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Journal of Cellular Biochemistry 111:742–754 (2010)

Journal of Cellular Biochemistry

Proanthocyanidins From the American Cranberry (*Vaccinium macrocarpon*) Inhibit Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 Activity in Human Prostate Cancer Cells via Alterations in Multiple Cellular Signalling Pathways

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ABSTRACT

Prostate cancer is one of the most common cancers in the Western world, and it is believed that an individual's diet affects his risk of developing cancer. There has been an interest in examining phytochemicals, the secondary metabolites of plants, in order to determine their potential anti-cancer activities *in vitro* and *in vivo*. In this study we document the effects of proanthocyanidins (PACs) from the American Cranberry (*Vaccinium macrocarpon*) on matrix metalloproteinase (MMP) activity in DU145 human prostate cancer cells. Cranberry PACs decreased cellular viability of DU145 cells at a concentration of 25 μg/ml by 30% after 6 h of treatment. Treatment of DU145 cells with PACs resulted in an inhibition of both MMPs 2 and 9 activity. PACs increased the expression of TIMP-2, a known inhibitor of MMP activity, and decreased the expression of EMMPRIN, an inducer of MMP expression. PACs decreased the expression of PI-3 kinase and AKT proteins, and increased the phosphorylation of both p38 and ERK1/2. Cranberry PACs also decreased the translocation of the NF-κB p65 protein to the nucleus. Cranberry PACs increased c-jun and decreased c-fos protein levels. These results suggest that cranberry PACs decreases MMP activity through the induction and/or inhibition of specific temporal MMP regulators, and by affecting either the phosphorylation status and/or expression of MAP kinase, PI-3 kinase, NF-κB and AP-1 pathway proteins. This study further demonstrates that cranberry PACs are a strong candidate for further research as novel anti-cancer agents. J. Cell. Biochem. 111: 742–754, 2010.

KEY WORDS: MATRIX METALLOPROTEINASES; PROSTATE CANCER; CRANBERRY PROANTHOCYANIDINS; CELLULAR SIGNALLING PATHWAYS

Prostate cancer is one of the most common forms of cancer in North American men, with over 210,000 diagnosed cases in 2008. Despite improvements in therapy and treatment over the last few decades, this disease still accounts for over 32,000 deaths per year In North America according to the American and Canadian Cancer Societies [2008]. This disease reaches a critical point in development when it becomes metastatic and spreads to multiple sites in the body, primarily bone and lung tissue [Bubendorf et al., 2000]. The earlier stages of prostate cancer are androgen dependent, meaning that this cancer requires androgen in order to grow. This characteristic is often used as a treatment by means of androgen ablation by physical or chemical castration. However, the heterogeneity of cancerous cells allows for the development and spread of androgen-independent cancerous cells which do not

Grant sponsor: National Cancer Institute of Canada (N.C.I.C.); Grant sponsor: Canadian Cancer Society; Grant sponsor: P.E.I. Health Research Program; Grant number: 17799.

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Received 10 May 2010; Accepted 24 June 2010 • DOI 10.1002/jcb.22761 • © 2010 Wiley-Liss, Inc.
Published online 12 July 2010 in Wiley Online Library (wileyonlinelibrary.com).

respond to androgen deprivation therapy [Abate-Shen and Shen, 2000]. Because this form of treatment eventually falters, new forms of prostate cancer treatments are required.

One of the critical steps during metastasis of prostate cancer is the penetration of the cancerous cells into vasculature, allowing it an avenue to spread to other parts of the body. A family of proteins called the matrix metalloproteinases (MMPs) allows cancerous cells to do just that [Yousif et al., 2002]. These proteins degrade the surrounding extracellular matrix and permit the cancer to access lymph tissue and vasculature. The gelatinases, specifically MMP 2 and MMP-9, degrade gelatin found in the basement membrane of tissues. The expression of these gelatinases has been reported to be up-regulated in a variety of different cancerous cells [Bachmeier et al., 2001; Gimeno-Garcíaa et al., 2006], and this is a possible reason why these cancers are able to spread throughout the body [Björklund and Koivunen, 2005].

Dietary considerations may have an effect/influence on prostate cancer. Certain foods have been associated with a decreased risk of developing cancer, notably fruits and vegetables [Steinmetz and Potter, 1991; Cohen et al., 2000]. It has been shown that these foods contain many 'bioactive' compounds, which both in vitro and in vivo have profound effects on the behaviour of cancerous cells. Some of these effects include increasing rates of apoptosis, decreasing incidences of metastasis and arresting the cell cycle [Liu et al., 2007; Gallo et al., 2008; Pan et al., 2008].

One food which has demonstrated many 'bioactive' properties is the American cranberry (*Vaccinium macrocarpon*). The phenolic constituents of cranberry fruit include anthocyanins, flavonols, polyflavan-3-ols (proanthocyanidins—PACs) and phenolic acid derivatives. These have been shown to be powerful anti-oxidants [Vinson et al., 2008], and the PACs have also been shown to prevent adherence of potentially harmful bacteria [Lavigne et al., 2008; Matsushima et al., 2008]. Recently, researchers have demonstrated the potential anti-cancer properties of the American cranberry and its effects upon cancerous cells. These effects include reduction of proliferation of cancerous cells, inhibition of cyclooxygenase enzyme activity and expression, and an induction of apoptosis [Neto, 2007]. One family of 'bioactive' compounds which are found in high concentrations in cranberries are flavonoids. These compounds have also been shown to affect the behaviour of cancer cells. Recently, reports have discussed the effects of various flavonoids including PAC oligomers on MMP expression and activity *in vitro* [Vayalil et al., 2004; Matchett et al., 2005]. Previously, we described the effects of a flavonoid-rich whole cranberry extract on MMP-2/-9 activities in DU145 human prostate adenocarcinoma cells *in vitro* [MacLean et al., 2007]. The purpose of this study is to examine the effect of a PAC-enriched extract from cranberry fruit on MMP activity and associated signalling pathways in DU145 human prostate cancer cells.

MATERIALS AND METHODS

All antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following antibodies: anti-TIMP-1, anti-TIMP-2, anti-EMMPRIN, goat polyclonal anti-actin, anti-Akt 1, anti-pJNK, anti-JNK 1, anti-pIκBα, anti-histone H2, rabbit polyclonal anti-p38, anti-pp38, anti-pERK1/2, anti-ERK 1, anti-ERK 2, anti-pAkt 1, PI-3-kinase p110, anti-NF-κB (p65), anti-JNK 2 and mouse monoclonal anti-IκBα and PI-3-kinase p85α, respectively, were used. All other chemicals and materials were purchased as indicated.

PREPARATION OF CRANBERRY EXTRACTS

The PAC-enriched cranberry extract(s) used in this study were prepared at University of Massachusetts-Dartmouth. Early Black cranberry fruit was harvested in October 2007 in Wareham, MA, flash frozen with liquid nitrogen and stored at −20°C until used. The PAC-enriched fraction used in this study was prepared as follows. One kilogram of fruit was extracted with 300 ml aliquots of a mixture of 40/40/19/1 methanol/acetone/water/formic acid at room temperature, each time pulsing with a Waring blender for 5 min, then after 30 min filtering and collecting the filtrate, repeating until the pulp was nearly colourless. The combined filtrates were concentrated in vacuo and freeze dried. Free sugars were removed to produce a concentrated whole crude extract by chromatography on Diaion HP-20 (4.5 cm × 30 cm column). The sample was applied to the column in aqueous solution, allowed to adsorb, then washed with several column volumes of distilled water to remove free sugars. The column was eluted with methanol until no pink colour remained and then rinsed with acetone. The organic extracts were combined, concentrated and freeze-dried to produce 10.5 g of crude cranberry polyphenolic extract. Phenolic constituents, including anthocyanins, flavonols and PACs were separated by chromatography on Sephadex LH-20 (3.0 cm × 22 cm column) as follows. Crude extract (500 mg) was dissolved in distilled water and applied to the column. The column was eluted first with 70/30 methanol/water yielding a fraction containing predominantly phenolic acids, followed by a second fraction containing predominantly anthocyanins, and a third fraction containing primarily flavanol glycosides. The column was then eluted with 70/30 acetone/water to produce a fraction containing mainly PACs based on HPLC analysis. Approximately 195 mg of PAC-enriched fraction was obtained. The purity and composition of the PAC-enriched fraction were verified by reverse-phase HPLC analysis with diode-array detection on a Waters 515/996 system with Millenium software and analysis by MALDI-TOF MS as previously described [Neto et al., 2006].

