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Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways

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Polyphenols from fruits and vegetables exhibit anticancer properties both *in vitro* and *in vivo* and specialty potatoes are an excellent source of dietary polyphenols, including phenolic acids and anthocyanins. This study investigated the effects of specialty potato phenolics and their fractions on LNCaP (androgen dependent) and PC-3 (androgen independent) prostate cancer cells. Phenolic extracts from four specialty potato cultivars CO112F2-2, PATX99P32-2, ATTX98462-3 and ATTX98491-3 and organic acid, phenolic acid and anthocyanin fractions (AF) were used in this study. CO112F2-2 cultivar extracts and their AF at 5 μg chlorogenic acid eq/ml were more active and inhibited cell proliferation and increased the cyclin-dependent kinase inhibitor p27 levels in both LNCaP and PC-3 cells. Potato extract and AF induced apoptosis in both the cells; however, the effects were cell context dependent. Cell death pathways induced by potato extract and AF were associated with mitogen-activated protein kinase and c-jun N-terminal kinase activation, and these kinases activated caspase-independent apoptosis through nuclear translocation of endonuclease G (Endo G) and apoptosis-inducing factor in both cell lines. Induction of caspase-dependent apoptosis was also kinase dependent but was observed only in LNCaP cells. Kinase inhibitors reversed this nuclear translocation of endonuclease G and apoptosis-inducing factor. This is the first report showing that the cytotoxic activities of potato extract/AF in cancer cells were due to activation of caspase-independent apoptosis. Current studies are focused on identifying individual components of the AF responsible for the induction of cell death pathways in prostate and other cancer cell lines and developing potato cultivars that overexpress these active compounds.

Introduction

Prostate cancer is the third leading cause of cancer deaths in men in the USA, and it is estimated that there will be 234 460 new cases and 27 350 additional deaths in 2006 due to prostate cancer (1). Epidemiological studies have shown that diets rich in fruits and vegetables have preventive effects on chronic diseases including prostate cancer (2–4). The preventive effects associated with vegetable consumption can be attributed in part to their content of antioxidant polyphenols because oxidative stress plays a role in tumor development (5,6). Chronic inflammation, uncontrolled proliferation of prostatic epithelial cells and inappropriate regulation of apoptosis may also play an important role in prostate cancer promotion and progression (7,8). Polyphenols also exhibit anti-inflamatory, antiproliferative and proapoptotic properties in addition to antioxidant activity, suggesting their role as chemopreventive agents (9,10).

A number of studies have examined the anticancer effects of polyphenols such as phenolic acids and anthocyanins. Caffeic acid phenyl ester suppressed proliferation of HO-1 (human melanoma cells) and GBM-18 (human glioblastoma) (11). Ferulic and chlorogenic acid (100 mg/kg) decrease lung tumors in A/J mice by 30–40% (12). The chemopreventive mechanisms of anthocyanins include scavenging free radicals, reducing cell proliferation, up-regulating/inducing apoptosis and modulating mitogen-activated protein kinase (MAPK) activities (13). Anthocyanins and their aglycones such as cyanidin, delphinidin, malvidin, pelargonidin and peonidin exhibit antiproliferative and proapoptotic properties in gastric adenocarcinoma (14), HT-29 and Caco-2 (colon cancer) (15) and bovine aortic endothelial cells (13), and also protect against esophageal cancer in rodents (16).

Specialty (colored) potato (*Solanum tuberosum* L.) contains significant levels of phenolic acids and anthocyanins (17,18) and there is high consumption of this vegetable, with ∼135 pounds per capita in the USA. Earlier studies (19–22) have focused on identification and quantification of polyphenols and their antioxidant properties in potatoes. Little information is available on the antiproliferative and proapoptotic properties of potato polyphenols against prostate cancer and the molecular mechanisms involved in this process.

In this study, we have investigated the effects of specialty potato extracts and their fractions on PC-3 (androgen independent) and LNCaP (androgen dependent) prostate cancer cells. Polyphenols from four specialty potato cultivars, CO112F2-2, PATX99P32-2, ATTX98462-3 and ATTX98491-3, were used. The CO112F2-2 extracts showed potential growth inhibition, and extracts were then fractionated into anthocyanin, phenolic acid fraction (PF) and organic acids. The anthocyanin fraction (AF) showed potent antiproliferative properties and increased levels of the cyclin-dependent kinase inhibitor p27 in both LNCaP and PC-3 cell lines. The AF induced apoptosis in both LNCaP and PC-3 cells; however, the effects were cell context dependent. In PC-3 cells, the AF induced caspase-independent apoptosis associated with nuclear translocation of endonuclease G (Endo G) and apoptosis-inducing factor (AIF), whereas both caspase-dependent and caspase-independent pathways were induced in LNCaP cells. These findings suggest that the cytotoxic effects involve induction of apoptosis rather than necrosis through caspase dependent and independent pathways. In addition, pretreatment of cells with c-jun N-terminal kinase and MAPK inhibitors blocked apoptotic pathways in both cell lines.

Abbreviations: AF, anthocyanin fraction; AIF, apoptosis-inducing factor; AR, androgen receptor; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; Endo G, endonuclease G; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetyl cysteine; PF, phenolic acid fraction; TUNEL, terminal deoxynucleotidyl transferase mediated dutp nick end labeling; WST, water-soluble tetrazolium; PARP, poly ADP ribose polymerase; ERK, extracellularly regulated kinase.

Materials and methods

Chemicals

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, Folin-Ciocalteu reagent, dimethyl sulfoxide (DMSO) and *N*-acetyl cysteine (NAC) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol, methanol and ethyl acetate were purchased from VWR International (Bristol, CT). Trolox and chlorogenic acid were obtained from Sigma (St Louis, MO), and malvidin was purchased from the Indofine Chemical Company (Hillsborough, NJ). The 4-(3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate [water-soluble tetrazolium (WST)-1], cell death detection ELISA kit and In situ cell death detection POD kit [Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL) assay] were obtained from Roche Applied Sciences (Indianapolis, IN). Antibodies for p27, cyclin D1, pc-jun, p-erk, c-jun, erk, glucose-related protein 78, AIF and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Endo G was from Prosci (Poway, CA).

Cleaved PARP, cleaved caspases 3, 9 and cytochrome C were obtained from Cell Signaling Technology (Beverly, MA). Western lightning™ chemiluminiscence reagents were from PerkinElmer Life Sciences (Boston, MA).

Selection and sample preparation and fractionation

Four different specialty potato selections (CO112F2-2P/P, ATTX98462-3RY/Y, PATX99P32-2RY/YR, ATTX98491-3YR/Y) were grown near McCook, TX. Tubers from each selection were harvested and transported at ambient temperature to Texas A&M University, College Station. Uniformly sized tubers without any defects were washed, diced into 0.5 cm cubes and freeze dried. These freeze-dried samples were stored at −20°C for further analysis.

Freeze-dried tuber samples (0.5 g) were homogenized with 15 ml of 85:15 ethanol:water using an Ultra-Turrax Tissumizer T25 (Cincinnati, OH) (30 000*g*) and stored for 12–15 h at −20°C. Samples collected after centrifugation were concentrated and diluted to 5 ml using 0.01% aqueous HCl (whole extract). The whole extract was passed through the C-18 Sep-Pak cartridges (Waters, Milford, MA) preconditioned with 0.01% acidified methanol to absorb phenolics and anthocyanins (23,24). Sugars and organic acids were eluted from the column with 0.01% aqueous HCl and collected as the organic acid fraction. Phenolics other than anthocyanins were collected by eluting the column with ethyl acetate (PF), and the AF was obtained by eluting the column with 0.01% methanolic HCl. The fractions were concentrated (under nitrogen gas) and reconstituted with either alcohol or DMSO.

