Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E–deficient mice[1,2]

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ABSTRACT

Background: Dietary supplementation with nutrients rich in antioxidants is associated with inhibition of atherogenic modifications to LDL, macrophage foam cell formation, and atherosclerosis. Pomegranates are a source of polyphenols and other antioxidants.

Objective: We analyzed, in healthy male volunteers and in atherosclerotic apolipoprotein E–deficient (E^0) mice, the effect of pomegranate juice consumption on lipoprotein oxidation, aggregation, and retention; macrophage atherogenicity; platelet aggregation; and atherosclerosis.

Design: Potent antioxidative effects of pomegranate juice against lipid peroxidation in whole plasma and in isolated lipoproteins (HDL and LDL) were assessed in humans and in E^0 mice after pomegranate juice consumption for ≤ 2 and 14 wk, respectively.

Results: In humans, pomegranate juice consumption decreased LDL susceptibility to aggregation and retention and increased the activity of serum paraoxonase (an HDL-associated esterase that can protect against lipid peroxidation) by 20%. In E^0 mice, oxidation of LDL by peritoneal macrophages was reduced by up to 90% after pomegranate juice consumption and this effect was associated with reduced cellular lipid peroxidation and superoxide release. The uptake of oxidized LDL and native LDL by mouse peritoneal macrophages obtained after pomegranate juice administration was reduced by 20%. Finally, pomegranate juice supplementation of E^0 mice reduced the size of their atherosclerotic lesions by 44% and also the number of foam cells compared with control E^0 mice supplemented with water.

Conclusion: Pomegranate juice had potent antiatherogenic effects in healthy humans and in atherosclerotic mice that may be attributable to its antioxidative properties.   *Am J Clin Nutr* 2000;71:1062–76.

KEY WORDS Pomegranate juice, flavonoids, lipid peroxidation, LDL, HDL macrophage, paraoxonase, platelet, atherosclerosis, antioxidants, men, atherosclerotic apolipoprotein E–deficient mice

INTRODUCTION

Major risk factors for atherosclerosis include high plasma LDL concentrations and LDL modifications such as its retention, oxidation, and aggregation (1–5). Blood platelet activation also contributes to accelerated atherosclerosis (6–8). Oxidative modification of LDL is thought to play a key role during early atherogenesis. Oxidized LDL is taken up by macrophages at an enhanced rate via their scavenger receptors (9), leading to the formation of lipid-laden foam cells, the hallmark of the early atherosclerosis (10). Cells of the arterial wall, including endothelial cells, smooth muscle cells, and macrophages, can oxidize LDL in vitro in the presence of catalytic amounts of transition metal ions (11–13). Although increased resistance of LDL to oxidation was observed after treatment with various synthetic pharmaceutical agents (14–17), an effort is being made to identify natural food products that can offer antioxidant protection against LDL oxidation. We showed previously the beneficial effects against LDL oxidation of dietary supplementation with β-carotene (18, 19), lycopene (20), vitamin E (21), and flavonoids from red wine (22, 23), licorice (24), or olive oil (25).

The pomegranate tree, which is said to have flourished in the garden of Eden, has been used extensively in the folk medicine of many cultures. In ancient Greek mythology, pomegranates were known as the "fruit of the dead" and in the ancient Hebrew tradition, pomegranates adorned the vestments of the high priest. The Babylonians regarded pomegranate seeds as an agent of resurrection, the Persians believed the seeds conferred invincibility on the battlefield, and for the ancient Chinese the seeds symbolized longevity and immortality. Edible parts of pomegranate fruit (about 50% of total fruit weight) comprise 80% juice and 20% seeds. Fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids. Pomegranate seeds are a rich source of crude fibers, pectin, and sugars. Dried pomegranate seeds contain the steroid estrogen estrone (26, 27), the isoflavone phytoestrogens genistein and daidzein, and the phytoestrogen coumestrol (28). In pomegranate