CELL CULTURE AND TREATMENT WITH PROANTHOCYANIDINS

Human DU145 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100 mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in alpha MEM (Gibco, Burlington, ON), supplemented with 10% foetal bovine serum (FBS) (Sigma, Oakville, ON) and 1% antibiotic–antimycotic (Gibco). Cells (4×10^5) were cultured on the tissue culture dishes and were initially incubated for 24 h at 37°C in the presence of 5% CO_2. After this 24-h incubation period, the media was removed and replaced with serum free alpha MEM supplemented with 5 μg/ml of transferrin (Sigma) and 2.5 μg/ml of insulin (Sigma). These plates were again incubated for 24 h at 37°C in the presence of 5% CO_2. After this incubation period, cells were treated with various concentrations of PACs ranging from 1 to 25 μg/ml for times ranging from half an hour to 6 h. PACs were

dissolved in dimethyl sulfoxide (DMSO) as the vehicle prior to use. The control cells received only the vehicle. After treatment, the media were removed from the cells and stored at −80°C for further analysis. The cells were then washed with PBS, and were removed by trypsin (Sigma) diluted in phosphate-buffered saline (PBS). The cells were re-suspended with alpha MEM supplemented with 10% FBS and were centrifuged for 4 min at 500*g*. After centrifugation, the cell pellet was re-suspended with PBS and was transferred to a microcentrifuge tube and was centrifuged at 500*g* for 4 min. After centrifugation, this cell pellet was then stored at −80°C until analysed further.

ZYMOGRAPHY

Zymography, or gelatin-gel electrophoresis was performed to evaluate MMP activity essentially as previously described [Matchett et al., 2005]. A 100 μl aliquot of the conditioned media were mixed in a 4:1 ratio with sample buffer, which consisted of 10% SDS and 0.1% bromophenol blue in 0.3 M Tris–HCl (pH 6.8) (Sigma). (This aliquot is representative of the MMP 'activity' expressed by 400,000 cells per plate.) Aliquots of each sample were loaded into wells of a 5% stacking gel and resolved by electrophoresis at a constant current at ambient temperature. The 10% resolving gel contained 0.1% gelatin (Sigma). Following electrophoresis, the gel was then incubated with a solution containing 2% Triton X-100 (Sigma) and 0.5 M Tris–HCl (Sigma) for 1 h. After this first incubation, the gel was then placed in the second solution containing 0.05 M of Tris–HCl (Sigma) and incubated for 30 min. After this second incubation, the gel was placed in a solution containing 1% Triton X-100, 0.05 M Tris–HCl and 0.005 M $CaCl_2$ (Sigma) and incubated at 37°C for 24 h. Following this incubation period, the gels were stained with a 0.1% Brilliant Blue R-250 stain containing 50% methanol, 40% distilled water and 10% acetic acid. Once stained, the gel was then de-stained in a solution containing 75% distilled water, 20% methanol and 10% acetic acid. Pre-stained molecular weight markers (Biorad, Mississauga, ON) were also resolved on the same gel. The location of the pre-stained markers on the gel was documented prior to the staining of the gel with Brilliant Blue R-250. Gelatinase activity appeared as zones of clearing (due to gelatin degradation) against a blue background.

The identity of the MMPs was determined by comparison of the relative positions of the zones of clearing (due to gelatin degradation) with the documented positions on the gel of the molecular weight markers.

CYTOSOLIC PROTEIN FRACTION AND NUCLEAR PROTEIN FRACTION ISOLATION

Protein expression was determined in the whole cytosolic protein fraction. Briefly, the cell pellets were removed from −80°C and placed on ice. The pellets were then reconstituted in 100 μl of 10 mM Tris–HCl buffer (7.4 pH) containing 0.5 mM PMSF (Sigma). Once reconstituted, the cells were then briefly sonicated. The cell lysates were then centrifuged for 10 min at 9,300*g* at 4°C. Following this centrifugation, the supernatant was removed from the pellet and subsequently evaluated. The total protein content of the supernatant was determined by Biorad protein assay (Biorad) as per manufacturer's instructions.

This nuclear isolation procedure was essentially as previously described [Wang et al., 2009]. Briefly, the previously prepared cell samples were removed from −80°C and placed on ice. These cells were then incubated on ice in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF for 10 min. After 10 min the cells were centrifuged at 10,000*g* for 10 min at 4°C. Once centrifuged, the supernatant containing the cytosolic proteins was removed from the pellet and stored at −80°C. The remaining pellet was then re-suspended in a buffer containing 20 mM HEPES, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF. The contents were then incubated on ice for 40 min with vigorous shaking. Once 40 min had passed, the contents were then centrifuged at 10,000*g* for 15 min at 4°C. After centrifugation the supernatant (containing the nuclear proteins) was removed and stored at −80°C until used. The total protein content of the cytosolic and nuclear fractions was determined by Biorad protein assay.

IMMUNOBLOT ANALYSES

Equal amounts of protein extracts from whole, cytosolic, and nuclear fractions were mixed in a 3:1 ratio with standard Laemmili buffer consisting of 50 mM Tris–HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, 10% glycerol and 100 mM beta-mercaptoethanol. Once mixed, these samples were boiled for 3 min. Once boiled, they were resolved by electrophoresis through 10% SDS–PAGE gels and were transferred onto nitrocellulose membranes (Biorad). After transfer, the membranes were incubated in the presence of a 1% BSA (w/v) TBS–Tween (0.05%, v/v) solution for 1 h at room temperature. Then, the membranes were incubated with primary antibodies diluted to a 1:200 ratio (v/v). The membranes were incubated in the presence of the primary antibody for 24 h at 4°C. After incubation the membranes were then washed three times with TBS–Tween (0.05%) for 10 min and were then incubated in alkaline phosphatase-conjugated secondary antibodies (1:2,000 dilution) for 1 h. After incubation, the membranes were again washed three times with TBS–Tween (0.05%) for 10 min, washed briefly with distilled water, and then exposed to SigmaFast BCIP-NBT tablets (Sigma) in solution to visualize protein expression levels. Such Western blots were then analysed using Infinity Capture software (Lumenera Corp., Ottawa, ON), and densitometry was performed with ImageJ software (National Institute of Health, Bethesda, MA).

ALAMAR BLUE CYTOTOXICITY ASSAY

The Alamar Blue assay (Invitrogen, Burlington, ON) was performed to determine cellular viability after treatment with PACs at 25 μg/ml at 3 and 6 h and was performed as per manufacturer's instructions. Briefly, DU145 cells were subcultured into a 96-well plate at 5,000 cells/well. After 24 h of incubation, the media were replaced with 100 μl of serum-free media and was once again incubated for 24 h. After this incubation, the cells were treated with PACs for either 3 or 6 h, with a final concentration of DMSO within each well of 1%. The control for this experiment was DU145 cells treated with DMSO at a total concentration of 1% for 6 h. Following treatment, 10 μl of Alamar Blue was added to each well in order to reach a final volume of 10% Alamar Blue in each well. The cells were incubated for 3 h post-exposure of the cells to Alamar Blue. After this 3-h

incubation period, the resulting fluorescence was read with an excitation wavelength of 528 nm and an emission wavelength of 590 nm.