Antioxidant activity

Antioxidant activity was estimated using the DPPH assay. DPPH is a stable free radical which reacts with oxidants and exhibits decreased absorbance at 515 nm (25). Aliquots of the column fractions (150 µl) were incubated with 2850 µl of diluted DPPH solution for 24 h at 25°C, and the absorbance was measured at 515 nm. The blank or negative control contained all the reaction reagents except the sample. Trolox (6-hydroxy-2, 3, 7, 8-tetramethylchroman-2-carboxylic acid) was used as a positive control. The results were expressed as microgram of Trolox equivalents per gram fresh weight (µg Teq/gfw).

Total phenolic content

The total phenolic content was measured spectrometrically using the Folin-Ciocalteu colorimetric method (26). The sample (150 µl) was diluted with 2.4 ml water and incubated with 150 µl of 0.25 N Folin-Ciocalteu reagent and 300 µl of 1 N Na_2CO_3 for 2 h with shaking at 25°C. The absorbance was recorded at 725 nm, and results were expressed as microgram of chlorogenic acid equivalents per gfw (µg CGA eq/gfw). The phenolic composition of the fractions was identified using high-performance liquid chromatography with authentic reference standards.

Cell lines

Human prostate carcinoma cell lines PC-3 (androgen independent) and LNCaP (androgen dependent) were obtained from the American Type Culture Collection (Manassas, VA), and cells were maintained at 37°C in 5% CO_2 jacketed incubator in RPMI 1640 (Sigma) supplemented with 2.38 g/l *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid 2.0 g/l sodium bicarbonate, 0.11 g/l sodium pyruvate, 4.5 g/l glucose, 100 ml/l fetal bovine serum and 10 ml/l antibiotic antimycotic solution (Sigma).

Cell proliferation assay

Cells were plated at a density of 2×10^4 per well in 96-well plates. After 24 h, media was replaced with Dulbecco's modified Eagle's medium F-12 media containing 2.5% charcoal-stripped serum, and ethanol extracts of four different specialty potato varieties were tested at different concentrations. Every 24 h, cell proliferation was measured using the water-soluble tetrazolium assay which required preincubation of cells in media for 4 h with the tetrazolium salt water-soluble tetrazolium-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (10 µl per well) followed by measuring absorbance at 450 nm. Percent cell proliferation was calculated based on control absorbance (100%). Results are expressed as means ± SEs for at least three separate experiments for each treatment group.

Cell proliferation was also determined using a Z1 Coulter Counter. Cells were plated at a density of 2.5×10^4 cells per well in 12-well plates, and after 24 h, Dulbecco's modified Eagle's medium F-12 media containing 2.5% charcoal-stripped fetal bovine serum was used and cells were treated with DMSO (solvent control) and different concentrations of the whole potato extracts or fractions. Fresh media along with compounds was added every 48 h. Cells were counted after 24, 48 and 72 h using a Coulter Counter. Each experiment was carried out in triplicate, and results were expressed as means ± SEs.

Fluorescence-activated cell sorting analysis

LNCap and PC-3 cells were plated at a density of 15×10^5 cells per 100 mm plate, and after 24 h treatment with either the vehicle (DMSO) or whole potato extracts (5 or 10 µg/ml), cells were trypsinized and centrifuged. The pellet was resuspended with 1 ml of PI (Propidium Iodide) staining buffer containing 4 mM sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide and 200 µg/ml RNAse and incubated for 10 min at 37°C in the dark, and the final concentration of sodium chloride was adjusted to 0.15 M. Cells were analyzed using FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and CellQuest (Becton Dickinson Immunocytometry Systems) acquisition software. Results were reported as percent cells in each phase of the cell cycle.

Apoptosis

DNA fragmentation was measured using an ELISA assay (Cell Death Detection ELISA; Roche Diagnostics) in both LNCaP and PC-3 cell lines. Cells were seeded in a 48-well plate at a density of 2.5×10^4 cells per well. Cells were incubated for 24 h with whole potato extract or fractions, then scraped and pelleted by centrifugation. Cells were then lysed and diluted to 1×10^4 cells per ml with incubation buffer, and the color development was measured according to the manufacturer's manual. The supernatant from the pelleted cells was used to measure cytotoxicity using an lactate dehydrogenase cytotoxicity detection kit.

TUNEL assay

Cells (4×10^4) were seeded in four-chambered glass slides, and after treatment for 12 h, the in situ cell death detection POD (horse-radish peroxidase) kit was used for the TUNEL assay according to the instruction manual protocol for fixed cells. After the incubation of cells with POD and diaminobenzidene substrate, apoptotic DNA fragmentation was detected by visualizing labeled DNA using a light microscope. Slides incubated without TdT (terminal deoxynucleotidyl transferase) served as a negative control, and slides treated with 1000 U DNAse I/ml for 10 min before TdT exposure served as a positive control. The percentage of apoptotic cells was calculated by counting the stained cells in 12 fields, each containing 50 cells.

Western blot analysis

LNCaP and PC-3 cells were seeded at a density of 1.5×10^5 cells per ml in Dulbecco's modified Eagle's medium F-12 media with 2.5% charcoal-stripped fetal bovine serum for 24 h. Cells were treated with DMSO or the potato extract or fraction for 24 and 48 h. Protein was extracted into a high-salt buffer containing 1% proteinase inhibitor cocktail (Sigma-Aldrich), and proteins were quantified using the Bradford reagent. After boiling the sample for 3 min at 100°C, 60 µg of protein was loaded per lane on acrylamide gel (10%, 12% and 15%) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 120 V for 3–4 h. Proteins were transferred by wet blotting onto 0.2 µ polyvinylidene difluoride membrane (Bio-Rad, Hercules CA). Membranes were blocked using 5% milk in TBST for 30 min and incubated with primary antibody either in fresh 5% milk in TBST or 3% bovine serum albumin in TBST overnight at 4°C with gentle shaking and incubated with the secondary antibody (1:5000) in 5% milk TBST for 90 min. The concentrations of the primary antibodies ranged from 1:250 to 1:5000. The membrane was incubated with chemiluminescence solution (PerkinElmer Life Sciences) after washing for 1 min and the membrane was exposed to X-ray film and developed.

Statistical analysis

Significance among treatments was determined by analysis of variance and Fisher least significant difference at 5 and 1% significance level. The results are expressed as means ± SEs for three replicates for each treatment.

Results

Phenolic content and antioxidant activity

Previous studies show that potato extracts contain several classes of phytochemicals that exhibit anticancer activity, including phenolic acids, flavonoids, anthocyanins and carotenoids (18). Many of the phenolic components also exhibit antioxidant activity. The total phenolic content was estimated using the Folin-Ciocalteu reagent, and the rank order for the phenolic content of these extracts was CO112F2-2 ≈ PATX99P32-2 > ATTX98491-3 > ATTX98462-3. The antioxidant activity of the extracts determined using the DPPH assay showed that their relative activities were CO112F2-2 ≈ PATX99P32-2 > ATTX98491-3 > ATTX98462-3. These results show that there was a correlation between the antioxidant activity and phenolic content of the extracts, and this was similar to a previous report showing a positive correlation between antioxidant activity and phenolic content of potato extracts (18).