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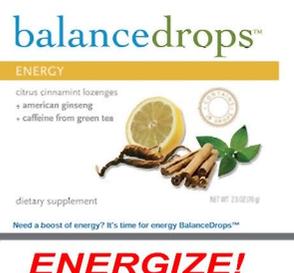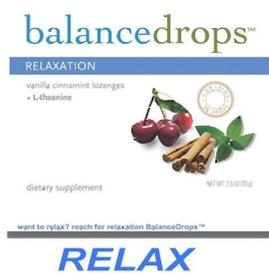
juice (PJ), fructose and glucose are present in similar quantities, calcium is 50% of its ash content, and the principal amino acids are glutamic and aspartic acids (29, 30). The soluble polyphenol content in PJ varies within the limits of 0.2–1.0%, depending on variety, and includes mainly anthocyanins (such as cyanidin-3-glucoside, cyanidin-3,5-diglucoside, and delphindin-3-glucoside), catechins, ellagic tannins, and gallic and ellagic acids (31). Fermented PJ and cold-pressed pomegranate seeds possess antioxidant activity and can reduce prostaglandin and leukotriene formation by inhibition of cyclooxygenases and lipoxygenases (32). The major purpose of the present study was to examine, for the first time, the in vitro and ex vivo effects of PJ on LDL atherogenic modifications including its retention, oxidation, and aggregation in humans and in atherosclerotic apolipoprotein E–deficient (E^0) mice.

SUBJECTS AND METHODS

Human studies

The antioxidative effect of PJ was tested ex vivo in 2 human studies. In the first study, 13 healthy, nonsmoking men aged 20–35 y who were taking no medication were supplemented with 50 mL PJ/d (1.5 mmol total polyphenols) for 2 wk. In the second study, performed for ≤10 wk, 3 subjects were supplemented with increasing doses of PJ (20–80 mL/d, equivalent to 0.54–2.16 mmol total polyphenols/d). Subjects were students or laboratory staff from the Technion Faculty of Medicine. Compliance with the PJ supplementation in all subjects was satisfactory as assessed by daily contact with the subjects. The subject's mean (±SD) body mass index (in kg/m^2) was 23.0 ± 1.5 and did not change significantly during the study. All subjects continued their habitual diets during the study. Blood samples were drawn after a 12-h fast before study entry and after 1 and 2 wk of PJ consumption. The studies were approved by the Helsinki Committee of the Rambam Medical Center, Israel Ministry of Health (no. 912).

Mouse study

E^0 mice were generously provided by Jan Breslow of Rockefeller University, New York. Gene targeting in mouse embryonic stem cells was used to create mice that lack apolipoprotein E (33). Thirty 6-wk-old E^0 mice were divided into 3 groups of 10. The 3 groups received 0, 6.25, or 12.5 μL PJ (equivalent to 0, 0.175, and 0.350 μmol total polyphenols) in their drinking water per mouse per day. Blood was taken at 6, 9, and 14 wk of age for plasma and LDL analyses. Peritoneal macrophages and aortas were obtained at the end of the study.

To evaluate the effect of PJ supplementation on the progression of atherosclerotic lesions, 3-wk-old E^0 mice were supplemented for 11 wk with 31 μL PJ (equivalent to 0.875 μmol total polyphenols) per mouse per day or given water alone (control mice). The entire aorta was dissected from each mouse at the end of the study.

Pomegranate processing

Pomegranates ('Wonderful' cultivar) were handpicked, washed, chilled to 4 °C, and stored in tanks. The fruit was then crushed, squeezed, and treated enzymatically with pectinase to yield the PJ and byproducts, which included the inner and outer peels and the seeds. Pectinase hydrolyzes α-1,4-galacturonide bonds in pectin and thus improves extraction and filtration and prevents the formation of pectin gels. The juice was filtered, pasteurized, concentrated, and stored at −18 °C.

For the aqueous extraction of the peels and seeds, 1 g peels and seeds was diluted in 5 mL water and then crushed, squeezed, and extensively vortex mixed. The extract was then centrifuged at 3000 × g for 20 min at 4 °C to remove any water-insoluble materials and the supernate was used in the analyses of LDL oxidation. Because ingredients other than polyphenols may also act as potent antioxidants, we also studied the effect of the extracts on the basis of weight. For this purpose, we lyophilized the extracts and redissolved the pellet in water to a concentration of 1 g/L and used this solution in analyses of LDL oxidation.

In addition to polyphenols, PJ contains several other antioxidants, including vitamin C, that may contribute to its antioxidative and antiatherogenic effects. Of the total polyphenols in PJ, flavonoids constitute ≈40% (including anthocyanins, catechins, and phenolics). Complex polyphenols are also present in PJ (including condensed and hydrolyzable tannins). We fractionated the PJ by HPLC (Sep-Pak; Waters Corp, Milford, MA) and obtained active antioxidant fractions detected at 254 nm (data not shown). These fractions were found to be easily solubilized in methanol or ethyl acetate but not in water. These observations suggest that the active antioxidant compounds in PJ are not proteins, sugars, or lipids, but may be complex polyphenols that can be absorbed in humans.

Polyphenol measurement

The total polyphenol concentration of the PJ was determined spectrophotometrically with phosphomolybdic phosphotungstic acid reagent (31) and quercetin as a standard.

