

Pomegranate juice protects nitric oxide against oxidative destruction and enhances the biological actions of nitric oxide

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Received 11 August 2005; revised 28 February 2006

Available online 19 April 2006

Abstract

Pomegranate juice (PJ), which is a rich source of potent flavonoid antioxidants, was tested for its capacity to protect nitric oxide (NO) against oxidative destruction and enhance the biological actions of NO. Employing chemiluminescence headspace analysis, PJ was found to be a potent inhibitor of superoxide anion-mediated disappearance of NO. PJ was much more potent than Concord grape juice, blueberry juice, red wine, ascorbic acid, and DL- α -tocopherol. As little as 3 μ l of a 6-fold dilution of PJ, in a reaction volume of 5000 μ l, produced a marked antioxidant effect, whereas 300 μ l of undiluted blueberry juice or nearly 1000 μ l of undiluted Concord grape juice were required to produce similar effects. PJ and other antioxidant-containing products were found to augment the anti-proliferative action of NO (DETA/NO) on vascular smooth muscle cell (rat aorta) proliferation. PJ was much more effective than the other products tested and elicited no effects when tested alone in the absence of added NO. Similarly, neither PJ nor the other products enhanced the anti-proliferative action of α -difluoromethylornithine, a stable substance that inhibits cell growth by NO-independent mechanisms. In order to determine whether PJ is capable of increasing the production of NO by vascular endothelial cells, PJ was tested for its capacity to upregulate and/or activate endothelial NO synthase (eNOS) in bovine pulmonary artery endothelial cells. PJ elicited no effects on eNOS protein expression or catalytic activity. Moreover, PJ did not enhance promoter activity in the eNOS gene (COS-7 cells transfected with eNOS). These observations indicate that PJ possesses potent antioxidant activity that results in marked protection of NO against oxidative destruction, thereby resulting in augmentation of the biological actions of NO.

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Keywords: Nitric oxide; Antioxidant activity; Polyphenols; Flavonoids; Vascular smooth muscle proliferation

Pomegranate juice (PJ) is a rich source of potent polyphenolic, flavonoid antioxidants (anthocyanins), which have been shown to possess anti-atherogenic properties [1]. Anthocyanins are glycosides of anthocyanidin and are the most abundant water-soluble plant pigments responsible for the red, blue, and purple colors of fruits. Flavonoids are potent antioxidants and their activity is

directly related to their unique chemical structures [2–4]. These substances are potent inhibitors of LDL (low-density lipoprotein) oxidation and are avid scavengers of reactive oxygen species that are generated both intracellularly and extracellularly [1]. The pomegranate fruit has been used extensively as a folk medicine in many cultures [5,6]. The fresh juice contains about 1.5% by weight of flavonoids, polyphenols, pectin, and ascorbic acid. The soluble polyphenol content is between 0.2 and 1%, depending on variety and includes mainly anthocyanins [7–9]. The seeds and peel also contain polyphenols and tannins. The total content of anthocyanins is higher in PJ than in any other fruit juice tested for antioxidant activity, thereby reflecting its

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¹ This work was funded by a grant from the Lynda and Stewart Resnick Revocable Trust to L.J.I. and C.N. Lynda and Stewart Resnick own the POM Wonderful Company, for which L.J.I. is a consultant.

high degree of antioxidant activity. For example, PJ was shown to possess greater antioxidant activity than that present in green tea or red wine, and the antioxidant activity was higher in juice extracted from whole pressed pomegranates than the juice obtained solely from arils [10].

One of the principal mechanisms for the rapid inactivation of NO in mammalian tissues is reaction with reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$) [11]. Under normal healthy conditions, sufficient endothelium-derived NO is produced and maintained to elicit its normal physiological actions. However, under conditions of oxidative stress, accelerated destruction of NO occurs, which is generally associated with the initiation and development of a host of cardiovascular diseases [12–14]. Since NO itself is a potent antioxidant and anti-inflammatory agent by virtue of its capacity to react with and remove certain reactive oxygen species, a decrease in endothelial NO levels means further oxidative stress and further destruction of NO. This vicious cycle of oxidative stress and diminished NO action can lead to such cardiovascular diseases as atherosclerosis [12–14]. In addition, perturbed shear stress represents a hemodynamic force of blood flow that can trigger signal transduction mechanisms that lead to increased oxidative stress and endothelial dysfunction attributed to diminished NO levels and actions [15]. NO can control vascular oxidative stress and the expression of redox-regulated genes [15]. An increase in endothelial NO can overcome and override the detrimental influence of oxidative stress on endothelial cell function [12–15]. For example, the systemic administration of supplemental quantities of arginine and antioxidants have been shown to slow or reverse the progression of atherosclerosis [15–25].

Antioxidants are well known to enhance the biological actions of NO by protecting the NO against oxidative destruction by reactive oxygen species [11]. Since PJ is a rich source of antioxidants, the objective of this study was to compare PJ with other products possessing antioxidant activity for their protective effects on NO subjected to conditions of oxidative stress. A chemiluminescence headspace assay was developed to measure antioxidant activity by monitoring the destruction or disappearance of NO in physiological salt solution by ($O_2^{\bullet-}$) generated by addition of pyrogallol. This is a unique antioxidant assay in that such a direct approach has not been reported previously. Moreover, antioxidant studies with PJ are relatively recent because PJ and the pomegranate fruit itself are rapidly gaining household popularity. In a second study, PJ was tested to determine whether it could enhance the biological activity of NO. More specifically, the model used was the inhibition of vascular smooth muscle cell proliferation by NO donor agents. One of the mechanisms by which NO is protective against atherosclerosis is the inhibition of vascular smooth muscle cell growth [26,27]. Finally, PJ was examined for its influence on endothelial NO synthase (eNOS) protein expression, eNOS catalytic activity, and eNOS gene promoter activity.