Inhibition of prostate cancer cell growth by extracts of potato cultivars

The potential growth inhibitory effects of the extracts were investigated using androgen receptor (AR)-positive LNCaP and AR-negative PC-3 prostate cancer cell lines. Figure 1 illustrates the effects of different concentrations of the extracts (expressed as micrograms of chlorogenic acid eq/ml) on proliferation of LNCaP cells, and the results show that the IC50 values for the CO112F2-2 extract (2.5–5 μg/ml) were lower than that observed for the other extracts where IC50 values were 5–10 μg/ml. A comparable cell proliferation study was carried out in PC-3 cells, and the results (Figure 1) were similar to those observed in LNCaP cells and the extracts from the CO112F2-2 cultivar were the most potent inhibitors of cell proliferation. Results using the water-soluble tetrazolium assay in LNCaP and PC-3 cells were similar to those observed for cell proliferation, with the CO112F2-2 extract being the most potent.

LNCaP and PC-3 cells were grown in 2.5% charcoal-stripped serum for 24 h and treated with DMSO (solvent control), 5 and 10 μg/ml CO112F2-2 cultivar extract. The percentage distribution of cells in the G_0/G_1, S and G_2/M phases was determined by FACS analysis after 24 h (Figure 2). Both concentrations of the extract decreased the percentage of LNCaP cells in the S phase and increased the percentage in G_0–G_1. In contrast, even 10 μg/ml did not alter the percentage distribution of cells in the G_0–G_1, S or G_2–M phases in PC-3 cells, indicating cell context-dependent differences in the effects of these extracts on G_0/G_1–S phase cell cycle progression (Figure 2).

Inhibition of prostate cancer cell growth by fractions

In order to identify the active fraction, extracts from the CO112F2-2 cultivar were fractionated into organic acid fraction, PF, and AF using cartridges as described in the Materials and Methods and previous studies (23,24). Figure 3 illustrates the effects of different fractions on

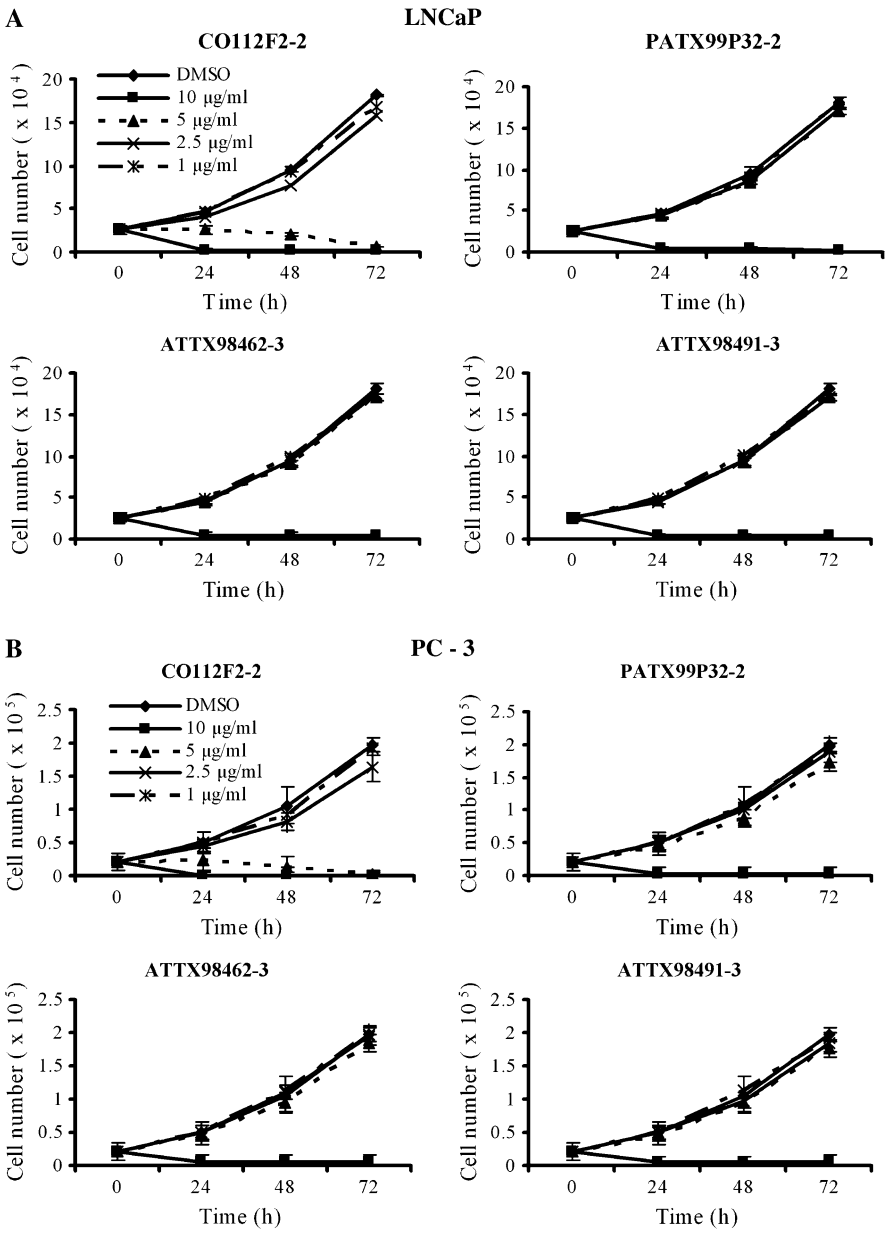

Fig. 1. Growth inhibition of (**A**) LNCaP and (**B**) PC-3 cells treated with potato extracts from cultivars CO112F2-2, PATX99P32-2, ATTX98462-3 and ATTX98491-3 at 1–10 μg chlorogenic acid eq/ml for 72 h and cell numbers were determined using a Coulter counter. Results are expressed as means ± SEs for three experiments at each time point.

proliferation of LNCaP and PC-3 cells. The effects of the organic acid fraction and PF were not significantly different from the solvent control as inhibitors of cell proliferation, even after treatment for 72 h. In contrast, IC50 values for the AF were 2.5–5 μg/ml, indicating that the AF was the most active component of the potato extracts for inhibition of LNCaP and PC-3 cell proliferation.

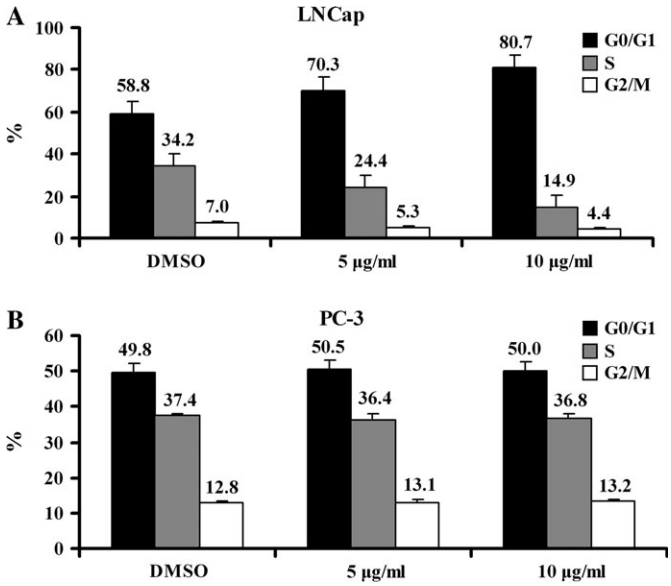

Fig. 2. FACS analysis. (**A**) LNCaP and (**B**) PC-3 cells were treated for 24 h with 5 and 10 μg/ml potato extract (CO112F2-2) and analyzed by FACS analysis as described in Materials and Methods. Results are presented as means ± SEs for three replicate experiments for each treatment group.