STATISTICAL ANALYSIS

Statistical analysis for each assay was done using GraphPad Prism 4.03 for Windows (GraphPad Software, Inc., San Diego, CA). Results from each zymogram and cytotoxicity assay were compared using a one-way ANOVA with a Tukey's post hoc test, and results were considered statistically significant at $P < 0.05$. Results from the densitometric analyses of each Western blot were compared using an unpaired t-test, and results were considered statistically significant at $P < 0.05$.

RESULTS

CHARACTERIZATION OF THE PROANTHOCYANIDIN-ENRICHED CRANBERRY FRACTION

MALDI-TOF MS analysis of the PAC-enriched cranberry fraction (PACs) confirmed the presence of A-linked PACs as reported for cranberry PAC preparations [Neto et al., 2006]. The masses detected represented epicatechin dimer (M + Cs = 709 amu), trimer (M + Cs = 997 amu), tetramer (M = Cs = 1285), each containing one A-type linkage, and larger oligomers (data not shown).

CRANBERRY PROANTHOCYANIDINS SIGNIFICANTLY DECREASE DU145 CELL VIABILITY AT 25 μG/ML AFTER 6 H, BUT NOT AFTER 3 H

A cytotoxicity assay using Alamar Blue to assess cellular viability in DU145 prostate cancer cells in response to treatment with PACs was performed. As shown in Figure 1, treatment of DU145 cells with PACs (25 μg/ml) for 6 h results in 30% inhibition of cellular viability (which was significantly different from control (vehicle-treated) cells, $P < 0.001$). However, after only 3 h of treatment with PACs, cell viability decreases by only 8%, which was not statistically significant when compared to control ($P > 0.05$) (Fig. 1). A concentration of PACs (25 μg/ml) was chosen for further study as this concentration of PACs was determined to be the effective concentration which resulted in a substantial effect on the behaviour of DU145 cells which warranted and permitted further investigations. PACs (at 50 μg/ml) treatment of DU145 cells was shown to be very cytotoxic to the cells and very few viable cells remained thereby negating further study at this concentration (data not shown). Hence, PACs (25 μg/ml) was used for the studies described.

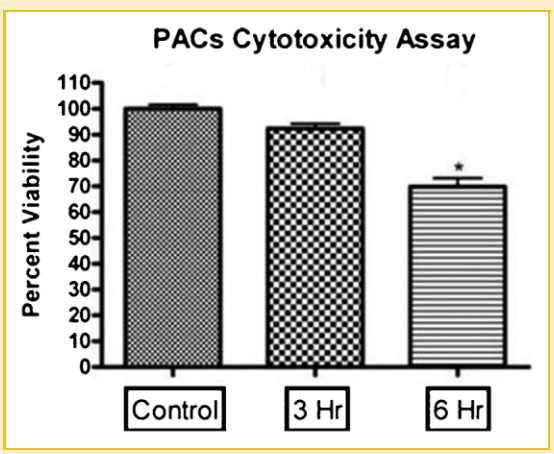

Fig. 1. PACs treatment of DU145 cells can affect cellular viability. DU145 cell viability was evaluated by Alamar Blue assay following treatment of cells with 25 μg/ml of PACs for 3 and 6 h, respectively. Control cells received 1% DMSO (vehicle) alone for 6 h. Fluorescence was read at 528 excitation and 590 emission wavelengths. Control cells were determined to represent 100% viability. Treatment of DU145 cells with 25 μg/ml of cranberry PACs for 6 h significantly decreased the viability of DU145 cells relative to control cells ($P < 0.05$). The results presented represent values obtained from eight separate experiments.

CRANBERRY PROANTHOCYANIDINS DECREASE MMP-2 AND MMP-9 ACTIVITY IN DU145 CELLS IN VITRO

To investigate the effects of PACs upon MMP activity, gelatin gel electrophoresis was performed with the cell media after treatment with PACs, with doses ranging from 0 to 25 μg/ml and times ranging from half an hour to 6 h. As shown in Figure 2A, MMP-2 and MMP-9 activity decreases after 3-h treatments with PACs at concentrations of 10 and 25 μg/ml, respectively. After performing this experiment, a time-course experiment was performed to determine how early the inhibition of MMP activity occurs after treatment with PACs. DU145 cells were treated with cranberry PACs at a concentration of 25 μg/ml for half an hour, 1 h and 3 h. As shown in Figure 2B, decreases in MMP-2 and MMP-9 activity were seen as early as 30 min post-treatment with PACs.

CRANBERRY PROANTHOCYANIDINS DECREASE THE EXPRESSION OF MMP INDUCER EMMPRIN, AND INCREASE THE EXPRESSION OF TIMP-2, AN MMP INHIBITOR

Having shown that treatment of DU145 cells with PACs results in inhibition of both MMP-2 and MMP-9 activities, we next evaluated the effects of PACs on the expression of a number of key factors known to regulate the expression of MMP-2/-9. These regulatory factors included the extracellular matrix metalloproteinase inducer (EMMPRIN) and two tissue inhibitors of matrix metalloproteinases (TIMPs), TIMP-1 and TIMP-2. As shown in Figure 3A,B, there was a statistically significant increase in the protein expression levels of TIMP-2 and a statistically significant decrease in the protein expression levels of both TIMP-1 and EMMPRIN, respectively, after treatment of DU145 cells with cranberry PACs (25 μg/ml) for 3 h.

CRANBERRY PROANTHOCYANIDINS AFFECT THE EXPRESSION OF MAPKS PATHWAY PROTEINS AND THE EXPRESSION OF PI3-KINASE PATHWAY PROTEINS

To further characterize the mechanisms whereby cranberry PACs can inhibit MMP-2/-9 expression levels, the effects of PACs on key cellular signal transduction proteins were determined. A number of cellular signalling proteins including those associated with the mitogen activated protein kinases (MAPKs) pathway and the phosphatidylinositol-3 kinase (PI3-kinase) pathway which subsequently activate AP-1 and NF-κB have been implicated in the regulation of the expression of MMPs [Lee et al., 2009]. Western blot analyses were performed with whole cell lysates from DU145 cells

