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Pomegranate Juice, Total Pomegranate Ellagitannins, and Punicalagin Suppress Inflammatory Cell Signaling in Colon Cancer Cells

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Phytochemicals from fruits such as the pomegranate (*Punica granatum* L) may inhibit cancer cell proliferation and apoptosis through the modulation of cellular transcription factors and signaling proteins. In previous studies, pomegranate juice (PJ) and its ellagitannins inhibited proliferation and induced apoptosis in HT-29 colon cancer cells. The present study examined the effects of PJ on inflammatory cell signaling proteins in the HT-29 human colon cancer cell line. At a concentration of 50 mg/L PJ significantly suppressed TNFα-induced COX-2 protein expression by 79% (SE = 0.042), total pomegranate tannin extract (TPT) 55% (SE = 0.049), and punicalagin 48% (SE = 0.022). Additionally, PJ reduced phosphorylation of the p65 subunit and binding to the NFκB response element 6.4-fold. TPT suppressed NFκB binding 10-fold, punicalagin 3.6-fold, whereas ellagic acid (EA) (another pomegranate polyphenol) was ineffective. PJ also abolished TNFα-induced AKT activation, needed for NFκB activity. Therefore, the polyphenolic phytochemicals in the pomegranate can play an important role in the modulation of inflammatory cell signaling in colon cancer cells.

KEYWORDS: Cancer; pomegranate; AKT; COX-2; NFκB

INTRODUCTION

Cellular signaling activated by proinflammatory conditions can lead to the initiation and progression of cancer by inducing DNA damage and epigenetic changes, increased sensitivity to growth factors and abnormalities in the expression and activity of transcription factors that control cell cycle progression and survival (*1*). Specifically, chronic inflammation of the colon has been shown to increase the risk for colon cancer (*2*, *3*) making inflammatory signaling pathways a target for cancer prevention and treatment. Epidemiological studies suggest that the consumption of a diet rich in phytochemicals can reduce the risk of cancer (*4*). Fruits and vegetables, which contain a diverse range of phytochemicals, are suggested to have properties important to the prevention of cancer including antioxidant, antiinflammatory, and antiproliferative activities as well as modulatory effects on subcellular signaling pathways, induction of cell cycle arrest, and apoptosis (*5*−*7*).

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and in processed forms as juice, jam, and wine (*8*). Commercial pomegranate juice (PJ) shows potent antioxidant and antiatherosclerotic properties attributed to its high content of polyphenols including ellagitannins, ellagic acid, and other flavonoids (quercetin, kaempferol, and luteolin glycosides) (*9*−*13*). The most abundant of these polyphenols is punicalagin, an ellagitannin implicated as the bioactive constituent responsible for >50% of the juice's potent antioxidant activity. Punicalagin is abundant in the fruit husk and during processing is extracted into PJ in significant quantities reaching levels of >2 g/L juice (*14*).

The cyclooxygenase (COX) enzymes (COX-1 and COX-2) are expressed in a variety of cell lines and are responsible for the conversion of free fatty acids to prostanoids. COX-1 produces prostanoids that aid in the regulation of normal tissue homeostasis, whereas COX-2 expression results in prostanoids that induce inflammation. For this reason, COX-2 overexpression has been implicated in cancer initiation and progression. Solid tumors tend to produce high levels of prostanoids and exhibit increased levels of COX-2 expression (*1*). Tumor cells that do not express COX-2 proliferate more slowly in vivo (*15*), and COX-2 specific inhibitors decrease the proliferation of a number of cancer cell lines in vitro (*16*−*19*). COX-2 also inhibits apoptosis, which can aid in tumor cell survival (*20*−*22*). In vivo, COX-2 inhibitors have shown effectiveness in the inhibition of tumor production in mice and in clinical trials with familial adenomatous polyposis (FAP) patients (*23*, *24*).