FACS analysis was also used to determine the distribution of both LNCaP and PC-3 cell lines in the G_0–G_1, S and G_2–M phases after treatment of the cells with 5 or 10 μg/ml AF for 24 h. The AF significantly increased the percentage of LNCaP cells in the G_0–G_1 phase and decreased in the S phase; however, as observed with the whole-cell extract (Figure 3), the AF did not affect G_0/G_1–S phase progression in PC-3 cells.

Modulation of cell cycle proteins by the extracts of potato or fractions

The effects of whole potato extracts and the AF on cyclin D1 and p27 protein expression were investigated in LNCaP and PC-3 cells. The results show that the extract and AF considerably increased p27 protein levels in both LNCaP and PC-3 cell lines after treatment for 6–24 or 18–24 h, respectively (Figure 3). In contrast, none of the treatments affected levels of cyclin D1 protein.

Effects of extracts of potato or fractions on apoptosis

The potato extracts and AF inhibited cell proliferation and at higher concentrations or after treatment for 72 h, dead cells were observed; therefore, we further investigated their cytotoxic effects in a series of assays. The effects of the potato extract or AF (5 or 10 μg/ml) on prostate cancer cells were investigated using a cell death detection ELISA kit (Figure 4). The potato extract and AF significantly induced apoptosis in both cell lines compared with untreated cells, and the apoptotic responses were similar to those observed for camptothecin, a known apoptotic inducing agent. The TUNEL assay was used to confirm the induction of apoptosis by the potato extract and AF. The brown staining characteristic of DNA fragmentation was observed

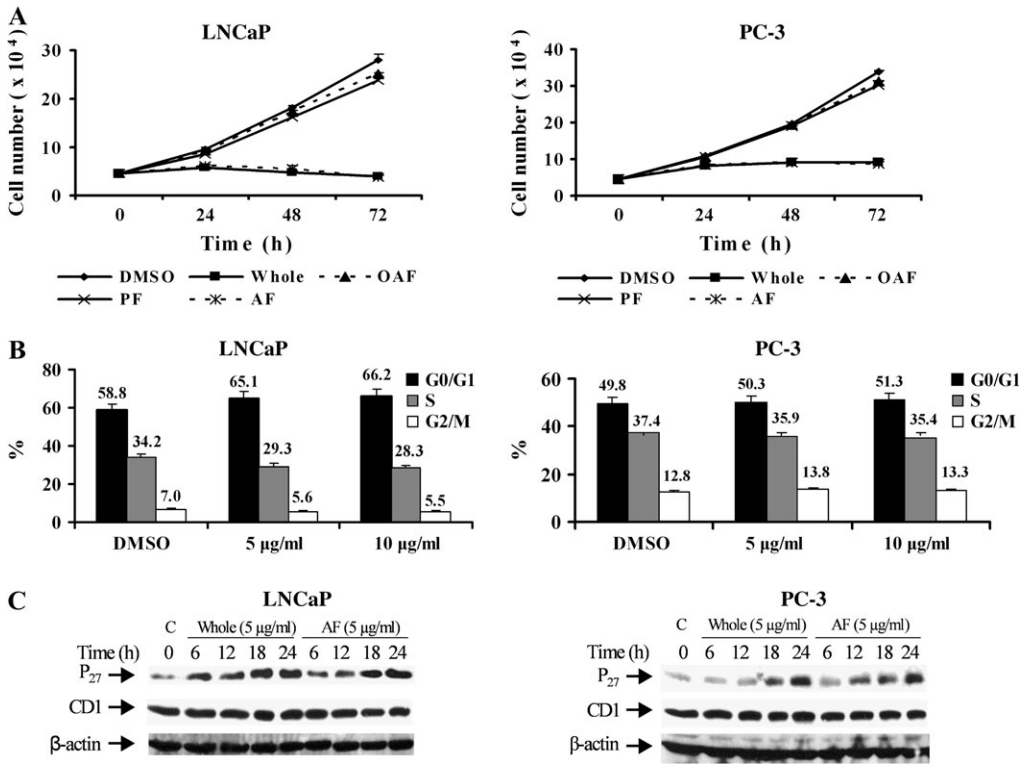

Fig. 3. Effects of potato extracts on cell growth inhibition, cell cycle progression and cell cycle proteins. (**A**) Inhibition of cell growth. LNCaP and PC-3 cells were treated with potato extract, organic acid fraction, PF and AF of potato cultivar CO112F2-2 at 5 μg/ml for 72 h. Cell numbers were determined using a Coulter counter. Results are expressed as means ± SEs for three experiments. (**B**) FACS analysis. LNCaP and PC-3 cells were treated for 24 h with 5, 10 μg/ml AF and analyzed by FACS analysis as described in Materials and Methods. Results are presented as means ± SEs for a single experiment. (**C**) Western blot analysis for p27 and cyclin D1 expression. LNCaP and PC-3 cells were treated with 5 μg/ml potato extract or AF for 6, 12, 18 and 24 h, and whole-cell lysates were analyzed by western blotting as indicated in the Materials and Methods. p27 protein levels increased in both LNCaP and PC-3 cell lines after treatment for 6–24 or 18–24 h, respectively. CD 1 protein levels were similar in all the treatments. Similar results were observed in duplicate experiments.

(Figure 4C), and quantification of these results showed (Figure 4D) significant induction of apoptosis in LNCaP and PC-3 cells after treatment with 5 μg/ml extract and AF.

The effects of potato extracts and the AF on caspase-dependent PARP cleavage and activation (cleavage) of caspases are illustrated in Figure 5. Cleaved PARP, cleaved caspase 3 and cleaved caspase 9 proteins were increased in LNCaP cells treated with potato extract or AF for 12, 18 and 24 h (Figure 5A). Bax protein levels were also induced, and these results confirm that the potato extracts and AF are potent inducers of apoptosis in LNCaP cells. In contrast, none of the treatments significantly affected the levels of cleaved PARP, caspase 3 and caspase 9 proteins (Figure 5A) even after treatment for 72 h (Supplementary Figure 1 is available at *Carcinogenesis* Online), whereas Bax protein levels significantly increased in PC-3 cells treated with the whole extract and AF.

Mitochondrial factors such as AIF and Endo G are also proapoptotic and contribute to caspase-independent cell death after being released from mitochondria and translocated into the nucleus (27). After treatment with the whole potato extract or AF, nuclear AIF and Endo G protein levels were increased in LNCaP cells and PC-3 cells (Figure 5B), demonstrating induction of caspase-independent apoptosis in both prostate cancer cell lines.