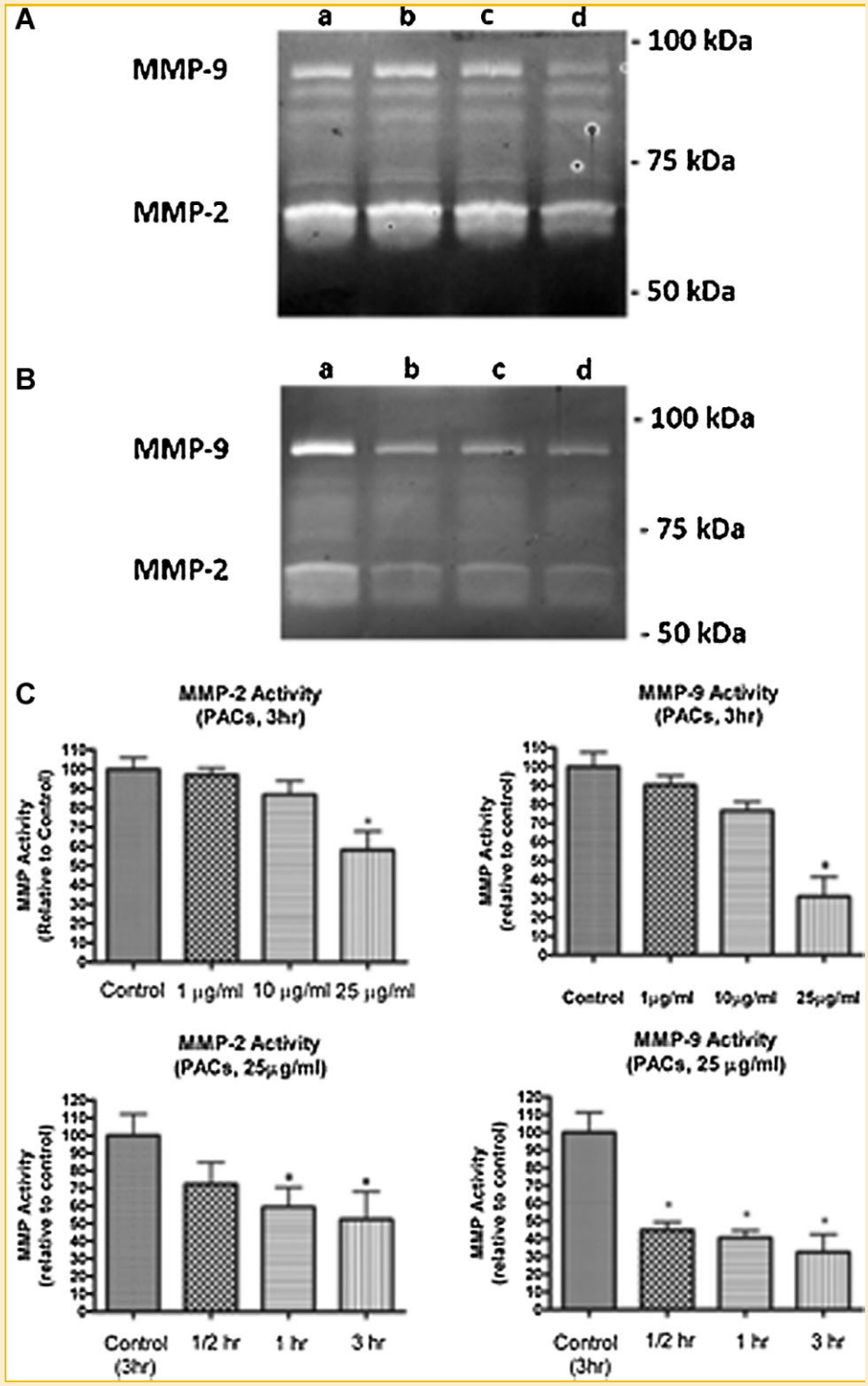

Fig. 2. Cranberry PACs decrease MMP-2 and MMP-9 activity. Gelatin gel electrophoresis was performed on aliquots of conditioned medium obtained from DU145 cells following exposure to PACs. Results obtained are representative of three independent experiments with assay duplicates. A: MMP-2 and MMP-9 activity decreases in response to PACs treatment: control cells (1% DMSO for 3 h) (a), and cells exposed to PACs (1 μg/ml) for 3 h (b), to PACs (10 μg/ml) for 3 h (c) and to PACs (25 μg/ml) for 3 h (d). B: MMP-2 and MMP-9 activity decreases in a time-dependent manner in response to PACs (25 μg/ml): control cells (cells treated with 1% DMSO for 3 h) (a), cells treated with 25 μg/ml PACs for 30 min (b), is cells for 1 h (c) and for 3 h (d). C: Histograms representing densitometric analyses of MMP-2 and MMP-9 activity levels. Asterisks denote a statistically significant difference from control ($P < 0.05$). Results presented are representative of findings from three independent experiments with assay duplicates.

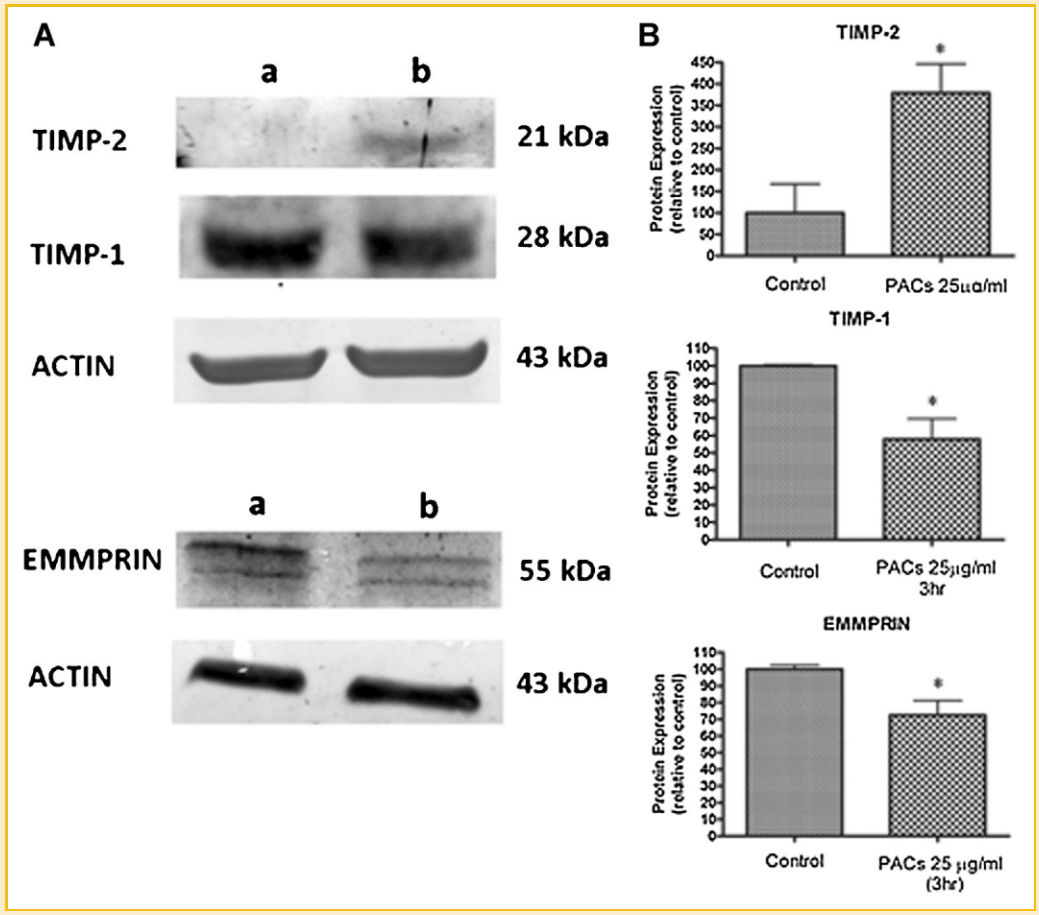

Fig. 3. Cranberry PACs increase TIMP-2 protein expression levels and decrease TIMP-1 and EMMPRIN protein expression levels in DU145 cells. A: TIMP-2, TIMP-1, EMMPRIN and actin protein levels, respectively, in vehicle-treated cells (a) and in PACs (25μg/ml)-treated (3 h duration) cells (b). Actin was used as a loading control. Each blot shown is representative of results obtained from three separate experiments with assay duplicates. B: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$).

following 3 h treatment with PACs (25 μg/ml) and protein expression levels of a number of proteins evaluated. As shown in Figure 4A,C, results indicated that after treatment of DU145 cells with PACs, the phosphorylation status of p38, ERK1 and ERK2 increased. These increases were statistically significant ($P < 0.05$). Additionally, as shown in Figure 4B,C, increased JNK1 and decreased JNK2 protein expression levels occurred in response to PACs with corresponding alterations in both pJNK1 and pJNK2.