Experimental procedures

Materials

PJ concentrate (Wonderful variety, POM Wonderful, Los Angeles, CA) was used in this study as described previously [28]. Briefly, whole pomegranates were pressed to yield the juice from the entire fruit and then treated with pectinase to improve extraction of flavonoids. The PJ was filtered, pasteurized, concentrated 5-fold, and stored at -20°C until use. Commercially available Concord grape juice was purchased, refrigerated, and used undiluted within three days. Fresh blueberry juice was prepared by pressing freshly purchased whole berries, allowing the particulate matter to sediment at 25°C , decanting and refrigerating the upper layer, and testing within three days. Orange juice was prepared by pressing whole Valencia oranges, allowing the particulate matter to sediment at 25°C , decanting and refrigerating the upper layer, and testing within three days. The red wine used was a commercially available pinot noir (14% alcohol) from a vineyard in the central valley of California, and was tested 10 min after removing the cork. The white wine used was a commercially available chardonnay (13.5% alcohol) from a vineyard in the central valley region of California, and was tested 10 min after removing the cork. As a control for the alcohol content of the wines, 14% (v/v) ethanol was tested.

Measurement of antioxidant activity

The antioxidant activity of PJ and other substances was assessed as protection against the oxidative destruction of authentic NO in physiological salt solution. The NO levels were determined by chemiluminescence detection, as we reported previously [29,30]. Briefly, reactions were conducted in 50 mM, Tris-HCl, pH 7.4, containing 0.9% NaCl in a final volume of 5 ml under stirring at 37°C for 10 min. Reactions were conducted in sealed glass vessels in an atmosphere of air, which was connected to a Dasibi (model 2108) chemiluminescence detector. Reaction mixtures were purged with air at a constant flow rate of 200 ml/min. The headspace above the reaction mixture was thereby swept into the chemiluminescence detector at 200 ml/min. Signals from the detector were analyzed with a Hewlett Packard HP 3396 Series II Integrator and recorded as the area under the curve (see [30]). Pyrogallol was used as a ($O_2^{\bullet-}$) generator and was added as indicated at a final concentration of $10\ \mu\text{M}$ immediately prior to addition of $1\ \mu\text{M}$ DEA/NO. DEA/NO is a popular NO donor agent that possesses a short half-life (2–3 min in water) and releases NO quickly [31]. When PJ and other products were tested, each was added just prior to addition of pyrogallol. All substances were added by injection into the reaction mixture through a side port on the reaction vessel with the aid of a gas-tight syringe.

Measurement of cell proliferation

Rat aortic smooth muscle cells (RASMC) were a generous gift from Dr. Steven Gross (Cornell Medical College, NY), and were grown as described previously [26]. Subcultured strains were used between passages 15 and 25. Exponentially growing RASMC were trypsinized and resuspended in fresh DMEM–Hepes medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were seeded in 12-ml plates at a density of 3×10^3 cells/cm² and incubated at 37 °C in a humidified atmosphere of 5% CO₂–95% air. After 24 h, the cells were washed twice with PBS. The growth medium was replaced with 1 ml of arginine-free DMEM–Hepes supplemented with 5% FBS and 50 µM L-arginine, and any test agents and 0.5 µCi [³H]thymidine (6.7 Ci/mmol; NEN) were added. In determining the rates of DNA synthesis, a modification of the [methyl-³H]thymidine incorporation procedure described previously [32] was used. Cell proliferation data are expressed as percent of control, as described previously.

Cell culture using BPAEC and COS-7 cells

Bovine pulmonary artery endothelial cells (BPAEC) were obtained from Cambrex (Santa Rosa, CA) and were used between passages 3 and 10. COS-7 cells were obtained from ATCC. Both cell types were cultured at 37 °C in a humidified atmosphere of 5% CO₂ using Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Western blot analysis

Two days before each experiment, 1×10^6 BPAEC were seeded in 6-well plates. After overnight incubation (adhesion), the medium was changed to phenol red-free DMEM containing 10% (v/v) charcoal-treated serum. The indicated concentration of PJ (L, 600-fold dilution from PJ concentrate; H, 300-fold dilution from PJ concentrate) was incubated with cells for 12, 24 or 48 h. In order to detect eNOS protein, 10 µg of total protein were separated by exposure to 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Gels were transferred to a PVDF-membrane, and then placed in blocking solution (Tris-buffered saline (10 mM Tris–HCl, pH 8.0) containing 0.05% Tween 20 and 5% nonfat milk) for 1 h. Blots were incubated for 1 h with anti-eNOS monoclonal antibody (BD Transduction Laboratories, KY) or anti-actin monoclonal antibody (Chemicon, CA), washed with Tris-buffered saline containing 0.05% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibody. Bound IgG was visualized using an enhanced chemiluminescence detection system (Pierce, IL) according to the manufacturer's protocol. Band intensities were quantified with NIH-image software.