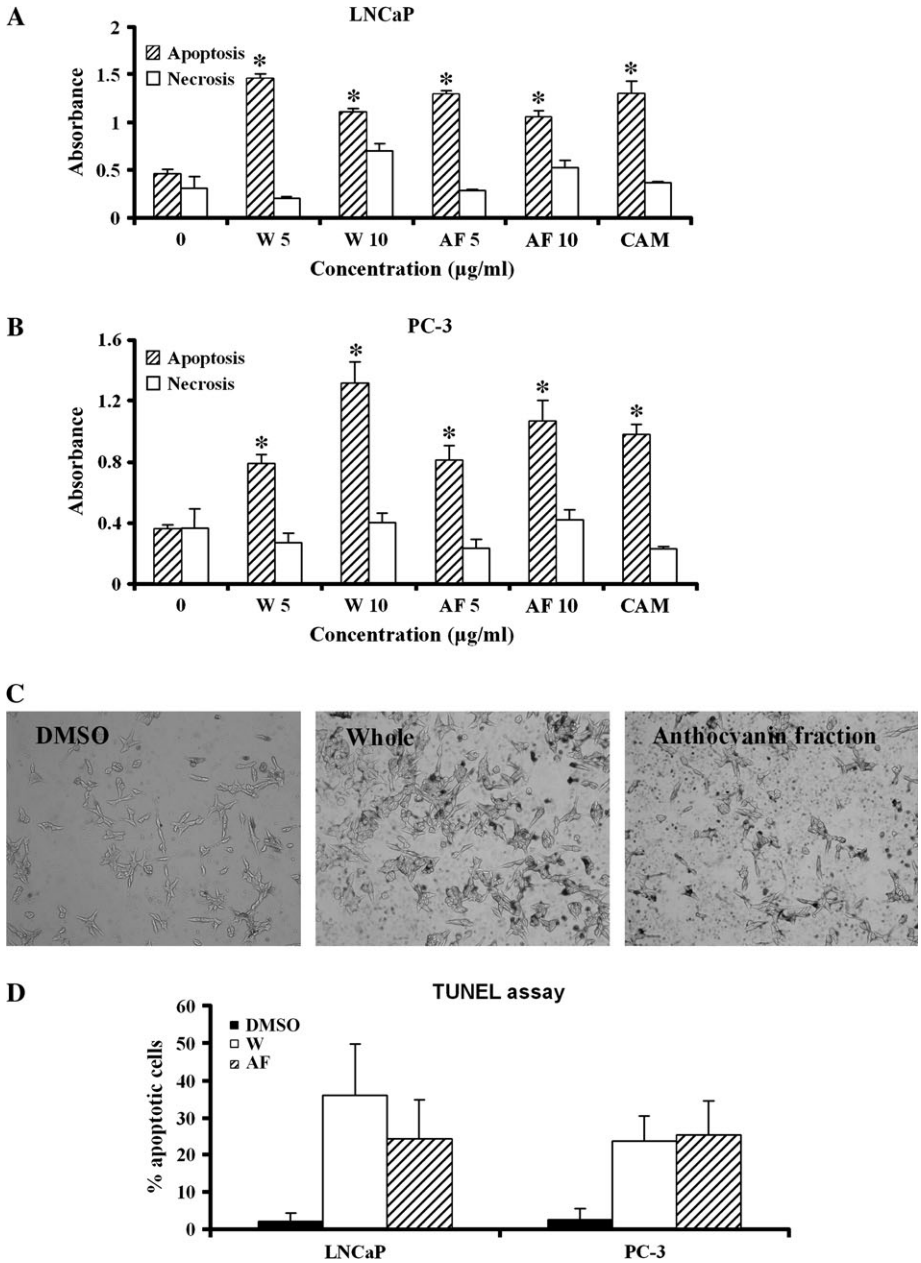

Fig. 4. Induction of apoptosis by potato extract and AF. Induction of apoptosis in (**A**) LNCaP and (**B**) PC-3 cells using a cell death ELISA kit. LNCaP and PC-3 cells were treated with potato extract and AF at 5, 10 μg/ml and camptothecin at 5 μM for 24 h. Absorbance is a measure of apoptosis (Cell death detection ELISA kit) and necrosis (lactate dehydrogenase assay). Results are presented as means ± SEs of three independent experiments. Significant (Fisher least significant difference at $P < 0.05$) induction of apoptosis by treatment groups is indicated by *. (**C**) TUNEL assay for apoptosis. LNCaP and PC-3 cells were treated with potato extract and AF for 12 h and analyzed for apoptosis using an in situ cell death detection POD kit (TUNEL assay) as described in Materials and Methods. Dark nuclear staining shows apoptotic cells observed under light microscope after adding POD and diaminobenzidene substrate to treated LNCaP cells. (**D**) Quantification of TUNEL assay. Percent apoptotic cells were calculated based on stained cells in 12 fields, each field with 50 cells. W: potato extract (5 μg/ml), AF: anthocyanin fraction (5 μg/ml); * represents significant difference against control (DMSO) as determined by Fisher least significant difference ($P < 0.05$).

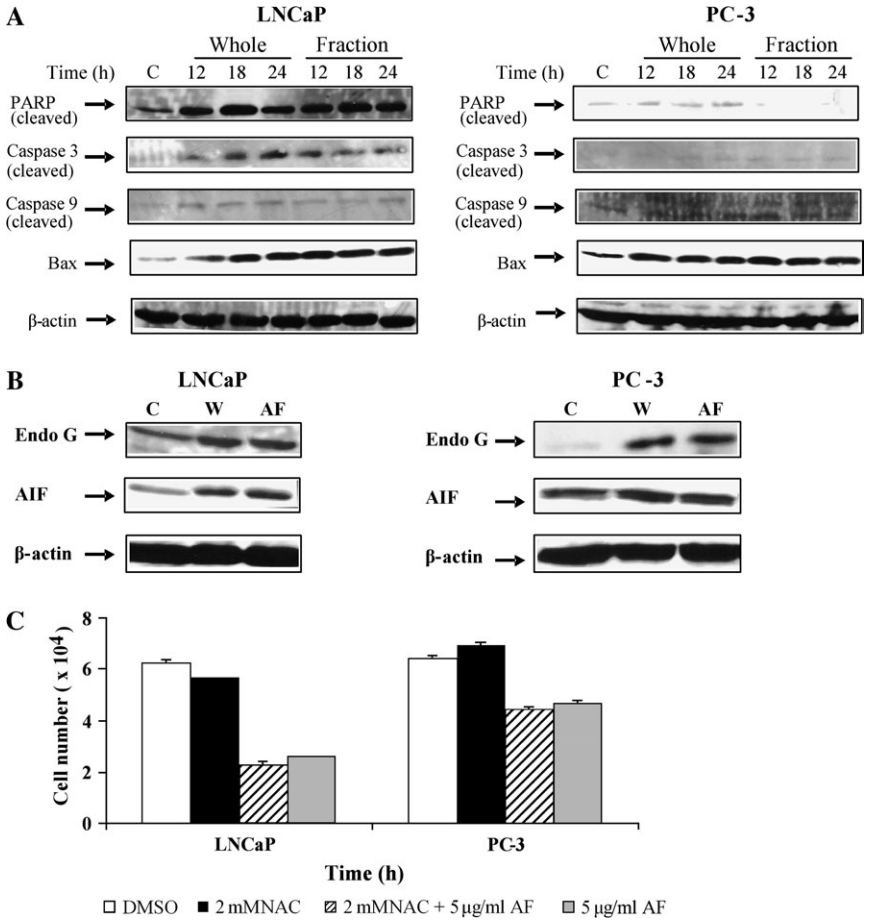

Fig. 5. Modulation of apoptotic proteins by potato extract (W) and AF in LNCaP and PC-3 cells and the effect of NAC on AF-induced responses. (**A**) Induction of caspase-dependent pathways. Both cell lines were treated with whole extract (5 μg/ml) or the AF (5 μg/ml) for 12, 18 and 24 h. Whole-cell lysates were analyzed by western blotting as described in Materials and Methods. Similar results were obtained in duplicate experiments. (**B**) Nuclear uptake of Endo G and AIF. Both cell lines were treated with potato extract or AF at 5 μg/ml for 24 h, and nuclear, cytoplasmic and mitochondrial proteins were separated using a mitochondria isolation kit coupled with cytoplasmic and nucleus isolation kits. The nuclear protein fraction was analyzed by western blot analysis as described in Materials and Methods. Similar results were observed in duplicate experiments. (**C**) Effects of NAC. PC-3 and LNCaP cells were treated with 5 μg/ml of AF for 72 h after pretreatment with NAC (2 mM) for 30 min. Cell numbers were analyzed using a Coulter counter. Results represent mean ± SE for duplicate experiment.