Plasma lipid peroxidation

In the in vitro studies, human plasma obtained from a healthy volunteer was diluted (2×) with phosphate-buffered saline (PBS) and increasing concentrations of PJ polyphenols (0–1.5 μmol/L) were added to the plasma. In the in vivo studies, plasma was obtained from studied subjects before and after 2 wk of PJ consumption and from E^0 mice at 0, 9, and 14 wk of PJ consumption.

In both studies, plasma was incubated in the absence or presence of 100 mmol/L of the free radical generator 2,2′-azobis-2-amidinopropane hydrochloride (AAPH; Wako Chemical Industries Ltd, Osaka, Japan) for 2 h at 37 °C. AAPH is a water-soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate. Plasma lipid peroxidation was determined by measuring the amount generated of thiobarbituric acid–reactive substances (TBARS) (34) and lipid peroxides (35).

Serum paraoxonase (arylesterase activity)

Arylesterase activity was measured by using phenylacetate as the substrate. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 5 μL serum, 1.0 mmol phenylacetate/L, and 0.9 mmol CaCl$_2$/L in 20 mmol tris-HCl/L, pH 8.0. Nonenzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. The extinction coefficient at 270 nm (E_{270}) for the reaction was 1310 (mol/L)$^{-1} \cdot$cm^{-1}. One unit of arylesterase activity is equal to 1 mmol phenylacetate hydrolyzed·min$^{-1} \cdot$L^{-1} (36). In the in vitro study, increasing concentrations of PJ were incubated with normal serum for 10 min before the analysis of arylesterase activity.

Total plasma antioxidant status

Total antioxidant status was measured in plasma with a commercially available kit (catalog no. NX 2332; Randox Laboratories Limited, Antrim, United Kingdom) applicable for COBAS MIRA (Hoffmann-La Roche, Basel, Switzerland). Plasma was incubated with ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulfonate)], a peroxidase (metmyoglobin), and hydrogen peroxide to produce a radical cation. The resulting product has a relatively stable blue-green color, which was measured at 600 nm. Antioxidants in the added sample suppress this color production in proportion to their concentration (37).

Lipoprotein isolation

For the in vitro studies, LDL was isolated from plasma collected from healthy, normolipidemic volunteers. For the ex vivo studies, human plasma was collected before study entry (baseline) and after 1 and 2 wk of PJ administration. In the mouse study, LDL was isolated from blood samples drawn before and after 9 and 14 wk of PJ administration.

Plasma samples were stored at 4°C for 2 wk until all 3 samples had been collected. LDL and HDL were then isolated from the plasma samples. No significant differences were found in the basal oxidative state (no oxidant added) of the lipoproteins. The lipoproteins (LDL and HDL) were prepared by discontinuous density gradient ultracentrifugation as described previously (38). The lipoproteins were washed at a density of 1.063 kg/L and dialyzed against 150 mmol NaCl/L and 1 mmol Na_2EDTA/L (pH 7.4) at 4°C. The LDL and HDL fractions were then sterilized by filtration (0.45 μm), kept under nitrogen in the dark at 4°C, and used within 2 wk. The lipoprotein protein concentration was measured with the Folin phenol reagent (39). Before oxidation, LDL and HDL were dialyzed against EDTA-free, PBS solution (pH 7.4) at 4°C.

Lipoprotein oxidation

LDL or HDL (100 mg protein/L) was incubated with 5 μmol $CuSO_4$/L for 3 h at room temperature. The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm (40). Incubations were carried out in the spectrophotometer cuvette (Ultraspec 3000; Pharmacia LKB, Biochrom Ltd, Cambridge, United Kingdom). The initial background optical density of the samples ranged from 0.1 to 0.2 as recorded at 234 nm. After initial absorbance was recorded, the spectrophotometer was set to zero against a blank and the increase in absorbance during LDL or HDL oxidation was recorded every 10 min. The lag time required for the initiation of lipoprotein oxidation was calculated from the oxidation curve.

LDL aggregation and retention

To determine LDL aggregation, LDL (100 mg protein/L) was mixed by vortex at a fixed strength and absorbance at 680 nm was monitored every 10 s against a blank solution (41). For the measurement of LDL retention in the ex vivo studies, human LDL isolated before and after 1 or 2 wk of PJ supplementation was used. In the in vitro studies, LDL was preincubated with increasing concentrations of PJ (up to 3.5 μmol polyphenols/L) for 1 h at 37°C. LDL (200 mg lipoprotein protein/L) was then incubated with chondroitin sulfate (100 mg/L) for 30 min at room temperature. The lipoprotein was precipi-

tated with a commercial kit for an HDL-cholesterol reagent (phosphotungstic acid-$MgCl_2$; Sigma, St Louis) that precipitated all the LDL present in the samples and was then centrifuged for 10 min at 2000 × g at 25°C (42).