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/nuclear factor kappa-B (NFκB) pathway positively

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affects COX-2 expression. PI3K directly activates AKT, which in turn phosphorylates and activates the IκB kinase (IKK) leading to NFκB activation. PI3K activity has been associated with colon cancer where phosphatase and tensin homologue gene (PTEN) mutations occur, as PTEN inhibits PI3K (*25*). Increased PI3K activity has been found in colon cancer cell specimens (*26*) and adenocarcinoma cell lines (*27*). In addition, inhibition of PI3K in colon and ovarian cancer cell lines leads to inhibition of cell proliferation (*28*). In HT-29 cells, the activation of NFκB by TNFα resulted in an increase in COX-2 protein expression, an effect that was inhibited by nonsteroidal antiinflammatory drugs (NSAIDS) (*29*). Consistent with the finding that the COX-2 promoter contains two NFκB binding sites (*30*), TNFα-regulated COX-2 protein expression in HT-29 colon cancer cells was found to be dependent upon NFκB activation (*31*).

Little information is available on the effects of pomegranate extracts on the cellular signaling pathways involved in cancer initiation and progression. The purpose of this study was to evaluate the effect of PJ, total pomegranate tannins (TPT), purified punicalagin, and ellagic acid (EA) on COX-2 and the signaling pathways leading to its expression in HT-29 colon cancer cell lines. Our results will further our understanding of how natural products may modulate cancer cell signaling and indicate possibilities for future investigation.

MATERIALS AND METHODS

Chemicals. NS398 was obtained from Sigma-Aldrich Co. (St. Louis, MO), wortmannin, the AKT kinase assay kit, phospho-p65 NFκB, total p65 NFκB, and antirabbit and antimouse antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the COX-2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TNFα and the cell titer glow assay were obtained from Promega Corp. (Madison, WI). McCoy's 5A medium was purchased from American Type Culture Collection (ATCC) (Manassas, VA), phosphate-buffered solution (PBS) was purchased from Invitrogen (Carlsbad, CA), and western blotting supplies were obtained from Biorad Laboratories (Hercules, CA).

Pomegranate Extract. Pomegranate juice (POM Wonderful LLC, Los Angeles, CA) is commercially available for human consumption and was used in concentrate form (contains 1.74 g/L punicalagin). Ellagitannins were purified from fruit husk as previously reported and analyzed for purity by HPLC and liquid chromatography electrospray ionization mass spectroscopy (LC−ESI/MS) (*14*). Concentrations of punicalagin and TPT were normalized to deliver equivalent amounts as those found in PJ.

Cell Culture. HT-29 colon cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were cultured in McCoy's 5A medium containing 10% FBS and 1% penicillin/streptomycin. Human myeloid KBM-5 cells were originally obtained from ATCC and were cultured in Iscove's modified Dulbecco's medium with 15% FBS. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and maintained in the linear stage of growth.

Cell Viability Assay. Proliferation was measured using the CellTiter-Glo luminescent cell viability assay (technical bulletin no. 288, Promega, Madison, WI). HT-29 cells were plated in 96-well plates at a density of 10 000 cells/well and incubated for 24 h. Cells were treated with either 100 μL of control media, vehicle (<0.3% DMSO), or the COX-2 specific inhibitor NS398 at concentrations from 20 to 80 mmol/L. After 48 h, plates were equilibrated at room temperature for 30 min, 100 μL of the assay reagent was added to each well, and cell lysis was induced on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescence signal, and results were read on an Orion microplate luminometer (Bertholds Detection Systems, Pforzheim, Germany). All plates had control wells containing medium without cells to obtain a value for background luminescence. Data are expressed as the percentage of untreated cells (treatment value − blank/vehicle value − blank) mean ± 95% CI for at least three replications.

Immunologic Detection of Proteins. HT-29 cells were plated in 100 mm dishes at a density of 500 000 cells/dish and incubated for 48 h. Media was then replaced with serum-free media for 24 h. Cells were pretreated for 1 h with vehicle control (<0.3% DMSO), wortmannin (0.2 μmol/L), PJ, TPT, or punicalagin at the indicated concentrations. TNFα (20 mg/L) was added directly to the dishes, and the cells were incubated for 30 min for NFκB and 24 h for COX-2 studies. After incubation with test products, the cells were rinsed in cold PBS and scraped into 450 μL of laemmli sample buffer. Cell samples were sheared using an insulin syringe, centrifuged at 14 000*g* for 5 min, and the supernatant was collected for analysis. Proteins were separated by SDS−PAGE, transferred to nitrocellulose membranes, and probed with either COX-2 antibody (1:100 dilution) or phospho and total p65 NFκB (1:1000 dilution). Bands were visualized via chemiluminescence using HRP-conjugated secondary antibodies. Bands were quantified using Biorad Quantity One software in conjunction with the Biorad densitometer GS-710 imaging system. Data are expressed as the percent decrease from TNFα-treated cells (mean ± 95% CI) and are representative of at least three separate experiments.