These results show that the potato extracts and AF induced intrinsic apoptotic pathways in LNCaP and PC-3 cells; however, the mechanisms associated with these responses require further investigation. Anthocyanins are antioxidants and may modulate cellular redox reactions which can activate apoptosis (28,29); therefore, we investigated the effects of NAC as a potential inhibitor of extract/AF-induced apoptosis. NAC did not affect the growth inhibitory effects of the AF (Figure 5C) in PC-3 cells, suggesting that the induction of oxidative stress and/or redox cycling was not associated with the proapoptotic responses induced by AF. Previous studies show that induction of ER stress (30,31) and activation of kinase pathways such as JNK (28,32) and MAPK (13) have also been linked to induction of apoptosis. Results summarized in Figure 6A show that glucose-related protein 78, a prototypical marker of ER stress (33,34) is not induced by AF in LNCaP or PC3 cells; however, there was a time-dependent increase in both c-jun and erk phosphorylation which is indicative of JNK and MAPK activation. Since both of these pathways have previously been associated with induction of apoptosis (13,14), we further investigated the effects of JNK (SP600125) and MAPK (PD98059) inhibitors on induction of apoptosis by potato extracts and AF. The results in Figure 6B show that both PD98059 (PD) and SP600125 (SP) significantly decrease nuclear uptake of Endo G and AIF in LNCaP and PC-3 cells treated with the proapoptotic extract/fraction. Moreover, in LNCaP cells where the extract and AF strongly induce PARP cleavage, cotreatment with PD or SP also inhibited this response (Figure 6C), further demonstrating the important role of the JNK and MAPK pathways on the proapoptotic activity of potato extracts and the AF in prostate cancer cells. We also investigated the effects of SP and PD on the antiproliferative activity of extracts and AF on growth of PC-3 (Figure 6D) and LNCaP (Figure 6E) cells. Although SP alone inhibited growth of PC-3 cells, the results show that both SP and PD significantly reversed the growth inhibitory effects of the potato extract and AF in both prostate cancer cell lines. The general caspase inhibitor z-VAD-fmk did not significantly decrease the whole extract and AF induced nuclear uptake of Endo G and AIF in LNCaP and PC-3 cells (Figure 6F). These data demonstrate for the first time that the proapoptotic activity of potato extracts and the AF are associated with activation of the JNK and MAPK-signaling pathways which are necessary for induction of caspase-dependent apoptosis and nuclear uptake of the proapototic factors Endo G and AIF (caspase independent).

Discussion

Consumption of fruits and vegetables has been associated with health benefits and protection from multiple diseases, including decreased incidence of some cancers. Fruit and vegetable intake were correlated with a decrease (non-significant) in the incidence of several chronic diseases, including cardiovascular disease in prospective studies which included the Nurses' Health (female) and Health Professionals Follow-up (male) studies (35). Several other reports correlated intake