After the supernate was discarded, the LDL in the precipitate was dissolved in 0.1 mol NaOH/L and analyzed for its glycosaminoglycan content by using the 1,9-dimethylmethylene blue spectrophotometric assay for sulfated glycosaminoglycans (43). Briefly, 2.5 mL ice-cold 1,9-dimethylmethylene blue working solution (46 μmol 1,9-dimethylmethylene blue/L, 40 mmol glycine/L, and 40 mmol NaCl/L in 5% ethanol, adjusted to pH 3.0) was added to 500 μL of the dissolved precipitate. Absorbency at 525 nm was then measured immediately. Chondroitin sulfate was used as a standard and was included within each series of assays. Similar preparations of LDL with no chondroitin sulfate added were used as a control. The glycosaminoglycan content of the control was subtracted from the glycosaminoglycan content of the LDL preparations incubated with chondroitin sulfate.

Mouse peritoneal macrophages

Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal fluid of each mouse 4 d after intraperitoneal injection of 3 mL thioglycolate (24 g/L in saline solution) (44). The harvested cells (10–20 × 10^6 per mouse) were washed and centrifuged 3 times with PBS at 1000 × g for 10 min at 25°C. The cells were then resuspended to a concentration of 1 × 10^9/L in DMEM (Biological Industries, Beit Ahemeq, Israel) containing 10% horse serum (heat-inactivated at 56°C for 30 min), 100 000 U penicillin/L, 100 mg streptomycin/L, and 2 mmol glutamine/L.

The cell suspension was dispensed into 35-mm plastic petri dishes and incubated in a humidified incubator (5% CO_2, 95% air) for 2 h. The dishes were washed once with 5 mL DMEM to remove nonadherent cells and the monolayer was incubated further under similar conditions for 18 h until various macrophage functions were analyzed.

Macrophage glutathione content

Cells (2 × 10^9/L PBS) were sonicated twice, for 20 s each time, at 80 W. The cellular protein content was determined by using the Folin phenol reagent method (39). For total glutathione analysis, 5% sulfosalicylic acid was added to the supernate of the sonicated cells (1:2, by vol) and the samples were then centrifuged at 20 000 × g for 10 min at 4°C. The glutathione content of all samples was measured in the supernate with the 5,5-dithiobis-2-nitrobenzoic acid–glutathione reductase (NADPH) recycling assay (45).

Superoxide anion release

The production of superoxide anion by MPMs was measured as the superoxide dismutase–inhibitable reduction of acetyl ferricytochrome c (46). Cells (2 × 10^6 per well) were suspended in 1 mL Hanks' balanced salts solution (Biological Industries) containing acetyl ferricytochrome c (150 μmol/L). Superoxide production by the cells was stimulated by the addition of LDL (100 mg protein/L) and 5 μmol $CuSO_4$/L for 1 h.

To some control samples, 30 mg superoxide dismutase/L was added. The amount of superoxide release was measured in the medium and was expressed as nmol superoxides/mg cell protein by using an extinction coefficient of $E_{550} = 21$ $(mmol/L)^{-1} \cdot cm^{-1}$.

LDL oxidation by macrophages

MPMs (2×10^6 per 35-mm dish) were incubated with LDL (100 mg protein/L) in RPMI medium (phenol-free; Biological Industries) in the presence of 2 μmol $CuSO_4$/L for 6 h. LDL was also incubated under similar conditions in the absence of cells. The extent of LDL oxidation was measured directly in the medium (after centrifugation at $1000 \times g$ for 10 min at 25 °C to spin down detached cells) by the TBARS assay (34). Macrophage-mediated oxidation of LDL was calculated by subtracting the oxidation rate in the absence of cells from that obtained in the presence of macrophages (47).