Immunoprecipitation and AKT Kinase Assay. HT-29 cells were plated in 100 mm dishes at a density of 500 000 cells/dish and incubated for 48 h. Media was replaced with serum-free media for 24 h. Cells were pretreated with vehicle control (<0.3% DMSO), wortmannin (0.2 μmol/L), or PJ at the indicated concentrations for 1 h, and TNFα (20 μg/L) for 30 min. The cells were then rinsed with cold PBS, and 500 μL of cold 1× cell lysis buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to each plate and incubated on ice for 5 min. The cells were scraped and lysed using an insulin syringe. Samples were centrifuged at 14 000*g* for 5 min, and the supernatant was collected for analysis. Immunoprecipitation for active AKT was accomplished by adding 20 μL of immobilized Akt antibody to 200 μL of cell lysate and incubating overnight at 4 °C with gentle rocking. The samples were microcentrifuged at 14 000*g* for 30 s at 4 °C. The pellet was washed with 500 μL of 1× cell lysis buffer, then 1× kinase buffer, and resuspended in 50 μL of 1× kinase buffer supplemented with 1 μL of 10 mmol/L ATP and 1 μg of glycogen synthase kinase-3 (GSK-3) fusion protein for 30 min. The reaction was terminated with 25 μL of 3× SDS sample buffer, and the mixture was vortexed and then microcentrifuged for 30 s at 14 000*g*. AKT activity was evaluated by SDS−PAGE utilizing phospho and total GSK-3 antibodies. Data are expressed as a percentage of TNFα-treated cells (mean ± 95% CI) and are representative of at least three separate experiments.

Electrophoretic Mobility Shift Assay (EMSA). KBM-5 cells were pretreated for 4 h with PJ, punicalagin, EA, or TPT, then stimulated with TNFα (0.1 nmol/L) for 30 min. Nuclear extracts were prepared and analyzed for NFκB activation by EMSA as previously described (*32*). Data are expressed as fold induction from untreated controls.

Statistical Analysis. The data presented are the means ± 95% confidence intervals of values from at least three independent experiments. To assess statistical significance, values were compared to controls using either the Student *t*-test or one-way ANOVA followed by Dunnett's test using Prism GraphPad 4 software. Significance was accepted at $p \leq 0.05$.

RESULTS

After 48 h of treatment with NS398, a dose-dependent decrease in cell viability was observed in HT-29 cells, with a maximum of 27% (95% CI = 25−30, $p \leq 0.01$) as compared to vehicle-treated controls. (**Figure 1**). Our results are consistent with other reports showing decreased cell viability by NS398 in colon cancer cell lines (*17*, *33*).

Treatment with PJ, TPT, and punicalagin significantly decreased TNFα-induced COX-2 protein expression in a dose-dependent manner (**Figure 2A−C**). At test concentrations normalized to deliver 50 mg/L punicalagin, COX-2 protein expression was significantly suppressed by PJ 79% (95% CI =

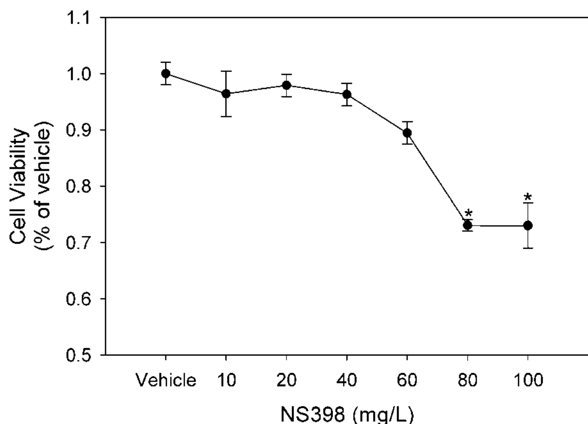

Figure 1. COX-2 inhibition decreases HT-29 colon cancer cell proliferation. Cells were treated with vehicle or NS-398 for 48 h. Cell proliferation was determined by the CellTiter-Glo luminescent cell viability assay. Data are expressed as a percentage of vehicle-treated controls, mean ± SE ($n = 3$). An asterisk indicates significant difference compared to vehicle controls ($p < 0.01$).