Measurement of eNOS catalytic activity

Two days before each experiment, 1×10^6 BPAEC were seeded in 6-well plates. After overnight incubation (adhesion), the medium was changed to phenol red-free DMEM containing 10% (v/v) charcoal-treated serum. The indicated concentration of PJ (L, 600-fold dilution from PJ concentrate; H, 300-fold dilution from PJ concentrate) was incubated with cells for 24 and 48 h. eNOS activity was measured by monitoring the conversion of [³H]arginine to [³H]citrulline using the NOS Assay Kit (Cayman Chemical, MI). BPAEC were washed with cold PBS, and harvested using a cell scraper and collected by centrifugation. Cell pellets were resuspended in homogenization buffer (25 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 1 mM EGTA) and centrifuged at 12,000 rpm for 5 min at 4 °C. The enzymatic reactions were conducted in 25 mM Tris–HCl, pH 7.4, containing 3 µM BH₄, 1 µM FAD, 1 µM FMN, 1 mM NADPH, 20 nCi/µl [³H]arginine, 600 µM CaCl₂, and 0.1 µM calmodulin at room temperature for 30 min. Enzyme reactions were terminated by addition of stop buffer (50 mM Hepes, pH 5.5, containing 5 mM EDTA) followed by addition of 100 µl of equilibrated resin to remove the unconverted [³H]arginine. The eluates were transferred to a scintillation vial and scintillation fluid was added. Radioactivity was quantified by liquid scintillation spectrometry. Total protein concentrations were measured by Dc protein assay (Bio-Rad, CA).

Transient transfection and luciferase assay

To investigate whether PJ enhances eNOS transcriptional activity, two days before each experiment, 2×10^5 COS-7 cells were seeded in 12-well plates. After overnight incubation (adhesion), the medium was changed to phenol red-free DMEM containing 10% (v/v) charcoal-treated serum. Transfections were performed with Polyfect (Qiagen, CA) according to the manufacturer's protocol. Briefly, COS-7 cells were transfected with 1 µg eNOS (–1193/+109)-luciferase cDNA and 10 ng β-galactosidase cDNA. At 24 h after transfection, cells were incubated with the indicated concentration of PJ for 24 h. Cells were washed with PBS and lysed with luciferase assay system lysis buffer. Luciferase activity was measured in cellular extracts using the luciferase assay system (Promega, WI). To correct for transfection efficiency, luciferase activity was normalized to the corresponding β-galactosidase activity, which was measured using the β-galactosidase assay system (Promega, WI).

Statistical analyses

Data for the chemiluminescence and cell proliferation experiments were analyzed statistically using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test to evaluate statistical significance

between any two groups. Probability values of <0.05 were taken to indicate statistical significance. Data were obtained from four (Western blot and eNOS activity) or three (luciferase activity) separate experiments. Each value represents the mean \pm SEM.

Results

Antioxidant activity of PJ and other products

The objective of this series of experiments was to determine the antioxidant activity of PJ as assessed by protection against superoxide anion-mediated destruction of NO. Chemiluminescence headspace analysis was used to monitor the disappearance of NO in the gas phase. Authentic NO was generated from DEA/NO, which has a half-life in aqueous solution at 25 °C of 2–3 min. Fig. 1 illustrates typical tracings obtained from the chemiluminescence detection of NO. The concentration of NO is a reflection of the area under the curve (AUC), which is calculated automatically by integration after the sample is assayed for exactly 10 min. AUC for authentic NO under control conditions was assigned an arbitrary value of 100. Pyrogallol catalyzes the production of ($O_2^{\cdot-}$) from the oxygen present in the aqueous buffer solution used in the assay. Addition of pyrogallol to buffer containing NO resulted in the rapid disappearance of the NO, as indicated by the much smaller AUC (Fig. 1). On the other hand, inclusion of PJ in the solution just prior to addition of pyrogallol and DEA/NO markedly protected against the pyrogallol-elicited dis-

appearance of NO. The concentration of PJ used in this experiment was 100 μ l of a 30-fold dilution of PJ concentrate (5-fold) added to a reaction volume of 5 ml. The 5-fold PJ concentrate was supplied by the manufacturer (POM Wonderful) for testing purposes. Thus, the final dilution of PJ in the reaction mixture was 300-fold. Three tablespoonfuls (45 ml) of PJ concentrate added to 6-oz (180 ml) of water (5-fold dilution) yields the same dilution as the commercially available PJ from POM Wonderful. Since the present experiments employed a 30-fold dilution rather than a 5-fold dilution of PJ concentrate, the concentration of antioxidants in a typical glass of PJ is approximately 6-fold higher than the stock solution used in the present experiments. Similar experiments using the undiluted PJ from POM Wonderful yielded virtually identical data (data not shown), indicating that the active antioxidants are chemically stable over the period of time since production of the undiluted juice (approximately 2- to 3-week).

Fig. 2A illustrates the concentration-dependent antioxidant effect of PJ against oxidative destruction of NO. PJ elicited significant antioxidant action at concentrations ranging from as little as 3 to 100 μ l of 30-fold dilution of PJ concentrate added to a 5 ml reaction volume. The antioxidant action of PJ was compared with that of certain other natural products containing antioxidant activity. Freshly expressed blueberry juice showed appreciable antioxidant activity in the present assay system (Fig. 2B). The quantity required for significant antioxidant activity was 300 μ l of undiluted juice. Therefore, PJ was much more potent than blueberry juice in protecting NO against oxidative destruction as determined in the present assay. Additional products were tested including Concord grape juice, orange juice, red wine, white wine, alcohol, vitamin C, and vitamin E. Fig. 3 illustrates the effects of some of these products. At test quantities of 300 μ l, only Concord grape juice and red wine showed significant antioxidant activity, whereas orange juice, white wine, and 14% alcohol were without effect. Volumes of 1000 μ l of the grape juice and red wine produced greater effects than the 300 μ l illustrated, but the larger volumes of orange juice, white wine, and 14% alcohol were still without effect (data not shown). Moreover, vitamin C (ascorbic acid) and vitamin E (DL- α -tocopherol), either individually (0.1 mM) or in combination (0.1 mM each) possessed no significant antioxidant activity in protecting NO against oxidative destruction (data not shown).