Cellular uptake of lipoproteins by macrophages

LDL was radioiodinated by the iodine monochloride method, as modified for lipoproteins (48). Radioiodinated oxidized LDL (Ox-[125I]LDL) was prepared from [125I]LDL that was dialyzed against PBS and then incubated with 5 μmol $CuSO_4$/L at 37 °C for 24 h. [125I]LDL or Ox-[125I]LDL (10 mg protein/L) was incubated with the cells at 37 °C for 5 h. Lipoprotein cellular degradation was measured in the collected medium as the trichloroacetic acid–soluble, nonlipid radioactivity that was not due to free iodide (49). Lipoprotein degradation in a cell-free system measured under identical conditions was minimal ($<10\%$) and was subtracted from the total degradation. The remaining cells were washed 3 times with cold PBS and dissolved in 0.1 mol NaOH/L for protein and cell-associated lipoprotein determination. Cellular binding of Ox-[125I]LDL was determined after the cells were incubated with increasing concentrations of Ox-[125I]LDL or [125I]LDL at 4 °C for 4 h. Then, the cells were washed with cold PBS on ice and dissolved in 0.1 mol NaOH/L, and samples were taken to measure radioactivity.

Platelet aggregation

For platelet studies, venous blood (10 mL) was collected with siliconized syringes into 3.8% sodium citrate at a ratio of 9:1 (by vol). Platelet-rich plasma was prepared by low-speed centrifugation ($100 \times g$ for 10 min) at 25 °C, and the remaining sample was recentrifuged at $1000 \times g$ for 10 min to obtain platelet-poor plasma (50). Collagen (Nycomed Arzneimittel, Munich, Germany) was used as the aggregating agent at a concentration of 2 mg/L because this concentration caused an aggregation amplitude of up to 75%. Platelet aggregation was determined at 37 °C in a PAP-4 computerized aggregometer (Chrono-Log Corp, Broomall, PA) using platelet-poor plasma as a reference system for platelet-rich plasma. In the in vitro studies, increasing concentrations of PJ were incubated for 10 min with 1 mL platelet-rich plasma and platelet aggregation was analyzed. Results are expressed as the slope of the aggregation curve in cm/min.

Free radical scavenging capacity

DPPH (1,1-diphenyl-2-picryl-hydrazyl) is a radical-generating substance that is widely used to monitor the free radical scavenging abilities of various antioxidants (51). To analyze free radical scavenging capacity, increasing concentrations of PJ (0–14 μmol polyphenols/L) were mixed with 3 mL of 0.1 mmol DPPH/L in ethanol. The time course for the change in optical density at 517 nm was monitored kinetically (51).

Histopathology of aortic atherosclerotic lesions

After E^0 mice were supplemented with PJ for 11 wk, they were anesthetized with ethyl ether. The heart and entire aorta were rapidly dissected out and immersion-fixed in 3% glutaraldehyde in 0.1 mol sodium cacodylate buffer/L with 0.01% $CaCl_2$, pH 7.4, at room temperature. After 1 h, the aortic arch was dissected away from the surrounding fatty tissue under a binocular stereomicroscope and the first 4 mm of the ascending aorta (beginning with the aortic valves) was removed and cut transversely with razor blades into four 1-mm blocks. The samples were stored in fixative at room temperature overnight. The samples were then rinsed and stored in 0.1 mol sodium cacodylate buffer/L containing 7.5% sucrose (wt:vol) before being treated with an unbuffered 1% aqueous solution of osmium tetroxide for 4 h. This treatment was followed by a cacodylate rinse and successive dehydration in 70%, 95%, and then 100% ethanol solutions before treatment with propylene oxide as an intermediate solvent and embedding in epoxy resin (Eponate 12; Pelco International, Redding, CA).

The blocks were orientated so that transverse section of the aorta could be cut. After heat polymerization (18 h at 60 °C), the blocks were trimmed and 1-μm sections were cut with glass or diamond knives on an LKB Nova ultramicrotome (LKB, Bromma, Sweden). When a sufficient number of semithin sections were obtained from all blocks, the remainders of the blocks were cut into much thicker sections (150–200 μm) for more macroscopic observation. The lipid content of the lesions of these thicker sections was stained an intense black from the prolonged osmium treatment, which permitted the lesion areas to be easily determined histomorphometrically. Only the area of the aortic arch was examined because previous and ongoing studies by us and others showed that this area is especially prone to atherosclerosis in E^0 mice and the areas are well defined with a clear starting point (aortic valves).

Histomorphometric determinations of lesion size were performed by using an Olympus Cue-2 image analysis system with appropriate morphometry software (Olympus Corporation, Lake Success, NY). The system consists of a Universal R photomicroscope ($\times$ 10 objective; Zeiss, Oberkochen, Germany) fitted with a WV-CD50 camera (Panasonic, Tokyo) with the video image seen on a 14-inch color monitor (Sony, Tokyo) and an IBM-compatible PC. Measurements were made in standardized "windows" (fields) with an area of 176 758 μm^2.