60−97, $p \leq 0.01$), TPT 55% (95% CI = 46−65, $p \leq 0.01$), and punicalagin alone 48% (95% CI = 22−75, $p \leq 0.01$) compared to that of TNFα-treated controls. Cells were also treated with wortmannin to evaluate the effect of PI3K inhibition on TNFα-induced COX-2 expression. PI3K inhibition by wortmannin decreased COX-2 expression by 20% compared to that of TNFα-treated controls (**Figure 2A**). Due to their lesser impact on COX-2 expression, TPT and punicalagin were tested at greater doses than PJ (up to 200 mg/L).

We investigated the effect of PJ on the activation of AKT and its downstream target NFκB, a transcription factor that positively affects COX-2 expression. Pretreatment with PJ significantly inhibited AKT activity in a dose-dependent manner, abolishing it completely at 50 mg/L (95% CI = 98−100.2, $p \leq 0.01$). PI3K inhibition with wortmannin also significantly inhibited AKT activity, illustrating the involvement of PI3K in its activation (**Figure 3**).

Pretreatment with 50 mg/L PJ reduced binding to the NFκB response element 6.4-fold (**Figure 5A**) and phosphorylation of the p65 subunit 92% (95% CI = 85−99, $p \leq 0.01$) compared to that of cells treated with TNFα alone (**Figure 4**). Purified TPT at a concentration of 100 mg/L suppressed NFκB binding 10-fold, punicalagin at 100 mg/L 3.6-fold, whereas 100 mg/L of EA (another pomegranate polyphenol formed from the hydrolysis of ellagitannins) had no effect on DNA binding (**Figure 5B−D**).