To further investigate the nature of the cellular signalling proteins involved in the PACs-mediated inhibition of MMP-2/-9, the expression of proteins in the PI-3 kinase pathway was evaluated. As such, Western blot analyses were performed and Akt (a protein in the PI-3 kinase pathway) protein levels were determined. As shown in Figure 5C,D, PACs treatment of DU145 cells resulted in a statistically significant decrease in both phosphorylated Akt and Akt proteins levels. To further investigate the mechanisms by which PACs act, Western blot analyses were performed to examine the expression of PI-3 kinases (p85 and p110 proteins). The expression of both PI-3 kinase p85 and PI-3 kinase p110 protein levels decreased in DU145 cells in response to treatment of these cells with PACs (Fig. 5A,B). The decreases were statistically significant.

CRANBERRY PROANTHOCYANIDINS DECREASE THE TRANSLOCATION OF P65 TO THE NUCLEUS, DECREASE THE PHOSPHORYLATION STATUS OF IκBα, ACTIVATE C-JUN AND INHIBIT C-FOS PROTEIN EXPRESSION

The pathways which can affect the expression of MMPs in cells can activate both AP-1 and NF-κB mediated events [Lee et al., 2009]. Since the expression of MMPs can be affected by the NF-κB pathway and NF-κB has been suggested to be a pivotal transcription factor in prostate cancer metastasis to bone [Andela et al., 2003] we next investigated whether or not, PACs may be also acting via this pathway to affect MMP expression levels in DU145 cells. To determine the amount of NF-κB (p65) protein in the nucleus, Western blot analyses were performed with nuclear extracts from untreated DU145 cells and from DU145 cells treated with PACs (25 μg/ml) for 3 h. To verify the integrity of the nuclear fraction extract prepared, the expression of a nuclear protein, H2B, which is a core histone protein was evaluated. Result indicated that there was a marked expression of H2B protein in the isolated nuclear fraction preparation and none detected in the cytosolic fraction which was also prepared. This result as shown in Figure 6A suggests that there is an enrichment of this protein in this fraction verifying

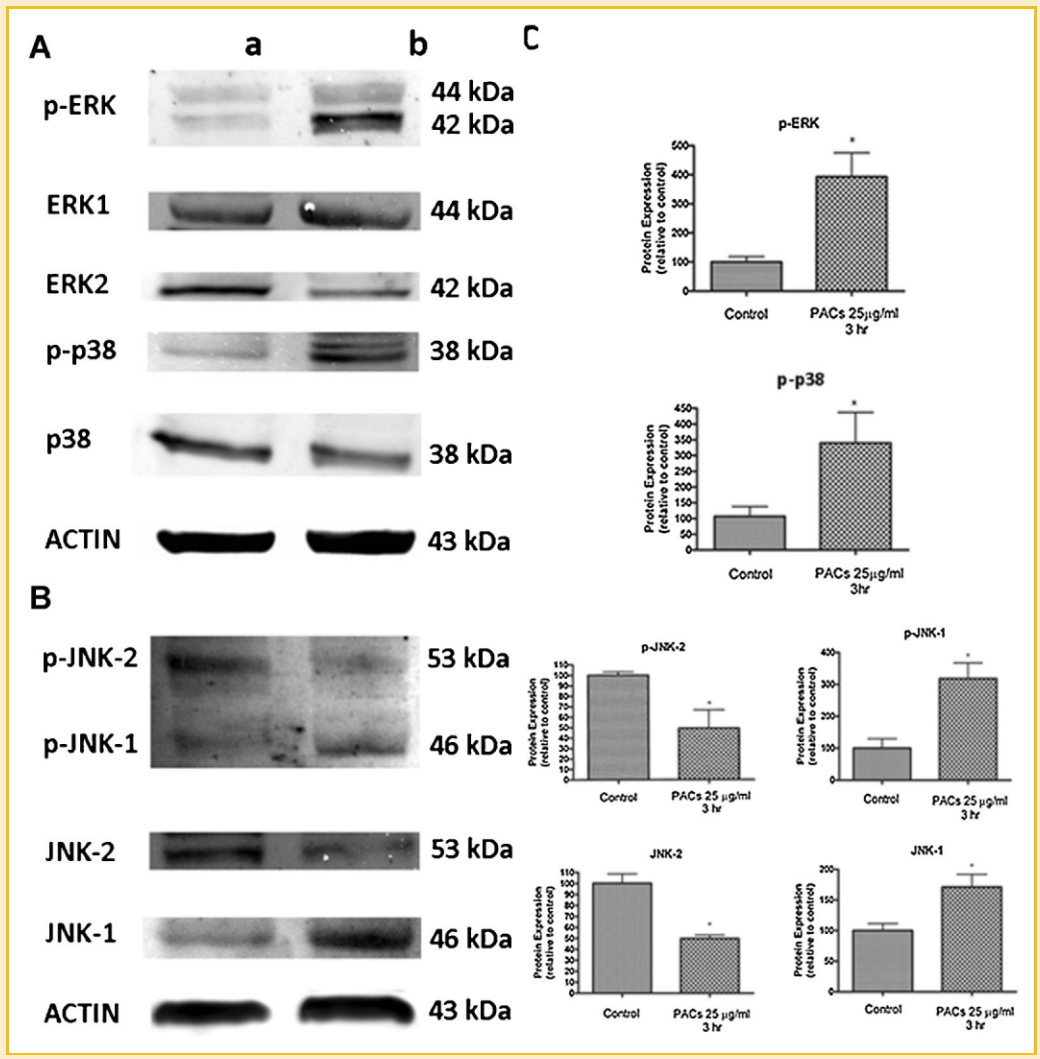

Fig. 4. Cranberry PACs affect the expression of MAPKs pathway proteins. A: pERK, ERK1, ERK2, p-p38, p38 and actin protein levels, respectively, in vehicle-treated control cells and in PACs (25μg/ml) (for 3 h)-treated cells (b). Actin was used as a loading control. Each blot shown is representative of results obtained from three separate experiments with assay duplicates. B: JNK-1, JNK-2, p-JNK-2, p-JNK-1 and actin protein levels, respectively, in vehicle-treated control cells (a) and in PACs (25 μg/ml) (for 3 h)-treated cells (b). C: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$).

the successful preparation of a nuclear fraction. Post-confirmation of the fidelity of the nuclear fraction prepared, the expression of NF-κB pathway proteins was evaluated. As shown in Figure 6B,C (and Fig. 6E), it was observed that there was a statistically significant decrease in translocation of NF-κB (p65) protein to the nucleus after treatment with PACs. A statistically significant decrease in p65 protein expression levels in the nuclear fraction was noted in response to PACs treatment whereas no statistically significant alteration in the p65 protein levels in the cytosolic fraction in response to PACs treatment occurred. This observation suggests that the relative amount of p65 found in the cytoplasm was not decreased in a manner which would account for the alterations in p65 protein levels noted in the nucleus in response to PACs treatment. This suggests that the nuclear protein levels were a result of decreases in p65 protein translocation. In order to further account for this decreased expression level of NF-κB (p65) protein which occurred in response to PACs treatment, Western blot analyses were performed and the protein expression levels of IκBα, which is an inhibitor of p65 translocation, were determined. As shown in Figure 6D (and Fig. 6E), results indicated that no change in the amount of IκBα occurred in response to PACs, but there was a statistically significant decrease in phosphorylated IκBα after treatment of cells with PACs. Additionally, as shown in Figure 6B (and Fig. 6E), it was observed that PACs treatment of DU145 cells resulted in increased expression of c-jun protein and decreased expression of c-fos protein. The changes in expression of these AP-1 transcription factors in response to PACs treatment were statistically significant.