Statistics

Student's paired *t* tests were performed for all statistical analyses. Results are given as means ± SDs. The degree of variation between experiments ranged from 7% to 9%. The computer

TABLE 1
Effect of pomegranate juice supplementation on plasma lipids and lipoproteins[1]

	Time after supplementation		
	0 (Before)	1 wk	2 wk
	mmol/L		
Total cholesterol	5.0 ± 0.4	5.2 ± 0.4	5.3 ± 0.3
LDL cholesterol	3.1 ± 0.3	3.2 ± 0.2	3.5 ± 0.3
VLDL cholesterol	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
HDL cholesterol	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Triacylglycerols	1.6 ± 0.2	1.7 ± 0.3	1.7 ± 0.1

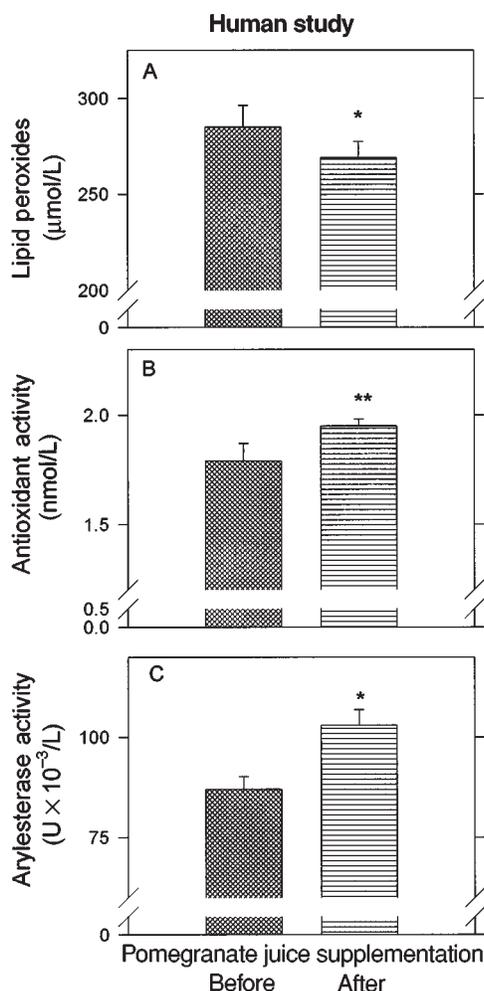[1] $\bar{x}$ ± SD; n = 13. There were no significant differences between time points.

FIGURE 1. Mean (±SD) effect of 2 wk of pomegranate juice supplementation on (A) the susceptibility of plasma to 2,2′-azobis-2-amidinopropane hydrochloride (AAPH)-induced lipid peroxidation, (B) plasma total antioxidant status, and (C) serum paraoxonase activity. The extent of plasma lipid peroxidation was determined by the lipid peroxides assay, plasma antioxidant status was measured with a commercially available kit, and serum paraoxonase activity was determined by measuring arylesterase activity. $n = 3$. *,**Significantly different from before supplementation: *$P < 0.01$, ** $P < 0.05$.

software program STATEASE (version 1.00; Data Plus Systems Inc, New York) was used for computation.

RESULTS

Ex vivo studies in humans and mice

Plasma lipid pattern

Administration of PJ to 13 healthy men for 2 wk had no significant effect on the plasma lipid profile, including total cholesterol, LDL-cholesterol, VLDL-cholesterol, HDL-cholesterol, and triacylglycerol concentrations (**Table 1**). There was also no significant effect of increasing PJ doses on blood chemistry and plasma lipid and lipoprotein patterns in 3 studied subjects, except that plasma glucose, cholesterol, and triacylglycerol con-

centrations were 10–15% higher after 1 wk of supplementation with the highest PJ dose (80 mL/d) (data not shown). Similarly, no significant effect of PJ consumption on plasma lipid concentrations was shown in E^0 mice (data not shown).

Plasma lipid peroxidation

Human plasma obtained after 2 wk of PJ consumption showed a small but significant 6% decreased susceptibility to AAPH-induced lipid peroxidation compared with plasma obtained before study entry (**Figure 1**A). Additionally, a significant 9% increase in plasma total antioxidant status was observed after 2 wk of PJ consumption (Figure 1B).