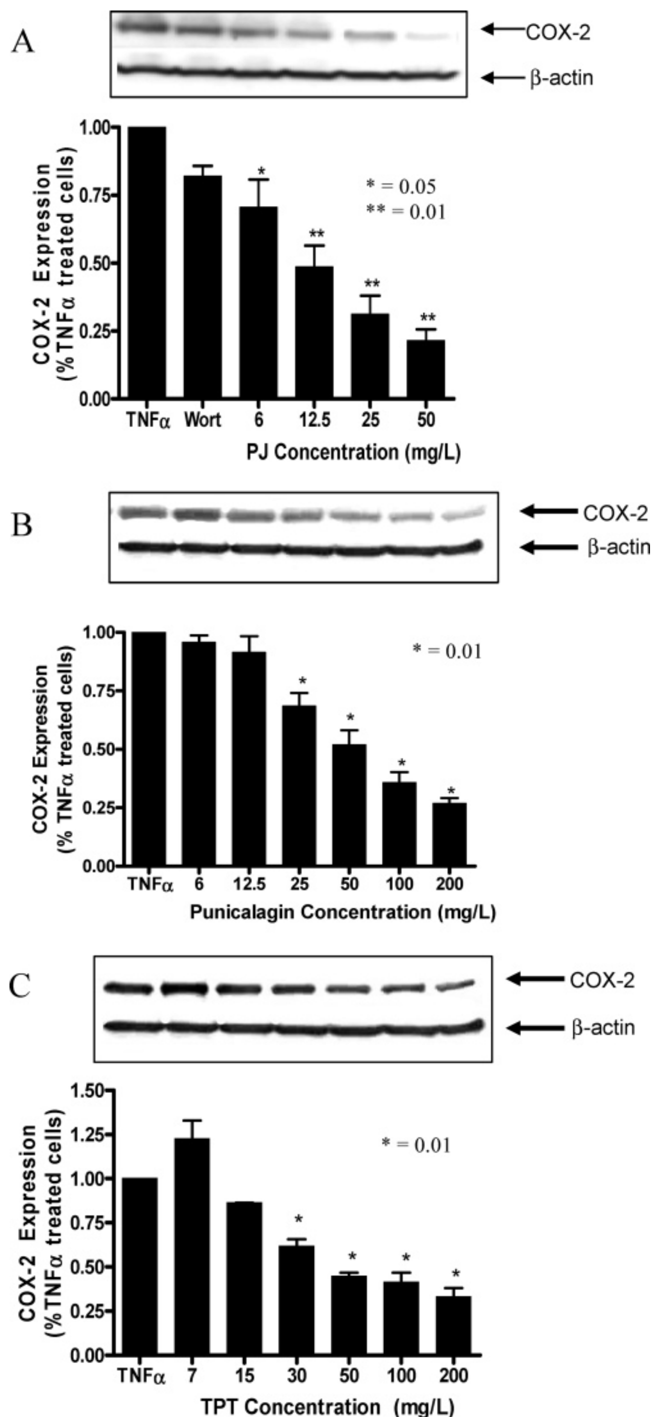

Figure 2. Effect of PJ, punicalagin, and TPT on TNFα-induced COX-2 protein expression in HT-29 colon cancer cells. Cells were pretreated with vehicle or PJ (**A**), punicalagin (**B**), or TPT (**C**) for 1 h then stimulated with TNFα (20 μg/L) for 24 h. COX-2 expression was determined by western blotting and chemiluminescence. Data were normalized to β-actin and expressed as a percentage of TNFα-treated cells, mean ± SE ($n = 3$). A single asterisk indicates significant difference ($p \leq 0.05$); a double asterisk indicates significant difference ($p \leq 0.01$) compared to TNFα-treated cells.

DISCUSSION

Previous studies in our laboratory have demonstrated a role for PJ and its purified ellagitannins in the inhibition of proliferation and induction of apoptosis in colon cancer cell lines (*11*). These observations are consistent with reported antiproliferative activity of pomegranate polyphenols in prostate and breast cancer cells (*34−36*). Studies have shown a correlation between enhanced COX-2 expression and an increase in cell proliferation (*37*). In the present study, we demonstrated inhibition of proliferation by treatment of HT-29 colon cancer cells with NS398, a COX-2 specific inhibitor. Therefore, we hypothesized that the modulation of COX-2 expression by PJ may be an important mechanism involved in its antiproliferative properties.

Pretreatment with PJ, TPT, and punicalagin decreased COX-2 expression in HT-29 cells in a dose-dependent manner. PJ proved to be more potent than purified TPT and punicalagin, most likely due to significant interactions with other bioactive polyphenols in PJ such as anthocyanins and flavonols. These data support the idea that separation of individual constituents from a whole entity such as PJ can often decrease overall activity due to an unanticipated requirement for other components.

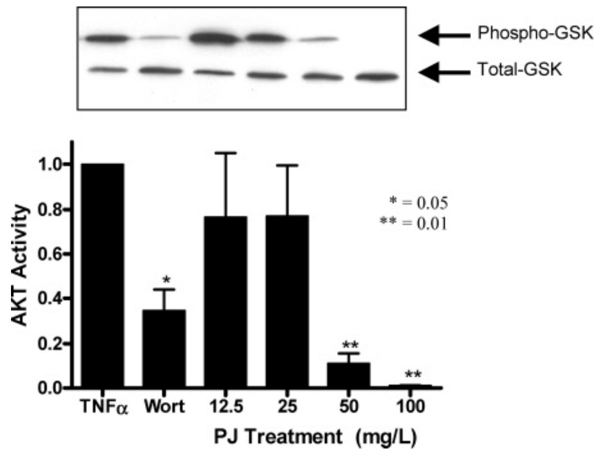

Figure 3. Effect of PJ on TNFα-induced AKT activity in HT-29 colon cancer cells. Cells were pretreated with vehicle, wortmannin (wort), or PJ for 1 h then stimulated with TNFα (20 μg/L) for 30 min. AKT kinase activity was determined by immunoprecipitation for active AKT followed by a kinase assay using a GSK-3 fusion protein. Results were visualized by western blotting and chemiluminescence. Data were normalized to total AKT and expressed as a percentage of TNFα-treated cells, mean ± SE ($n = 3$). A single asterisk indicates significant difference ($p \leq 0.05$); a double asterisk indicates significant difference ($p \leq 0.01$) compared to TNFα-treated cells.