DISCUSSION

In this study we examined the effects of cranberry PACs upon MMP activity in DU145 human prostate cancer cells. Cranberry PACs were able to inhibit MMP-2 and MMP-9 activity. This inhibition was part of

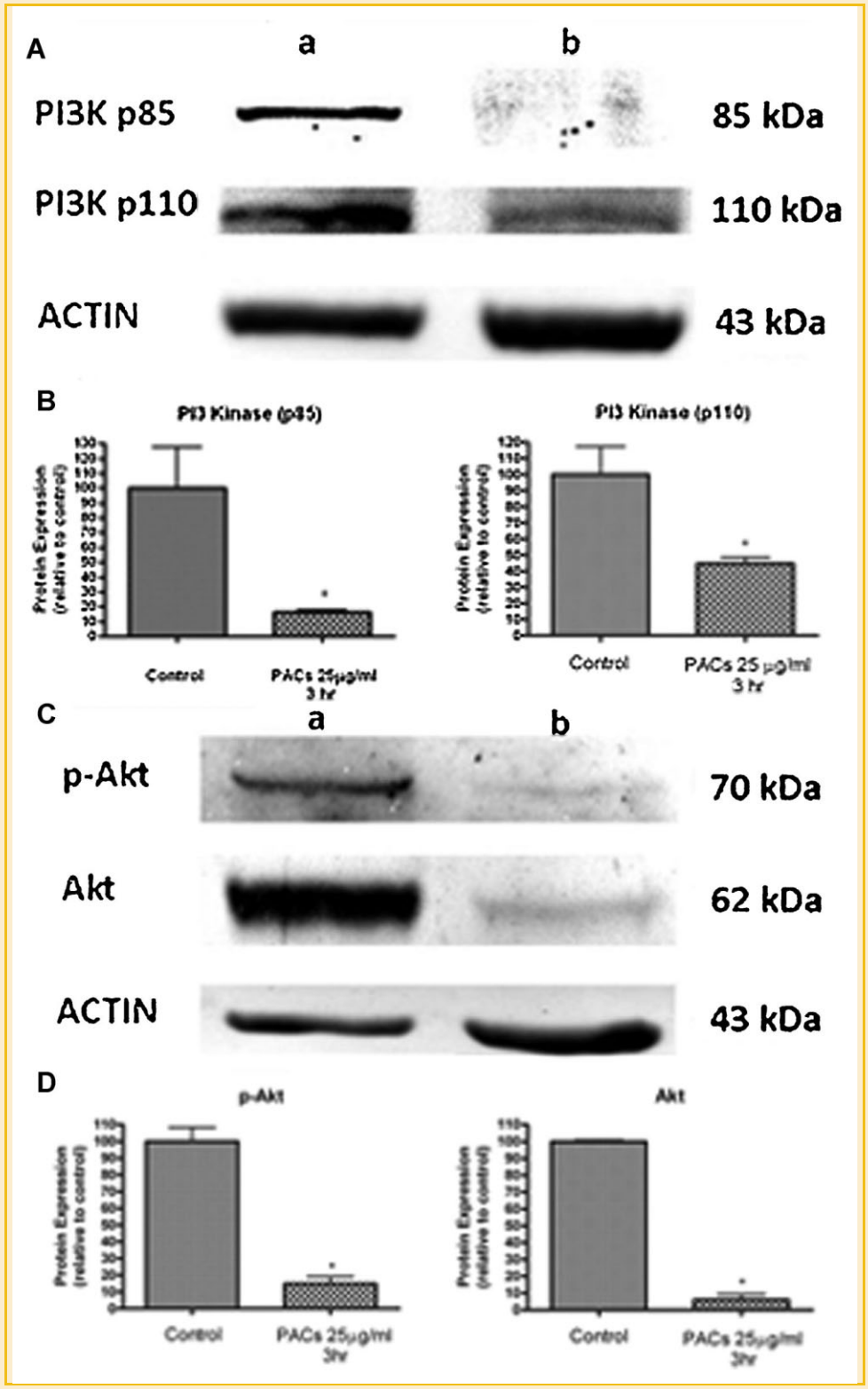

Fig. 5. Cranberry PACS affect the expression of PI-3 kinase pathway proteins. A: Western blot analysis of PI-3K p85, PI-3K p110 and actin protein expression levels in vehicle-treated cells (a) and in PACs (25 μg/ml)-treated (for 3 h) cells (b). Each blot shown is representative of results obtained from three separate experiments with assay duplicates. B: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$). C: Western blot analysis of pAkt, Akt and actin protein expression levels in cells as described above. Actin was used as a loading control. Each blot is representative of results obtained three separate experiments with assay duplicates. D: Histograms representing densitometric analyses of Western blots with asterisks denoting a statistically significant difference from control ($P < 0.05$).