To determine the effects of increased or decreased doses of PJ on AAPH-induced plasma lipid peroxidation and to analyze the ability of PJ to maintain its effect after juice consumption ended, 3 subjects were studied further. Supplementation with 20 mL PJ/d for 1 wk resulted in a significant 11% decrease in plasma lipid peroxide content. Supplementation with 50 mL PJ/d for 1 more week resulted in a further 21% decrease in
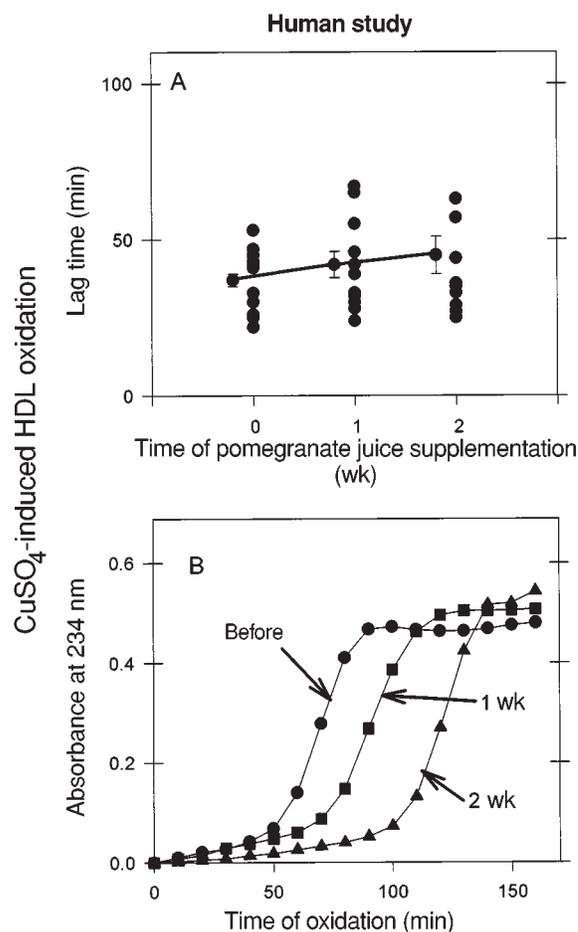

FIGURE 2. Effect of pomegranate juice (PJ) supplementation on the susceptibility of HDL to oxidation ex vivo. (A) Mean (±SD) lag time before the initiation of oxidation of HDLs obtained from 12 healthy volunteers before (0) or after 1 or 2 wk of PJ supplementation. HDL (100 mg protein/L) was incubated with 5 μmol $CuSO_4$/L for 3 h at room temperature. The formation of conjugated dienes was monitored kinetically at 234 nm. (B) A representative oxidation curve before and after 1 or 2 wk of PJ supplementation.
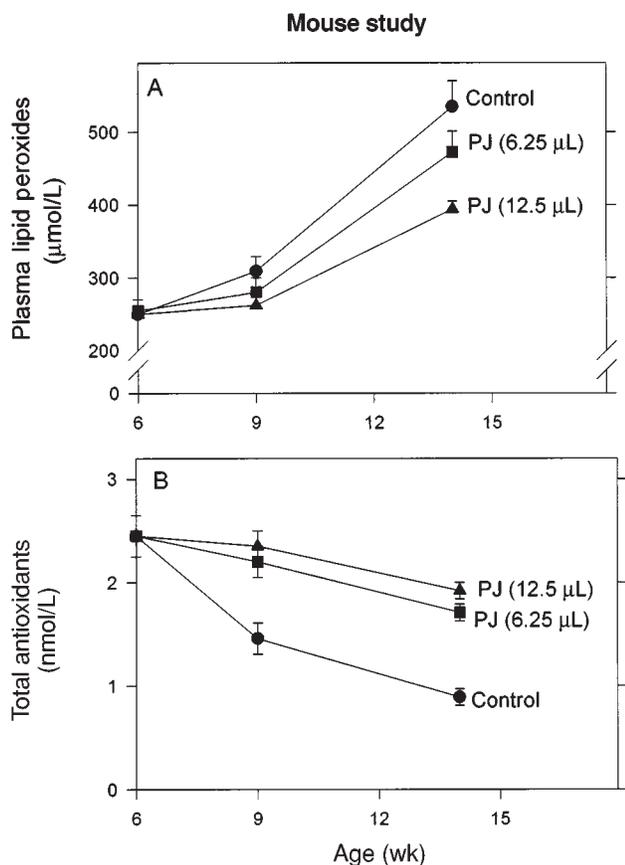
FIGURE 3. Mean (±SD) effect of supplementation of atherosclerotic apolipoprotein E–deficient (E^0) mice with placebo (control) or 6.25 or 12.5 μL pomegranate juice (PJ)/d (equivalent to 0, 0.175, or 0.350 μmol total polyphenols, respectively) on plasma oxidative stress during aging. Blood samples were drawn at 6, 9, and 14 wk. (A) The plasma oxidative state was determined by measuring the lipid peroxide concentrations in plasma samples. (B) The antioxidant status of the plasma samples was measured by using a commercial kit. $n = 3$.