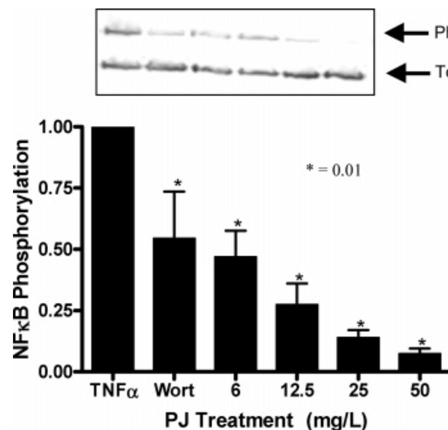

Figure 4. Effect of PJ on TNFα-induced NFκB (p65) phosphorylation (ser536) in HT-29 colon cancer cells. Cells were pretreated with vehicle, wortmannin (wort), or PJ for 1 h then stimulated with TNFα (20 μg/L) for 30 min. P65 phosphorylation was determined by western blotting and chemiluminescence using phospho and total p65 antibodies. Data were normalized to β-actin and expressed as a percentage of TNFα-treated cells, mean ± SE ($n = 3$). A single asterisk indicates significant difference ($p \leq 0.01$) compared to TNFα-treated cells.

COX-2 expression is mediated by signaling pathways such as PI3K/AKT/NFκB and mitogen-activated protein kinase (MAPK) (*29*). NFκB activity is modulated by PI3K signaling through AKT. In mesangial cells, the activation of PI3K resulted in the increased expression of COX-2 and an increase in cell proliferation (*37*) illustrating a specific relationship between PI3K and COX-2. Consistent with this observation, we have shown that pretreatment with PJ inhibits AKT activity, NFκB activation, and COX-2 expression in HT-29 cells.

Although some reports have concluded that COX-2 expression in HT-29 cells is NFκB dependent (*31*), our studies showed that inhibition of NFκB by wortmannin only partially decreased COX-2 expression. This result suggests that other signaling pathways may be essential, in concert with NFκB, in the modulation of COX-2 expression in HT-29 cells. The MAPK pathways (ERK1/2, p38, and SAPK) are a likely candidate for this role. MAPK has been shown to mediate COX-2 expression in a number of studies (*38*−*40*). In vitro, ERK and p38 have been shown to regulate NFκB activity (*30*). In addition, other studies suggest that both NFκB and MAPK may mediate COX-2 expression (*29*). However, the interrelationship of these signaling proteins has yet to be elucidated.