Inhibition of vascular smooth muscle cell proliferation

The purpose of these experiments was to ascertain the effectiveness of PJ and other antioxidant-containing products in augmenting the anti-proliferative action of NO in cell culture. We have reported previously that NO is a potent inhibitor of growth of rat aorta smooth muscle cells (RASMC) in vitro [26]. Employing the same cell culture system, the NO donor agent, DETA/NO (half-life of about 24-h in cell culture medium [31]), inhibited

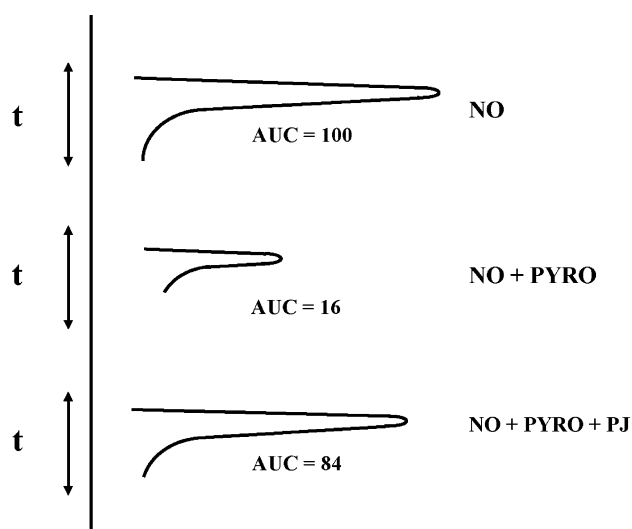


Fig. 1. Quantification of NO by chemiluminescence detection. Data represent actual experimental tracings obtained from a Hewlett Packard HP 3396 Series II Integrator. The top curve represents NO generated from DEA/NO (NO donor agent used) added to a final concentration of 1 μ M. The middle curve illustrates the destructive effect of 10 μ M pyrogallol (PYRO) on NO, added just prior to DEA/NO. The bottom curve shows the protective effect of 100 μ l of 30-fold dilution of PJ concentrate (PJ) against PYRO-elicited destruction of NO, when added just prior to PYRO and DEA/NO. AUC, area under the curve; t, time (10 min).

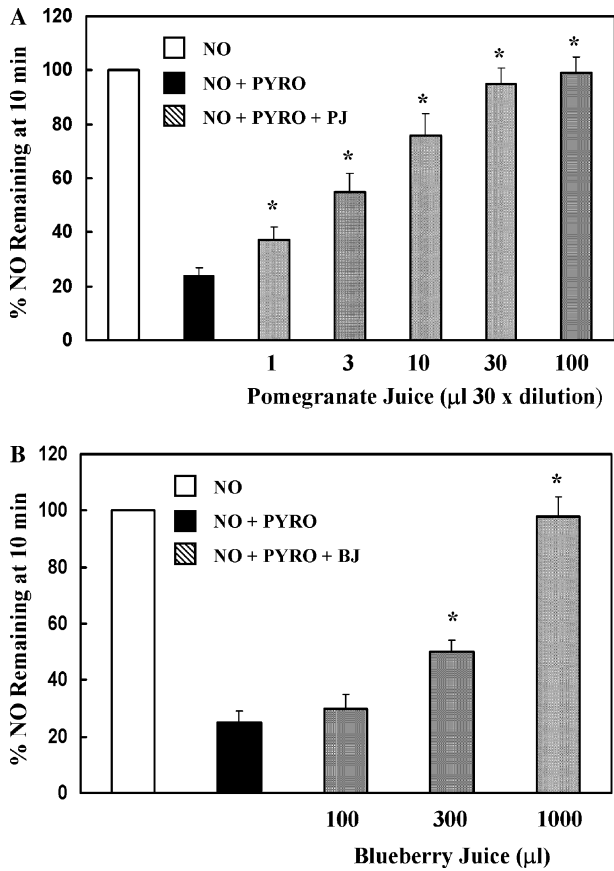


Fig. 2. (A) Antioxidant effect of PJ as determined by protection against oxidative destruction of NO. Experiments were conducted as described in Experimental procedures. PJ was tested at the indicated volumes of 30-fold dilution of concentrate in a reaction volume of 5 ml. Data are expressed as % NO remaining after 10 min of reaction. Data represent means \pm SEM of eight separate experiments. PYRO, pyrogallol (10 μ M). *Significant difference ($p < 0.05$) from NO + PYRO. (B) Antioxidant effect of blueberry juice as described in (A). Undiluted juice was tested in the volumes indicated. Data represent means \pm SEM of eight separate experiments. BJ, blueberry juice; PYRO, pyrogallol (10 μ M). *Significant difference ($p < 0.05$) from NO + PYRO.