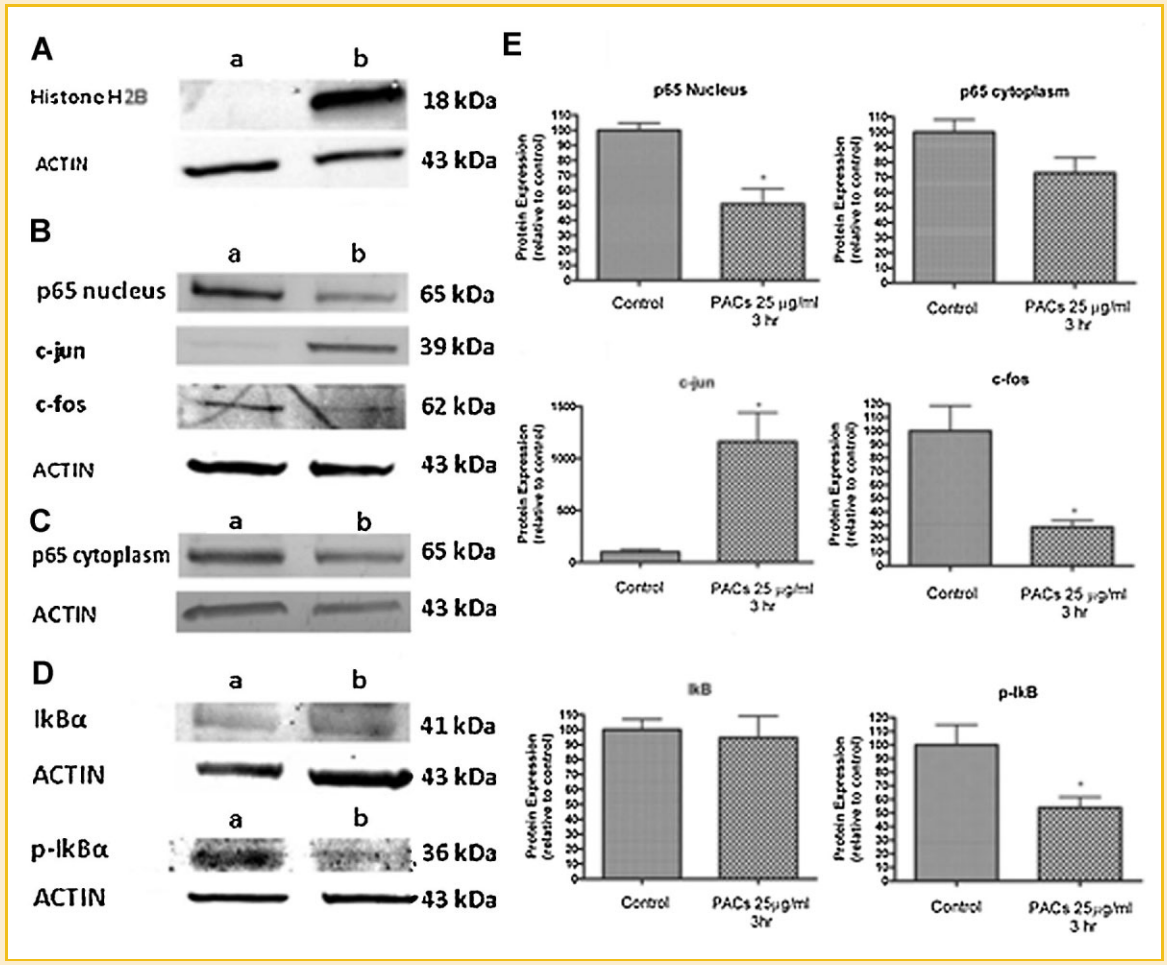

Fig. 6. Cranberry PACs affect protein expression levels of proteins in the NF-κB and AP1 pathways. A: Western blot analysis of histone H2B protein expression levels in cytosolic fraction (a) and in nuclear fraction (b) from DU145 cells treated with 25 μg/ml of PACs for 3 h. Nuclear-enriched cell lysates and cytosolic cell lysates (10 μg of protein each) were electrophoresed through a 10% SDS–PAGE gel and immunoblotted with anti-histone H2B. Actin was used as the loading control. Each blot is representative of three separate experiments with assay duplicates. B: Western blot analysis of p65, c-jun and c-fos protein expression levels in the nuclear enriched cell lysate isolated from vehicle treated control cells (a) and isolated from DU145 cells treated with 25 μg/ml PACs for 3 h (b). Actin was used as a loading control. Each blot shown is representative of three separate experiments with assay duplicates. C: Western blot analysis of p65 protein expression levels in the cytosolic cell lysate isolated from vehicle treated control cells (a) and isolated from cells treated with 25 μg/ml PACs for 3 h (b). Actin was used as a loading control. D: Western blot analysis of IκBα and pIκBα protein expression levels in cytosolic cell lysates isolated from vehicle treated control cells (a) and isolated from DU145 cells treated with 25 μg/ml of PACs for 3 h (b). Actin was used as a loading control. Each Western blot shown is representative of results obtained three separate experiments with assay duplicates. E: Histograms representing densitometric analysis of each of the Western blots of p65 (from nuclear and from cytosolic fractions), c-jun, c-fos, IκBα and p-IκBα with the asterisk denoting a statistically significant difference from control ($P < 0.05$).

the cytotoxic response associated with PACs treatment of DU145 cells. Cranberry PACs were shown to be cytotoxic to these cells post 6 h of treatment. However, most interestingly, PACs also were able to inhibit MMP-2 and MMP-9 activity in these cells at earlier times which occurred in the absence of cellular cytotoxicity in response to PACs treatment. This finding suggests that cranberry PACs may have the ability to modify MMP's behaviour directly. The findings that cranberry PACS can inhibit MMPs in DU145 cells are in agreement with studies which have shown that grape seed PACs can also inhibit MMPs in DU145 cells [Vayalil et al., 2004]. Similarly, cranberry PACs have been shown to inhibit MMP production and activity in macrophages stimulated with LPS [La et al., 2009]. Interestingly, in this study, the macrophages did not exhibit any significant changes in cellular viability after 24-h treatment with PACs at doses of 100 μg/ml [La et al., 2009]. This suggests that cranberry PACs may not be cytotoxic to untransformed cells, though further research is needed to determine the effects of cranberry phytochemicals upon untransformed human prostate cells. Furthermore, it would be important to determine the effects of PACs on other prostate cancer cell lines such as LNCaP cells or PC3 cells in order to ascertain if the effects seen in DU145 cells is a cell type/cell-specific response or if the cellular response to cranberry PACs treatment occurs in a multi-factorial manner and in the same way in different prostate cancer cells. Cocoa procyanidins have been shown to inhibit the expression and activation of MMP-2 in vascular smooth muscle cells by direct inhibition of MEK and MT1-MMP activities [Lee et al., 2008].

The cranberry PACs were able to inhibit the MMP-2 and MMP-9′ activities by affecting the biological signalling within these DU145

cells as PACs treatment affected the expression of a number of proteins in the MAPKs and PI-3 kinase signal transduction pathways and could also disrupt the translocation of NF-κB to the nucleus. Different flavonoids and their effects upon MMP activity have been recently examined and these studies have suggested that they limit the activity of MMPs [Ende and Gebhardt, 2004]. The current study suggests that the decrease in MMP activity seen in response to cranberry PACs treatment occurs in a multi-factorial manner. One of these factors is the decrease in translocation of NF-κB to the nucleus leading to a proposed decrease in the transcription rate of NF-κB mediated genes. When IκBα becomes phosphorylated, it is no longer able to inhibit the translocation of p65 to the nucleus. In this study, no change in the amount of IκBα occurred but there was a pronounced decrease in the amount of phosphorylated IκBα following treatment of DU145 cells with cranberry PACs. Such a decrease in phosphorylated IκBα in the cytosol in response to cranberry PACs treatment could potentially decrease the ability of p65 to translocate to the nucleus. In this regard, the effects of flavonoids upon various cancer cell lines, including DU145 human prostate cancer cells, have been examined and these studies have found a decrease in both NF-κB translocation and DNA binding after treatment with this class of compounds [Woo et al., 2006; Shen et al., 2010; Yun et al., 2009]. Recently, a study has shown that PACs from cranberries can affect both the expression and activity of select MMPs *in vitro* and in this study, cranberry derived PACs were able to inhibit the activation of p65 in human macrophages [La et al., 2009]. Interestingly, a study by Vayalil et al. [2004], which found that grape seed PACs decreased MMP-2 and -9 activities in DU145 cells, showed that this effect of grape seed PACs was linked to decreases in both NF-κB translocation and DNA binding which is agreement with the findings reported herein with cranberry PACs. The effects of grape seed PACs were also attributed to decreases in phosphorylation of three MAP kinase pathway proteins [Vayalil et al., 2004]. In our study, the cranberry PACs-mediated inhibition of MMP-2/-9 activities was associated with increases in the phosphorylation status of p38 and ERK1/2. This difference may be due to the fact that PACs from different sources contain different compounds and exert their effects upon the same cell line differently. For example, grape seed PACs have only B-type linkages between their monomers, whereas cranberries more commonly have A-type linkages [Neto, 2007]. Acacetin, a flavone has been shown to inhibit the invasion and migration of DU145 cells via inactivation of the p38 MAPK signalling pathway [Shen et al., 2010]. Alternatively, cranberry PACs may be inducing cellular stresses upon the cell earlier than grape seed PACs, thereby causing an initial increase in the phosphorylation of p38, which is activated during periods of cellular stress [Krishna and Narang, 2008]. The activation of p38 has been associated with programmed cell death, and in this case cranberry PACs may be activating p38 in order to induce apoptosis [Kima et al., 2007; Chen and Wong, 2008]. Previous research in our lab has also demonstrated that at 25 μg/ml concentration cranberry PACs caused DU145 cells to undergo apoptosis (unpublished observations). The activation of ERK1/2 in DU145 cells in response to cranberry PACs treatment is also very interesting as this pathway is commonly associated with cellular proliferation and growth. It is suggested that the activation of this particular pathway is due, in part, to some of the cellular stresses imposed upon the cell by the cranberry PACs treatment, and that activation of these proteins may be part of a compensatory response whereby the cells are attempting to maintain homeostasis. In this regard, in response to 2,3,5-*tris*-(glutathion-*S*-yl)hydroquinone (TGHQ), a compound which after treatment results in oxidative stress, the ERK1/2 pathway was induced in non-transformed epithelial cells [Ramachandiran et al., 2002]. The ERK1/2 activation by TGHQ was suggested to be a compensatory mechanism exerted by cells since blocking this pathway with a selective inhibitor prevented the cytoprotective effects of ERK1/2 activation and resulted in cell death [Ramachandiran et al., 2002]. Cranberry PACs treatment of DU145 cells also resulted in alterations in the expression levels of phosphorylated JNK1/2. The anti-metastatic potential of fisetin, a naturally occurring flavonoid, involves inactivation of the PI-3 kinase/Akt pathway and JNK signalling pathways with a concomitant down-regulation of MMP-2/9 expressions in prostate cancer PC-3 cells [Chien et al., 2010]. Grape seed extract has been shown to inhibit EGF-induced and constitutively active mitogenic signalling but activate JNK in DU145 cells and this was suggested to have a possible role in anti-proliferation and apoptosis in these cells [Tyagi et al., 2003].