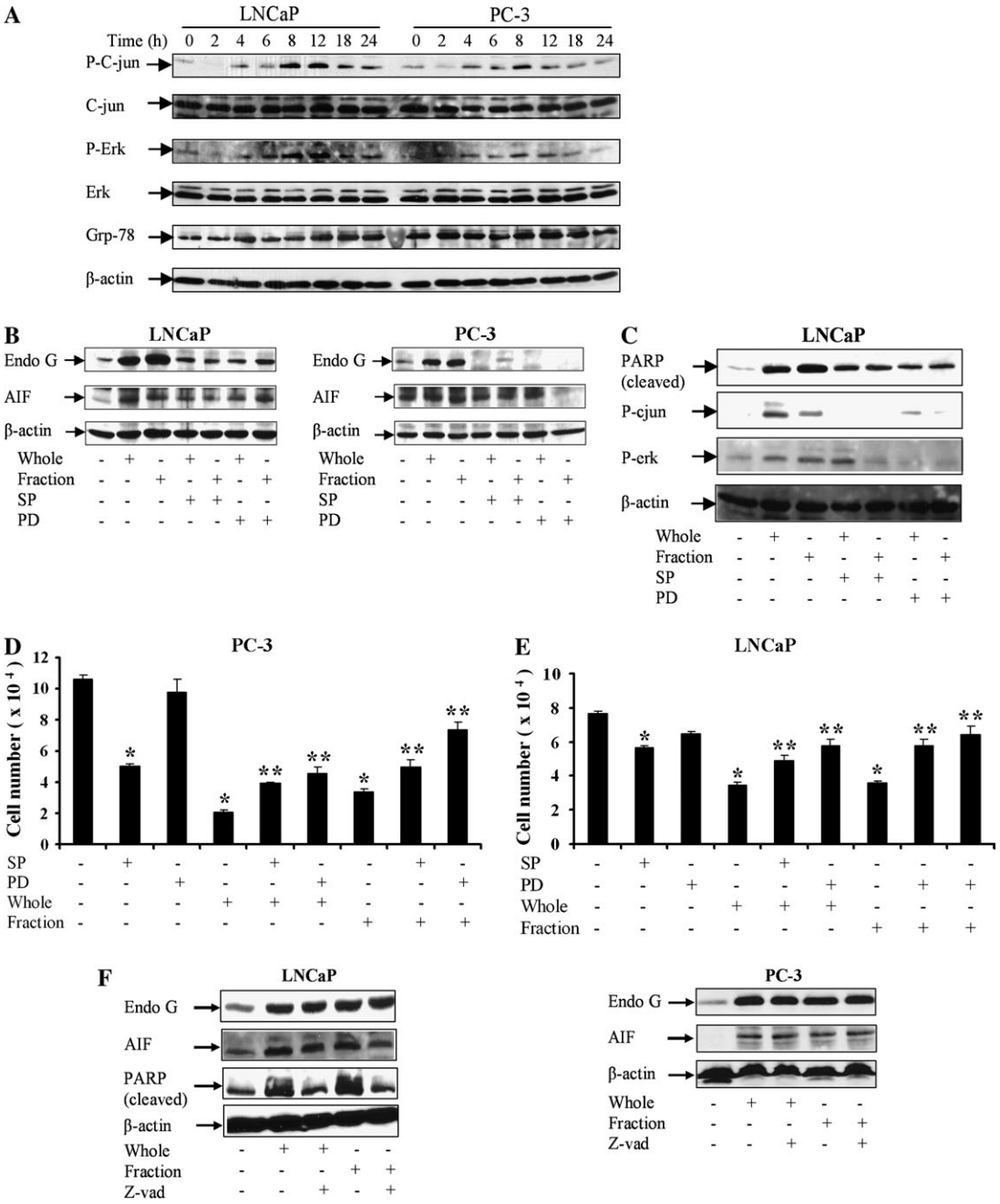

Fig. 6. Role of kinases and ER stress in potato extract/AF-induced apoptosis in LNCaP and PC-3 cells. (**A**) Activation of kinases and ER stress in LNCaP and PC-3 cells. Cells were treated with the extract/AF for 24 h and whole-cell lysates were analyzed by western blot analysis at regular time intervals. (**B**) Effects of PD and SP on nuclear uptake of Endo G and AIF. Cells pretreated with SP (JNK inhibitor—20 μM) or PD (MAPK inhibitor—20 μM) for 30 min were treated with extract/AF at 5 μg/ml for 24 h, and the nuclear fraction was analyzed by western blotting. Similar results were observed in duplicate experiments. (**C**) Effects of PD and AP on PARP cleavage in LNCaP cells. LNCaP cells were treated with 20 μM SP and PD for 30 min before treatment with the extract/AF for 24 h. Lysates were analyzed by western blot analysis. Similar results were observed in duplicate experiments. Effects of kinase inhibitors on potato extract or AF (5 μg/ml) induced cell growth inhibition in (**D**) PC-3 and (**E**) LNCaP cells. SP or PD pretreated cells (30 min) were treated with potato extract or AF for 24 h and cell numbers were determined by a Coulter counter. Results are presented as mean ± SE for duplicate experiments. Significant ($P < 0.05$) growth inhibition by extracts or kinase inhibitors alone are indicated * and significantly increased cell numbers in co-treatment groups are also indicated **. (**F**) Effects of z-VAD-fmk on nuclear uptake of Endo G and AIF in LNCaP and PC-3 cells and PARP cleavage in LNCaP cells. Cells were pretreated with z-VAD-fmk (caspase inhibitor—20 μM) for 30 min then treated with extract/AF at 5 μg/ml for 24 h, and the nuclear fraction was analyzed by western blotting. Similar results were observed in duplicate experiments.

of fruits and vegetables with decreased incidence of several cancers including prostate cancer (36–38). The health benefits and anticancer activities derived from consumption of vegetables and fruits are associated with different structural and functional classes of phytochemicals, many of which exhibit both chemopreventive and chemotherapeutic activity. Not surprisingly, natural products and their synthetic analogs are major sources for development of pharmaceuticals (39).

In this study, we have investigated the growth inhibitory/pro-apoptotic effects of extracts from various potato cultivars using AR-positive and AR-negative LNCaP and PC-3 prostate cancer cells, respectively. One of the objectives of this study was to identify specific

cultivars with enhanced activity against prostate cancer, and initial growth inhibition studies (Figures 1 and 2) clearly show that extracts from the CO112F2-2 cultivar were highly active. This extract also exhibited the highest total phenolic content and antioxidant activity among the cultivar extracts; however, extracts of the PATX99P32-2 cultivar also exhibited comparable antioxidant activity and phenolic content, but were less active in the growth inhibition studies (Figures 1 and 2). Fractionation of the CO112F2-2 cultivar extracts showed that the AF was the most active component, and both the potato extract and AF inhibited growth of LNCaP and PC-3 cells and blocked G_0/G_1–S phase progression only in the former cell line. Despite these differences, potato extract and AF induced the cyclin-dependent kinase inhibitor p27 in both cells (Figure 3).

The potato extract and AF inhibited growth of LNCaP and PC-3 cells, and after prolonged treatment (72 h and longer), there was a pronounced accumulation of dead cells. Studies in several laboratories have reported that anthocyanins and their glycosides induce death of many different cancer cell lines, and this has been linked to caspase-dependent apoptosis (3,14,15,40–45). Results of ELISA and TUNEL assays for apoptosis (Figure 4) demonstrate that the potato cultivar extract and AF also induced apoptosis in LNCaP and PC-3, and this is consistent with the proapototic effects of anthocyanins in other cancer cell lines (3,14,15,40–45). Moreover, the extract and AF induced PARP cleavage and activation of caspase 3 (cleavage) in LNCaP cells (Figure 5A), and these responses are typically observed for anthocyanin-induced caspase-dependent apoptosis in other cancer cell lines (3,14,15,40–45). In contrast, PARP cleavage and caspase 3 activation were not observed in AR-negative PC-3 cells (Figure 5A), demonstrating that anthocyanins differentially induced apoptosis in LNCaP (caspase dependent) and PC-3 cells where only a caspase-independent pathway appears to be activated in the latter cell line.

Endo G and AIF are proapototic mitochondrial proteins that can be released from the mitochondria by apoptosis-inducing agents or conditions (46–49). Both proteins are directly involved in DNA fragmentation and can be important mediators of caspase-independent cell death, although there are also reports that in some cells mitochondrial release of AIF and Endo G can be caspase dependent (46). Since PC-3 cells undergo caspase-independent cell death (Figure 5A), we further investigated the role of AIF and Endo G in mediating potato extract and AF-induced apoptosis in LNCaP and PC-3 cells (Figure 5B). The results show that both potato extracts and AF induced nuclear uptake of Endo G and AIF in PC-3 and LNCaP cells and demonstrate for the first time that anthocyanins induce caspase-independent apoptosis in prostate cancer cells. Interestingly, the caspase-independent pathway is the major route for cell killing in PC-3 cells, whereas LNCaP cell death induced by the potato extract was both caspase dependent and caspase independent.

Previous studies showed that the anthocyanin delphinidin induced Bax expression (45), and this was similar to potato extract/AF-induced up-regulation of Bax observed in this study (Figure 5A). Apoptosis induced by delphinidin in hepatoma cells was associated with induction of oxidative stress and was blocked by NAC and catalase (45). However, NAC did not affect AF-induced effects on PC-3 and LNCaP cell survival, suggesting that oxidative stress was not a factor in mediating apoptosis in prostate cancer cells. Several previous studies show that one of the underlying proapoptotic mechanisms activated by anthocyanins in cancer cell lines involved modulation of kinases (14,40,45). For example, delphinidin induced phosphorylation of JNK in hepatoma cells (45), malvidin decreased phospho-erk but increased phosphorylation of p38 in gastric adenocarcinoma cells (14) and hibiscus anthocyanins (extract) also activated p38 in promyelocytic leukemia (HL-60) cells (40). All of these anthocyanins inhibited cancer cell growth and induced apoptosis, and in both gastric and leukemia cells, the p38 inhibitor blocked anthocyanin-induced apoptosis (14,40). The AF from the potato extract induced phosphorylation of erk (Figure 6A) and c-jun, and both the JNK inhibitor SP6000125 and the MAPK inhibitor PD98056 blocked potato extract and/or AF-induced caspase-dependent apoptosis in LNCaP cells (Figure 6C) and caspase-independent Endo G and AIF nuclear translocation in PC-3 and LNCaP cells (Figure 6B). Thus, both apoptotic pathways were activated by the AF and extracts from potatoes, and these were dependent on upstream activation of MAPK and JNK pathways which appeared to act cooperatively since inhibition of the either kinase was sufficient to block apoptosis.

In summary, this study has identified a specialty potato cultivar (CO112F-2) that contains phytochemicals in the AF that inhibit LNCaP and PC-3 cell growth and induce apoptosis. Cell death pathways activated by the AF are associated with induction of MAPK and JNK in both cell lines, and these kinases cooperatively induce caspase-independent cell death in both cell lines and also caspase-dependent apoptosis in LNCaP cells. This study demonstrates for the first time that AF/potato extract induces mitochondrial release and nuclear uptake of the proapoptotic Endo G and AIF proteins. This represents an important and hitherto undetected mechanism of anticancer action for these phytochemicals in prostate cancer cells and has not been reported in other cancer cell lines. Current studies are focused on identifying individual components of the AF responsible for the induction of cell death pathways in prostate and other cancer cell lines. This will facilitate further development of potato cultivars which overexpress specific phytochemicals and thereby provide enhanced chemopreventive activity against cancer.