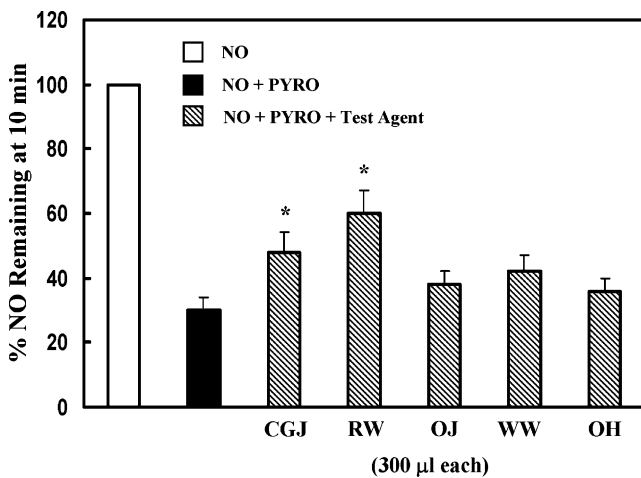


Fig. 3. Influence of Concord grape juice (CGJ), red wine (RW), orange juice (OJ), white wine (WW), and 14% (w/v) ethanol (OH) on oxidative destruction of NO. Experiments were conducted as described in Fig. 2. Volumes tested were fixed at 300 μ l of undiluted product. *Significant difference ($p < 0.05$) from NO + PYRO.

RASMC proliferation at concentrations of 10–30 μ M (Fig. 4A). The actual steady-state concentrations of pure NO in solution in the presence of these concentrations of DETA/NO are approximately 30–90 nM [31]. Addition of PJ to cell cultures produced a marked augmentation of the anti-proliferative action of NO (Fig. 4A). As little as 3 μ l of 30-fold dilution of PJ concentrate added to 1 ml of cell culture medium elicited significant effects. This represents a final dilution of PJ in the cell culture medium of nearly 2000. In fact, in the presence of added PJ, even the 3 μ M concentration of DETA/NO, which was inactive when tested alone, now elicited a substantial anti-proliferative effect. When tested alone, in the absence of added DETA/NO, the PJ was completely without effect on cell

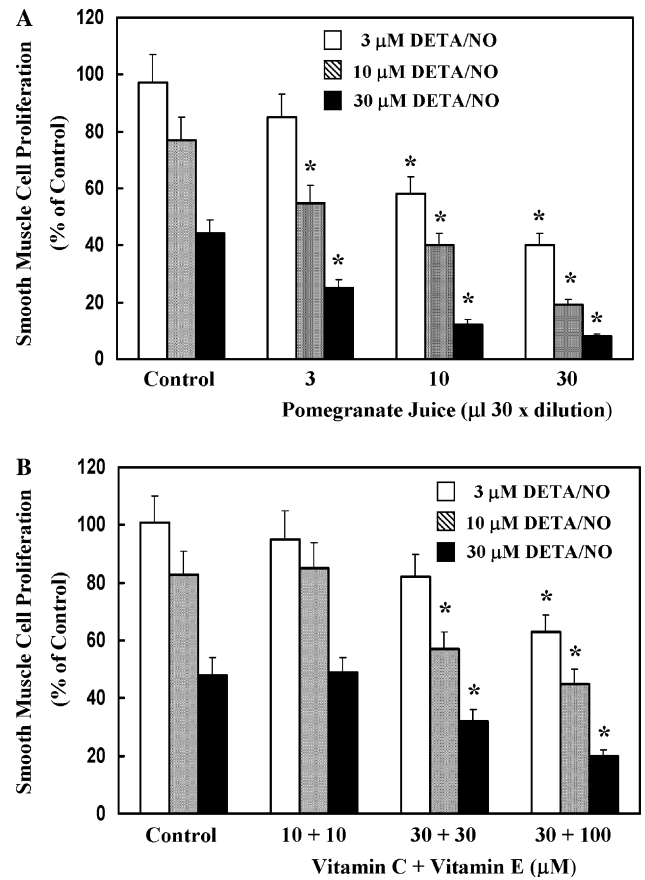


Fig. 4. (A) Inhibition of RASMC proliferation by NO and its augmentation by PJ. Cell proliferation was assessed by thymidine incorporation into DNA during the final 24 h of RASMC incubation, as described in Experimental procedures. Test agents were added to cells at the time of thymidine addition. DETA/NO was the NO donor agent used. Data are expressed as % of control, where control represents cells grown in the absence of added test agents. PJ was tested at 3, 10, and 30 μ l of 30-fold dilution of concentrate in a reaction volume of 1 ml. *Signifies statistically significant difference ($p < 0.05$) from corresponding control. Data represent means \pm SEM of duplicate determinations from four to five separate experiments. (B) Enhancement by combinations of vitamins C and E of inhibition of RASMC proliferation by NO. Cell proliferation was assessed as described in (A). Data are expressed as % of control, where control represents cells grown in the absence of added test agents. *Signifies statistically significant difference ($p < 0.05$) from corresponding control. Data represent means \pm SEM of duplicate determinations from three separate experiments.

proliferation at concentrations ranging from 3 to 100 μl (data not shown). These observations reveal the marked protective effect of PJ on the anti-proliferative action of NO in cell culture.

Concord grape juice and blueberry juice were each tested at 10, 30, and 100 μl of undiluted juice. The grape juice showed significant enhancement of the anti-proliferative action of NO only at the highest concentration tested, 100 μl , whereas the blueberry juice was active at 30 and 100 μl , but the effects were only about 50% of the effect for PJ (data not shown). Wines and alcohol could not be tested at concentrations above 10 μl because of the detrimental effect of alcohol on cell viability. These products were without effect on cell growth when tested at 3 and 10 μl volumes (data not shown). The combination of 10 μM vitamin C plus 10 μM vitamin E had no enhancing action on the anti-proliferative effect of NO, but higher

concentrations were effective (Fig. 4B). For example, the combination of 30 μM vitamin C plus 30 or 100 μM vitamin E showed a significant effect in enhancing the anti-proliferative effect of NO. Neither the juices nor the antioxidant vitamins showed activity when tested alone in the absence of DETA/NO.

