase inhibitor and caspase-9-specific inhibitor.

Because apigenin-induced apoptosis is also significantly attenuated in the presence of zVAD-fmk and zLEHD-fmk, we postulate involvement of the intrinsic caspase cascade in cell death caused by apigenin.

Recent studies from our laboratory have indicated that apigenin-induced cell death is initiated by generation of reactive oxygen species (ROS) (47). This conclusion is based on the following observations: (i) apigenin exposure causes generation of ROS in both 22Rv1 and PC-3 cells; (ii) apigenin-mediated disruption of mitochondrial membrane potential; (iii) apigenin-induced activation of p53 and its phosphorylation is significantly blocked by antioxidant N-acetylcysteine, p53 inhibitor pifithrin-α and enzyme, catalase; (iv) p53 antisense oligonucleotide treatment offers significant protection against apigenin-induced cell death and (v) pan-caspase inhibitor, z-VAD-FMK and caspase-3 inhibitor, DEVD-CHO partially rescued...
the cells from apigenin-mediated apoptosis. Involvement of ROS in the cellular effects of apigenin has also been postulated in other cellular systems (48, 49). However, the precise mechanism by which apigenin causes formation of ROS remains to be elucidated.

In our cell culture studies, impairment in cell survival and apoptosis induction by apigenin were observed at 10–40 μM concentrations, which provided mechanistic insights; however, demonstration that these effects are also operative in vivo is required to establish a potential for clinical development. Our in vivo studies using 20 and 50 μg/day apigenin administration to mice with prostate cancer xenografts confirmed that apigenin intake significantly inhibited tumor growth, without any apparent signs of toxicity. Consistent with the findings in cell culture, apigenin intake resulted in inactivation of Akt through its reduced phosphorylation at Ser473 and its downstream targets p-BAD at Ser136 and p-GSK3β, increase in the levels of cleaved caspase-9 and caspase-3 favoring apoptosis, compared with vehicle-treated animals. Based on these observations, it is apparent that apigenin most probably exerts its potential cancer preventive/therapeutic effects directly through the PI3K–Akt-signaling pathway. The demonstration of downregulation of constitutive Akt kinase activity by apigenin provides a rationale to explore its role as a preventive and perhaps as a chemotherapeutic agent or in combination to overcome therapeutic resistance in the management of prostate cancer.

Funding
United States Public Health Services (RO1 CA108512, RO3 CA094248, RO3 CA099049) Cancer Research and Prevention Foundation to S.G. Athymic Animal Core Facility of the Comprehensive Cancer Center of Case Western Reserve University and University Hospitals Case Medical Center (P30 CA43703).

Acknowledgements
The authors are thankful to Dr Pingfu Fu for performing statistical evaluations for this study.

Conflict of Interest Statement: None declared.

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Received May 30, 2008; revised July 25, 2008; accepted August 18, 2008