The Akt pathway is commonly associated with cellular survival and the evasion of apoptosis. The over-expression of these proteins has been found to occur in many patients with prostate cancer [Le Page et al., 2006]. In this study, in response to cranberry PACs treatment, both the phosphorylation status of Akt and expression of Akt were affected in DU145 cells, along with a decrease in expression of their upstream mediators, the PI-3 kinases. A decrease in the phosphorylation of PI-3K/Akt proteins has been shown to cause human prostate and colon cancer cells to undergo apoptosis [Hsu et al., 2000; Engelbrecht et al., 2007]. Quercetin, a flavonoid present in red wine has been shown to affect the tumour necrosis factor α-mediated up-regulation of MMP-9 expression by affecting the expression of PI-3 kinase [Huang et al., 2009]. The decrease in expression of PI-3 kinases by cranberry PAC treatment seen in our study may, in part, offer an explanation why the viability of the DU145 cells diminishes substantially after 6 h of treatment with cranberry PACs, as a dramatic decrease in expression of PI-3K/Akt proteins could cause human prostate cancer cells to undergo apoptosis. In this regard, a study examining the effects of grape skin extract treatment on human prostate cancer cells showed that the expression of phosphorylated Akt and Akt decreased [Hudson et al., 2007]. This study also suggested another possible explanation which was that treatment with grape skin extract could be affecting the ubiquitination and the subsequent degradation of this protein. Whether or not cranberry PACs affects the ubiquitination status of cellular proteins in DU145 cells remains to be determined. Cranberry PACs treatment of DU145 cells also resulted in alterations in the expression levels of phosphorylated JNK1/2. The anti-metastatic potential of the flavonoid, fisetin, involves inactivation of the PI-3 kinase/Akt pathway and JNK signalling pathways with a concomitant down-regulation of MMP-2/9 expressions in prostate cancer PC-3 cells [Chien et al., 2010].

Many different types of cancerous cells over-express the MMP inducer EMMPRIN, or extracellular matrix metalloproteinase inducer [Riethdorf et al., 2006], and the modulation of expression

of this protein has been suggested as a potential target for the treatment of cancer [Dean et al., 2009]. Conversely, the increased expression of certain inhibitors of MMP activity (notably the TIMPs, or tissue inhibitors of MMPs) has been associated with decreased vascular formation and decreased cellular migration and invasion within cancerous cells [Valente et al., 1998; Hinz and Ramer, 2008]. This study also showed that cranberry PACs can affect the expression of MMP-2 and MMP-9 activity by affecting the expression of known cellular modulators of MMP activity. In this regard, the expression of EMMPRIN (a documented activator of MMPs) decreased and the expression of TIMP-2 (a documented inhibitor of MMP activity) increased in DU145 cells in response to cranberry PACs. Such changes would affect and regulate the behaviour of MMP-2 and MMP-9 in response to cranberry PACs. Unexpectedly, the expression of TIMP-1 decreased after treatment with cranberry PACs. TIMP-1 is an inhibitor of MMP-9 activity and as such these findings suggest that the cranberry PACs-mediated inhibition of MMP-9 does not apparently involve an activation of TIMP-1 and occurs apparently independent of this fact. In this regard, the acquisition of more quantitative evidence/findings would allow the determination/evaluation of actual MMP/TIMP ratios which would potentially provide more information regarding the possible alterations of protease/anti-protease balance due to PACs treatment. Alternatively, the decreases in TIMP-1 protein expression levels in response to cranberry PACs treatment may represent part of the cell's attempts to maintain homeostasis in response to the cellular stresses imposed upon the cell by cranberry PACs. Additionally, TIMP-1 (as well as other TIMP proteins) not only act as inhibitors of MMPs but can also act as pleiotropic molecules in several physio-pathological conditions including prostate cancer. As such, the exact relationships which occur between MMPs and TIMPs in response to PACs remain to be determined and warrant further investigations to resolve the exact nature of this regulatory relationship.

Treatment of cells with PACs from the Japanese quince has also been shown to affect the activity of both MMP-2 and -9 in a dose-dependent manner [Strek et al., 2007]. This particular study hypothesized that these PACs isolated from the Japanese quince may be directly binding with the MMPs, therefore rendering them inactive. Baxter et al. [1997] have suggested that polyphenols can directly bind to proteins which have proline-rich regions in their structure. MMP-9 contains many proline-rich regions but whether or not cranberry PACs can affect MMP' activity in this manner is not known.

In summary, this study has demonstrated and characterized a novel relationship between cranberry PACs and the expression and regulation of MMPs in DU145 human prostate cancer cells. DU145 cells are hormone refractory and as such the response demonstrated may be reflective of a cell type-specific response. Future studies will be directed to examining the effects of cranberry PACs on MMP expression and regulation of MMPs in hormone sensitive prostate cancer cells such as LNCaP cells. It is possible that the response which occurs in such cells may be different or more pronounced. The DU145 cell line is one of the more aggressive prostate cancer cell lines as it is androgen independent. As many prostate cancers are relatively slow growing, it may be beneficial to gain insight into how cranberry PACs could affect less aggressive or androgen sensitive/dependent prostate cancer cells. Our previous studies have shown that a whole cranberry extract is capable of affecting the expression of MMPs in DU145 cells. This study has extended our previous observations and has identified the specific nature of the compounds from cranberry which can account for cranberry's effects on MMPs. It is possible that the PACs are not the only compounds found in cranberry capable of affecting MMP expression. Studies are currently ongoing to examine the effects of other enriched fractions isolated from cranberry on the expression and regulation of MMPs in DU145 cells. It is possible that the PACs fraction may be responsible entirely for the behaviours noted, or alternatively that PACs may act in concert with some or all of these other fractions in either an additive or synergistic manner to further affect/modify the expression of MMPs in DU145 cells. Such studies are currently ongoing. The effects of dietary cranberry on prostate cancer development in an animal model, to the best of our knowledge, have not yet been reported. Intraperitoneally injected cranberry extracts decreased the growth of DU145 prostate tumour cell explants in mice [Ferguson et al., 2006] and it is currently not known whether similar effects would occur as a result of ingestion of cranberry extracts. Such studies are currently ongoing. Finally, this present study contributes to our understanding of how a naturally occurring 'bioactive' is capable of regulating the behaviour of prostate cancer cells and as such provides further evidence in support of the potential health benefits associated with the American cranberry and further provides support for its inclusion as part of a healthy diet because of its possible chemo-preventative and its possible chemo-protective properties against prostate cancer.

ACKNOWLEDGMENTS

This work was funded by grants from the National Cancer Institute of Canada (N.C.I.C.) through the auspices of the Canadian Cancer Society and by the P.E.I. Health Research Program. Bob Deziel is a recipient of a Canadian Cancer Society [P.E.I. Division] Scholarship. The authors wish to acknowledge Christian Krueger and Jess Reed, University of Wisconsin-Madison for their assistance with the MALDI-TOF MS analysis of the proanthocyanidin fraction.

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