The enhancement of NO action by PJ is attributed to the antioxidant protective effects of PJ against oxidative destruction of NO. α -Difluoromethylornithine (DFMO) is a well known, chemically stable, inhibitor of cell growth whose mechanism of action is independent of NO. PJ did not increase the anti-proliferative action of DFMO (Fig. 5), thereby supporting the view that PJ works by augmenting the anti-proliferative action of NO.

Effects on eNOS protein expression, catalytic activity, and gene promoter activity

In view of the reported protective action of PJ against atherosclerosis in humans and animals [1], and that administration of arginine and antioxidants to animals causes upregulation of eNOS [15,24], PJ was examined for additional effects on NO production by vascular endothelial cells in culture. Fig. 6 illustrates that PJ was without appreciable influence on eNOS protein expression in bovine pulmonary artery endothelial cells (BPAEC) as determined by Western blot analysis. The concentrations of PJ in cell culture media were 300- and 600-fold dilutions from PJ concentrate. The 300-fold dilution is equivalent to the 100 μl PJ tested in the chemiluminescence assay (Figs 1 and 2A), and exposures of up to 48 h elicited no significant effects on eNOS protein expression. In addition, the influence of PJ on eNOS catalytic activity in BPAEC was examined by monitoring the conversion of L-arginine to L-citrulline in vitro. Cells were incubated with PJ for 24-h or 48-h and then the cells were harvested, homogenized, and assayed for eNOS activity. Fig. 7A illustrates that PJ

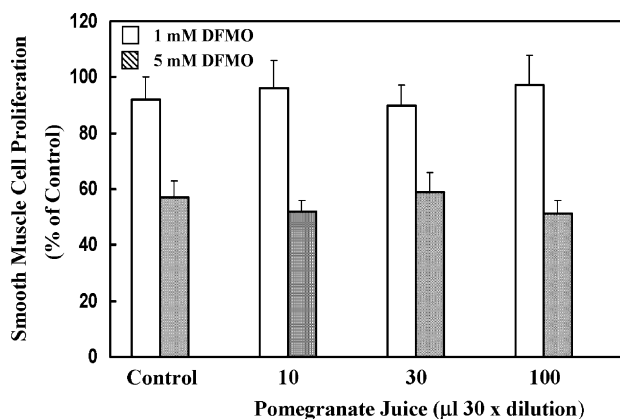


Fig. 5. Inhibition of RASMC proliferation by DFMO without enhancement by PJ. Cell proliferation was assessed as described in Fig. 4. Data are expressed as % of control, where control represents cells grown in the absence of added test agents. There was no significant effect of PJ. Data represent means \pm SEM of duplicate determinations from three separate experiments. DFMO signifies α -difluoromethylornithine.

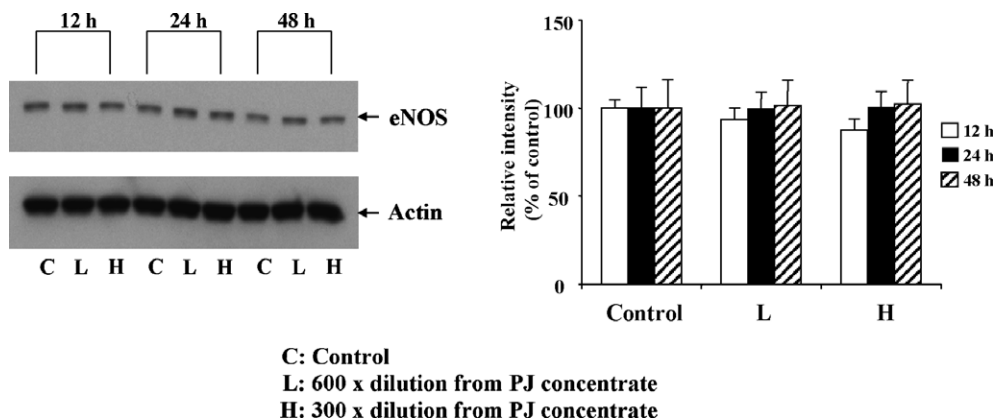


Fig. 6. Western blot analysis of eNOS protein expression in BPAEC and the influence of PJ. Experimental details are described in the text. The left panel illustrates typical Western blots. PJ at a 600-fold final dilution (L) and 300-fold final dilution (H) was incubated with BPAEC for 12, 24 or 48 h, as indicated. C signifies control (absence of PJ). Actin was measured as a control protein, which does not undergo protein expression changes in response to most agents. Data represent one of four separate experiments. The right panel shows the relative intensity of the Western blot bands, expressed as % of control, after calculation using NIH Image software. The data represent means \pm SEM of four separate experiments. There was no significant effect of PJ.