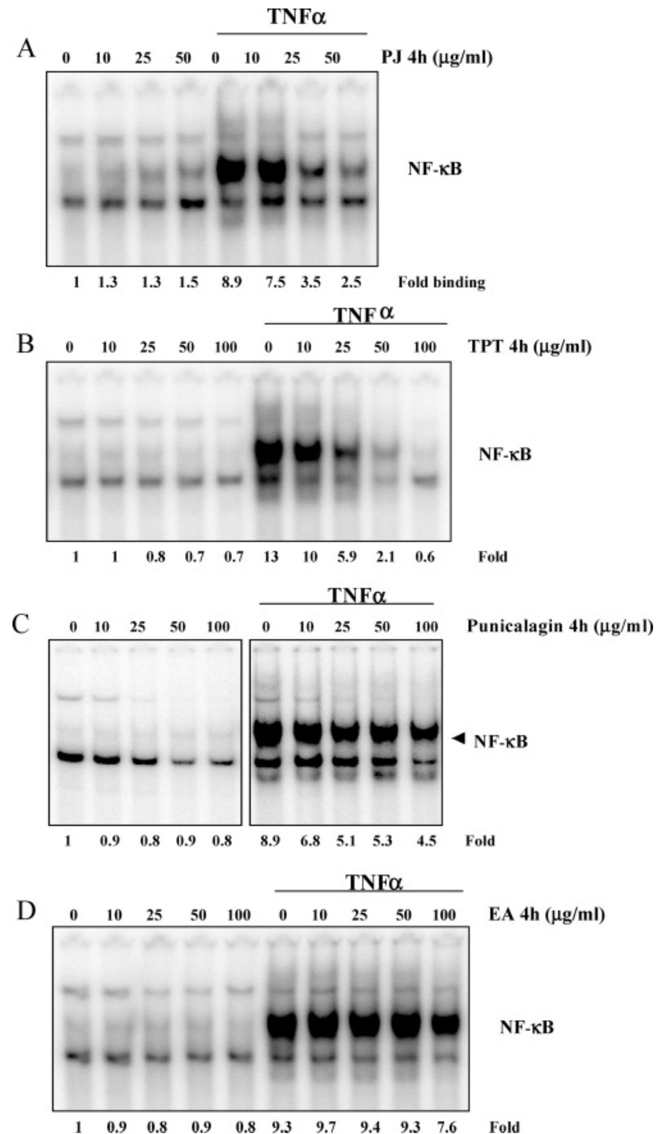

Figure 5. Effect of PJ, punicalagin, ellagic acid (EA), and total pomegranate tannins (TPT) on TNFα-induced NFκB (p65) DNA binding in KBM-5 human myeloid cells. Cells were pretreated with (**A**) PJ, (**B**) TPT, (**C**) punicalagin, or (**D**) ellagic acid (EA) for 4 h, stimulated with TNFα (0.1 nmol/L) for 30 min, and analyzed for NFκB p65 DNA binding by EMSA.

The control of COX-2 expression and related pathways by polyphenol-rich fruit extracts is not unprecedented, although studies on pomegranate polyphenols are limited. Topical application of pomegranate fruit extracts 30 min prior to TPA treatment of CD-1 mice resulted in inhibition of ERK1/2, p38, JNK1/2 activity, and COX-2 expression (*5*). Phytochemical-rich extracts of several cherries, berries, and crabapples have been shown to inhibit COX-1 and COX-2 (*41*), and lingonberry extracts have been shown to inhibit ERK1/2, p38, and MEK1/2 phosphorylation in UVB-treated JB6 P^+ mice (*42*). Curcumin, a polyphenol found in turmeric, has been shown to inhibit COX-2 gene expression in human colon cancer cell lines (*43*,

44). Tea polyphenols have also shown efficacy in inhibiting inflammatory signaling in human colon mucosa and cancer cells (*45*). These studies illustrate a role for phytochemical-rich dietary components in the modulation of proinflammatory signaling.

Although EA did not effectively prevent binding to the NFκB response element, in our previous study (*11*) EA showed significant inhibitory effects on the proliferation of several cancer cell lines. Therefore, its lack of effect on binding to the NFκB response element is not necessarily evidence of an absence of biologic activity of this phytochemical in cancer cell lines. The enhanced potency of PJ over its purified ellagitannins in our studies indicates significant interactions between the bioactive constituents present in PJ. Punicalagin is the major ellagitannin in PJ, however, other bioactive polyphenols such as the anthocyanins and flavonols are also abundant. Their presence is likely essential to the anticancer activity of PJ.

The bioavailability of flavonoids is as yet a relatively unexplored field. However, studies suggest that they are poorly absorbed in the upper gastrointestinal tract. Small intestinal absorption can range from 0% to 60% of the ingested dose, dependent upon the food source (*46*). Therefore, flavonoids may reach the colon either as unabsorbed forms or secreted as absorbed conjugates, which are then secreted in the bile. Whether the doses utilized in this study reflect that which would be found in the colon in vivo is yet to be elucidated. However, the inhibition of COX-2, AKT and NFκB provides insight into the anticancer mechanisms of the action of pomegranate juice in colon cancer cells and presents us with a direction for future studies into its role in the prevention and treatment of colon cancer.

ABBREVIATIONS USED

AKT, protein kinase B; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; FAP, familial adenomatous polyposis; GSK, glycogen synthase kinase; IKK, I-kappa B kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa-B; NS398, COX-2 inhibitor; NSAIDS, nonsteroidal antiinflammatory drugs; PBS, phosphate-buffered solution; PI3K, phosphatidyl inositol 3-kinase; PJ, pomegranate juice; PMSF, phenylmethanesulfonyl fluoride; PTEN, phosphatase and tensin homologue deleted on chromosome ten; TNFα, tumor necrosis factor α; TPT, total pomegranate tannins.

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