Supplementary material

Supplementary Figures can be found at http://carcin.oxfordjournals.org/

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References

1. American Cancer Society (2006) Overview: prostate cancer.
2. Veluri,R. *et al*. (2006) Fractionation of grape seed extract and identification of gallic acid as one of the major active constituents causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. *Carcinogenesis*, **27**, 1445–1453.
3. Yi,W. *et al*. (2005) Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *J. Agric. Food Chem.*, **53**, 7320–7329.
4. Matito,C. *et al*. (2003) Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr.*, **42**, 43–49.
5. Davis-Searles,P.R. *et al*. (2005) Milk thistle and prostate cancer: differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. *Cancer Res.*, **65**, 4448–4457.
6. Singh,R.P. *et al*. (2002) Flavonoid antioxidant silymarin and skin cancer. *Antioxid. Redox Signal.*, **4**, 655–663.
7. Ho,E. *et al*. (2004) Dietary influences on endocrine-inflammatory interactions in prostate cancer development. *Arch. Biochem. Biophys.*, **428**, 109–117.
8. Saddler,S.S. (2003) Potential mechanism of phytochemical induced apoptosis in human prostate cancer cells: genistein and β-lapachone. Florida Atlantic University, *MS Thesis* Boca Raton, Florida.
9. Yang,C.S. *et al*. (2001) Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.*, **21**, 381–406.
10. Nijveldt,R.J. *et al*. (2001) Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.*, **74**, 418–425.
11. Guarini,L. *et al*. (1992) Growth inhibition and modulation of antigenic phenotype in human melanoma and glioblastoma multiforme cells by caffeic acid phenethyl ester (CAPE). *Cell. Mol. Biol.*, **38**, 513–527.
12. Nichenametla,S.N. *et al*. (2006) A review of the effects and mechanisms of polyphenolics in cancer. *Crit. Rev. Food Sci. Nutr.*, **46**, 161–183.
13. Martin,S. *et al*. (2003) Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochem. Pharmacol.*, **65**, 669–675.

14. Shih,P.H. *et al*. (2005) Effects of anthocyanidin on the inhibition of proliferation and induction of apoptosis in human gastric adenocarcinoma cells. *Food Chem. Toxicol.*, **43**, 1557–1566.
15. Yi,W. *et al*. (2005) Study of anticancer activities of muscadine grape phenolics *in vitro*. *J. Agric. Food Chem.*, **53**, 8804–8812.
16. Stoner,G.D. *et al*. (2006) Protection against esophageal cancer in rodents with lyophilized berries: potential mechanisms. *Nutr. Cancer*, **54**, 33–46.
17. Al-Saikhan,M.S. *et al*. (1995) Antioxidant activity and total phenolics in different genotypes of potato (*Solanum tuberosum*, L). *J. Food Sci.*, **60**, 341–343.
18. Brown,C.R. (2005) Antioxidants in potato. *Am. J. Potato Res.*, **82**, 163–172.
19. Shakya,R. *et al*. (2006) Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography. *J. Agric. Food Chem.*, **54**, 5253–5260.
20. Nara,K. *et al*. (2006) Antioxidative activity of bound-form phenolics in potato peel. *Biosci. Biotechnol. Biochem.*, **70**, 1489–1491.
21. Al-Saikhan,M.S. (2000) Antioxidants, proteins, and carotenoids in potato.(*Solanum tuberosum*, L). Ph.D. *Department of Horticutural Sciences.* Texas A&M University, College Station.
22. Kanatt,S.R. *et al*. (2005) Potato peel extract-a natural antioxidant for retarding lipid peroxidation in radiation processed lamb meat. *J. Agric. Food Chem.*, **53**, 1499–1504.
23. Giusti,M.M. *et al*. (1999) Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.*, **47**, 4657–4664.
24. Giusti,M.M. *et al*. (1999) Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.*, **47**, 4631–4637.
25. Brand-Williams,W. *et al*. (1995) Use of free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.*, **28**, 25–30.
26. Singleton,V.L. *et al*. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth. Enzymol.*, **299**, 152–178.
27. Ory,B. *et al*. (2007) Zoledronic acid activates the DNA S phase checkpoint and induces osteosarcoma cell death characterized by AIF and EndoG translocation independently of p53 and Rb status. *Mol. Pharmacol.*, **71**, 333–343.
28. Hou,D.X. *et al*. (2003) Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: structure-activity relationship and mechanisms involved. *Int. J. Oncol.*, **23**, 705–712.
29. Hou,D.X. *et al*. (2005) Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Arch. Biochem. Biophys.*, **440**, 101–109.
30. Lei,P. *et al*. (2006) 1,1-Bis(3′-indolyl)-1-(p-substituted phenyl)methanes inhibit ovarian cancer cell growth through peroxisome proliferator-activated receptor-dependent and independent pathways. *Mol. Cancer Ther.*, **5**, 2324–2336.
31. Nakano,T. *et al*. (2006) Endoplasmic reticulum Ca2+ depletion induces endothelial cell apoptosis independently of caspase-12. *Cardiovasc. Res.*, **69**, 908–915.
32. Sato,M. *et al*. (2001) Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. *Free Radic. Biol. Med.*, **31**, 729–737.
33. Chae,H.J. *et al*. (2004) Ge-Jee-Bok-Ryung-Hwan induces apoptosis in human cervical carcinoma HeLa cells—an endoplasmic reticulum stress pathway. *Life Sci.*, **75**, 2997–3016.
34. Uramoto,H. *et al*. (2005) Expression of endoplasmic reticulum molecular chaperone Grp78 in human lung cancer and its clinical significance. *Lung Cancer*, **49**, 55–62.
35. Hung,H.C. *et al*. (2004) Fruit and vegetable intake and risk of major chronic disease. *J. Natl Cancer Inst.*, **96**, 1577–1584.
36. Cohen,J.H. *et al*. (2000) Fruit and vegetable intakes and prostate cancer risk. *J. Natl Cancer Inst.*, **92**, 61–68.
37. Kolonel,L.N. *et al*. (2000) Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 795–804.
38. Rashidkhani,B. *et al*. (2005) Fruits, vegetables and risk of renal cell carcinoma: a prospective study of Swedish women. *Int. J. Cancer*, **113**, 451–455.
39. Koehn,F.E. *et al*. (2005) The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discov.*, **4**, 206–220.
40. Chang,Y.C. *et al*. (2005) Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicol. Appl. Pharmacol.*, **205**, 201–212.
41. Chen,P.N. *et al*. (2005) Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth *in vivo*. *Nutr. Cancer*, **53**, 232–243.
42. Fimognari,C. *et al*. (2005) *In vitro* antitumor activity of cyanidin-3-O-beta-glucopyranoside. *Chemotherapy*, **51**, 332–335.
43. Guerra,M.C. *et al*. (2005) Cyanidin-3-O-beta-glucopyranoside, a natural free-radical scavenger against aflatoxin B1- and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). *Br. J. Nutr.*, **94**, 211–220.
44. Lazze,M.C. *et al*. (2004) Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines. *Carcinogenesis*, **25**, 1427–1433.
45. Yeh,C.T. *et al*. (2005) Induction of apoptosis by the anthocyanidins through regulation of Bcl-2 gene and activation of c-Jun N-terminal kinase cascade in hepatoma cells. *J. Agric. Food Chem.*, **53**, 1740–1749.
46. Arnoult,D. *et al*. (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J.*, **22**, 4385–4399.
47. Cande,C. *et al*. (2002) Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? *J. Cell Sci.*, **115**, 4727–4734.
48. Cande,C. *et al*. (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie*, **84**, 215–222.
49. Kang,Y.H. *et al*. (2004) Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. *Cancer Res.*, **64**, 8960–8967.

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