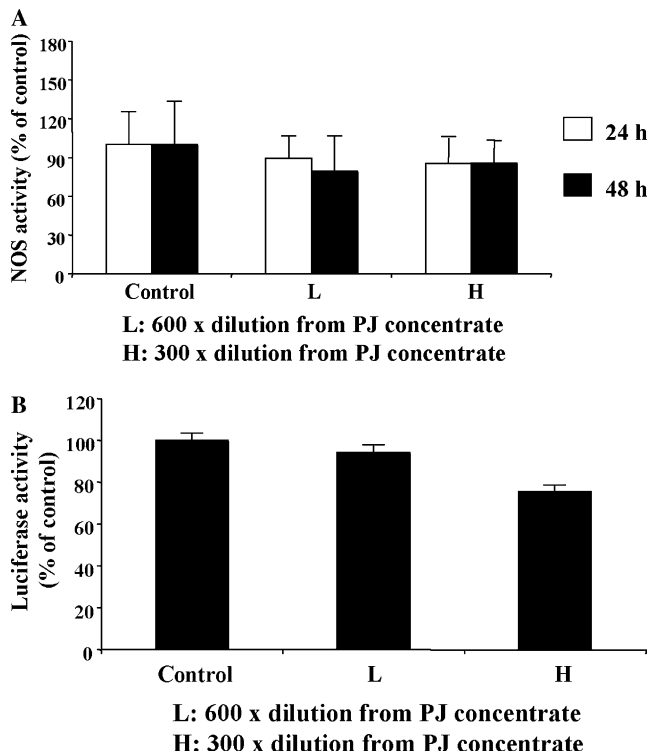


Fig. 7. (A) Influence of PJ on eNOS catalytic activity in BPAEC. eNOS enzymatic activity was measured by monitoring the conversion of L-arginine to L-citrulline, as described in Experimental procedures. PJ at a 600-fold final dilution (L) and 300-fold final dilution (H) was incubated with BPAEC for 24 or 48 h, as indicated. Data are expressed as % of control (absence of PJ), and represent means \pm SEM of four separate experiments. There was no significant effect of PJ. (B) Influence of PJ on the promoter activity of the eNOS gene in COS-7 cells. The transfection of eNOS into COS-7 cells and the determination of eNOS promoter activity by monitoring luciferase activity are described in Experimental procedures. PJ at a 600-fold final dilution (L) and 300-fold final dilution (H) was incubated with cells for 24 h. Data are expressed as % of control (absence of PJ), and represent means \pm SEM of three separate experiments. There was no significant effect of PJ.

did not appreciably inhibit eNOS activity at dilutions that had exhibited marked antioxidant and anti-proliferative activities. Finally, PJ was tested for its capacity to stimulate eNOS promoter activity in COS-7 cells that had been transfected with the eNOS gene. Fig. 7B illustrates that PJ did not significantly influence eNOS promoter activity. Therefore, it is clear from the experiments conducted that PJ, at the dilutions tested, does not increase the protein expression or activity of eNOS.

Discussion

The present study indicates that PJ (a) possesses potent antioxidant activity as measured by protection against ($O_2^{\bullet-}$)-mediated destruction of NO, (b) is capable of enhancing the action of NO to inhibit vascular smooth muscle cell proliferation, and (c) does not appear to affect the transcriptional expression or catalytic activity of endothelial NOS. Therefore, the antioxidant activity of PJ is potent enough to observe the enhancement of certain

well-known actions of NO, such as inhibition of vascular smooth muscle cell proliferation. PJ is a rich source of potent polyphenolic, flavonoid antioxidants that possess anti-atherogenic properties [1]. The flavonoids have been demonstrated to inhibit LDL cholesterol oxidation and are very efficient scavengers of reactive oxygen species, such as ($O_2^{\bullet-}$), that are generated both inside and outside cells [1]. Although we have not yet tested purified flavonoid substances in the assays described here, the antioxidant and pharmacological effects of PJ are likely attributed to the flavonoid substances present in the PJ. A detailed analysis of the component flavonoids, anthocyanins, and other antioxidants in PJ and in other fruit juices is still incomplete. Therefore, it is not known whether or not PJ contains antioxidants that are unique to PJ. It is clear, however, that the total content of polyphenol antioxidants is higher in PJ than in other fruit juices. Pomegranates may provide more antioxidant activity than other fruits merely because pomegranates contain substantially greater quantities of antioxidants.

The antioxidant assay employed in this study is a valid and unique procedure to assess the capacity of an antioxidant to protect NO against destruction and inactivation by ($O_2^{\bullet-}$), a common reactive oxygen species in cells and tissues. Indeed, the objective of this study was specifically to compare PJ with other antioxidant products for their capacity to retard the oxidative inactivation of NO and to augment certain biological actions of NO through this protective effect. Although one should probably not use the term potency when comparing crude fruit juices, it is possible to compare effectiveness among a variety of juices at equivalent dilutions. When potency is used in this study, it refers to antioxidant activity of equivalent dilutions of fruit juices. The data in this study are presented in such a way that reference can easily be made to the dilution of juice that would be typically ingested. This is true for the PJ, Concord grape juice, and orange juice tested. The blueberry juice tested may be an exception in that it was a freshly pressed and undiluted juice from whole blueberries (blueberry juice, either undiluted or concentrated, is not commonly available commercially). The antioxidant data from this study indicate that, although the grape juice and blueberry juice contained appreciable antioxidant activity, PJ was far more effective than the other fruit juices tested in protecting NO against inactivation by ($O_2^{\bullet-}$). For example, PJ produced significant antioxidant effects at dilutions of greater than 1000-fold. This high degree of antioxidant activity of PJ is likely attributed to the relatively large content of anthocyanin type polyphenolic flavonoids present in PJ. [7–9,11]. The antioxidant activity of the other fruit juices is also attributed to polyphenolic, flavonoid substances [2–4], but the total content of such flavonoids is substantially less than that found in PJ. Experiments are in progress to separate out the more prominent anthocyanins in order to test each one and compare them for antioxidant activity as conducted in the present study.

Red wine is well known to possess significant antioxidant activity that can be of potential therapeutic value in cardiovascular disease [33,34]. In the present study, red wine, but not white wine or alcohol, was shown to possess significant antioxidant activity. The effectiveness of the red wine was similar to that for some of the fruit juices tested, but was far less effective than PJ. The active antioxidant species found in red wine derives from the inner side of the peel or skin of the red grape, and consist of polyphenols, one of which is resveratrol [33]. Unpublished observations from this laboratory indicate that micromolar concentrations (10–30 μM) of resveratrol can protect NO against oxidative destruction and can augment the anti-proliferative action of NO. Neither the white wine tested nor 14% ethanol showed any antioxidant or pharmacological activity in the present study. The reason for this may be that the assay systems employed in this study are not sensitive enough to detect any effects of these substances. Nevertheless, the effects observed with the red wine (also 14% alcohol) clearly cannot be attributed to the alcohol content.

NO has been shown to be a potent inhibitor of vascular smooth muscle cell proliferation [26]. There may be multiple mechanisms involved in the expression of this important physiological effect [26,27,14], but one definite mechanism is a cyclic GMP-independent, S-nitrosylation-mediated inhibition of ornithine decarboxylase, the rate-limiting step in the production of polyamines for cell cycle progression [35,36]. Inhibition of vascular smooth muscle cell growth may represent one important mechanism by which NO elicits its anti-atherogenic or anti-atherosclerotic actions. The anti-atherogenic effects of L-arginine-containing dietary supplements that stimulate the production and/or actions of NO are well established [15–25]. In the present study, PJ and certain other antioxidant-containing products were shown to augment the anti-proliferative action of NO on rat aortic smooth muscle cells in cell culture. PJ was the most effective product tested and elicited significant effects at dilutions down to nearly 2000. The next most effective product tested was blueberry juice, but the dilutions required to elicit comparable effects to those of PJ were in the range of 10–30, as compared with nearly 2000 for PJ. Red wine was ineffective at dilutions of up to 100, and more concentrated solutions could not be tested because of the detrimental effect of the alcohol on cell viability. Ascorbic acid and DL- α -tocopherol in combination enhanced the NO-mediated inhibition of cell proliferation but only at relatively high concentrations. Therefore, as with the antioxidant assay, PJ was much more effective than the other antioxidant-containing products tested in augmenting the action of NO in slowing vascular smooth muscle cell proliferation. It is important to note that neither PJ nor any of the other products, when tested alone, directly influenced cell proliferation. The inhibitory effect was indirect in that the influence was on the direct action of NO. These observations support the view that PJ and the other active products tested augmented the action of NO via their antioxidant activity to protect and maintain

the functional levels of NO within the smooth muscle cells in culture.

In view of the findings that administration of arginine and antioxidants to animals causes upregulation of eNOS [15,24], PJ was examined for possible additional effects on NO production by vascular endothelial cells in culture. However, PJ did not influence either eNOS protein expression or catalytic activity. Similarly, PJ failed to stimulate eNOS promoter activity under the defined experimental conditions. The PJ was tested at dilutions that produced significant antioxidant and anti-proliferative effects in the other assay systems. These experiments were conducted with BPAEC and COS-7 cells in culture. BPAEC were used in this study because prior experiments in this laboratory revealed the presence of substantial quantities of eNOS protein. COS-7 cells were used instead of endothelial cells because we have had greater success in expressing relatively large amounts of eNOS gene and conducting eNOS promoter experiments in COS-7 cells than in BPAEC or other endothelial cells. The negative data obtained in these experiments may not reflect what can actually occur in the intact animal. The only conclusion that can be drawn from these studies is that PJ possesses extensive antioxidant activity that results in the augmentation of certain biological actions of NO.

Atherosclerosis is an inflammatory disease [37] characterized by vascular endothelial cell dysfunction and impaired production of NO [17,38–40]. NO is a widespread signaling molecule that functions in multiple ways to protect against the initiation and progression of atherosclerosis [16,18,41]. For example, NO aids in preventing the adhesion and aggregation of blood cells and platelets along the endothelial cell lining [16,18], and inhibits vascular smooth muscle cell proliferation [26]. NO elicits antioxidant and anti-inflammatory effects by scavenging certain reactive oxygen species [19,42,43], and prevents oxidation of LDL-cholesterol, thereby retarding the progression of atherosclerosis [17,44]. Similarly, other antioxidants can scavenge reactive oxygen species and thereby spare the destruction of NO and augment the biological actions of NO. For example, the potent antioxidants in PJ are capable of eliciting anti-atherosclerotic effects, including inhibition of oxidation of LDL-cholesterol [1]. Dietary consumption of flavonoids was shown to be inversely related to morbidity and mortality from coronary artery disease [45,46]. PJ consumption by humans was shown to lower the systemic blood pressure in hypertensive patients [47] and to slow the progression of atherosclerosis [1]. Anti-atherosclerotic effects of PJ were also observed in mice [1,28]. The inclusion of antioxidants (typically ascorbate and DL- α -tocopherol) into L-arginine-containing diets produced a synergistic anti-atherosclerotic effect over L-arginine alone [15–25]. These findings are consistent with the present findings that PJ shows antioxidant activity that is sufficient to protect NO against oxidative destruction and thereby enhance the biological actions of NO.

In summary, PJ contains antioxidants that are capable of markedly protecting NO against $O_2^{\bullet-}$ -mediated destruction, and augmenting certain biological actions of NO such as inhibition of vascular smooth muscle cell proliferation. Whether some of these antioxidants are unique to PJ or are just present in relatively large quantities remains to be determined. These observations support the conclusions from previous reports that PJ possesses potent antioxidant activity that is associated with anti-atherosclerotic effects in animals and humans.

Acknowledgment

This work was funded by a grant from the Lynda and Stewart Resnick Revocable Trust (to L.J. and C.N.).

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