plasma of control E^0 mice, increased gradually as the mice aged from 260 μmol/L plasma at 6 wk of age to 309 and 535 μmol/L plasma at 9 and 14 wk of age, respectively (**Figure 3**A). In mice fed PJ, plasma lipid peroxidation was markedly lower, and this effect was dependent on concentration. Similarly, serum total antioxidant status was higher in E^0 mice that consumed PJ than in control mice, and this effect was again concentration dependent (Figure 3B). The decrease in serum paraoxonase activity with age in the atherosclerotic E^0 mice, which are under excess oxidative stress (23), was not prevented by PJ consumption (data not shown).

LDL modifications

LDL oxidation. The susceptibility of LDL to copper ion–induced oxidation was gradually reduced with PJ consumption, as shown by a prolongation of the lag time required for the initiation of LDL oxidation of 29% and 43% after 1 and 2 wk of supplementation, respectively (from 35 ± 6 min before to 44 ± 6
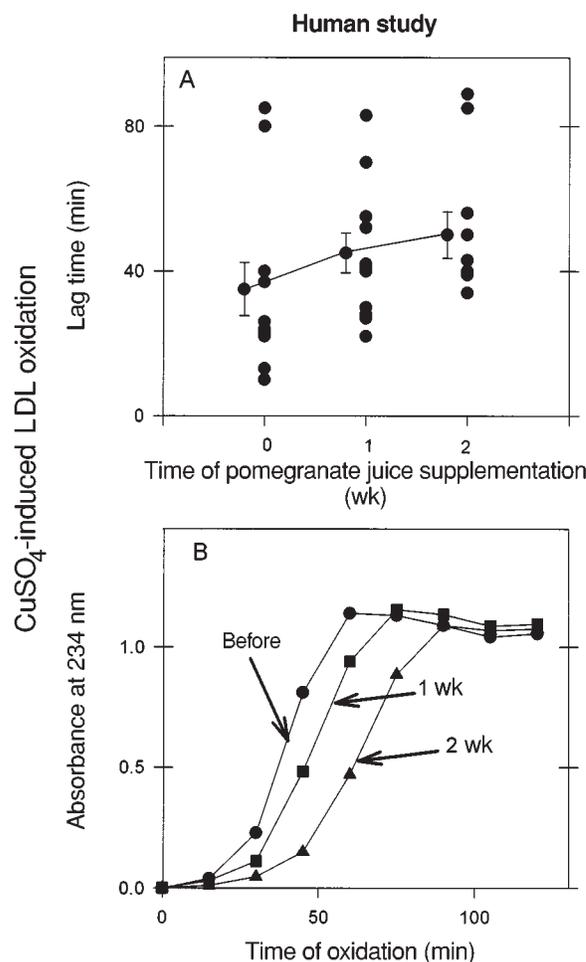

FIGURE 4. The effect of pomegranate juice (PJ) supplementation on the susceptibility of LDL to oxidation ex vivo. (A) Mean (±SD) lag time before the initiation of oxidation of LDLs obtained from 12 healthy volunteers before (0) or after 1 or 2 wk of PJ supplementation. LDL (100 mg protein/L) was incubated with 5 μmol $CuSO_4$/L for 3 h at room temperature. The formation of conjugated dienes was monitored kinetically at 234 nm. (B) Representative oxidation curve before and after 1 or 2 wk of PJ supplementation.

plasma lipid peroxidation, whereas an additional increase in PJ supplementation to 80 mL PJ/d for an additional week did not inhibit plasma susceptibility to lipid peroxidation further. The inhibitory effect of PJ consumption on plasma lipid peroxidation was maintained for 2 wk after PJ supplementation ended (data not shown).

Two weeks of PJ consumption also resulted in a significant 18% increase in serum paraoxonase (Figure 1C). Because serum paraoxonase is bound to HDL, we questioned whether the increased serum paraoxonase activity after PJ consumption was associated with increased resistance of HDL to oxidation. As shown in **Figure 2**, PJ consumption for 2 wk gradually and significantly ($P < 0.01$) increased the resistance of HDL to copper ion–induced oxidation. The lag time required for the initiation of HDL oxidation increased from 37 ± 2 min before supplementation to 45 ± 6 min after 2 wk. Also shown in Figure 2 is a representative kinetic analysis of copper ion–induced oxidation of HDL before study entry and after 1 and 2 wk of PJ consumption.

PJ also had antioxidative effects when administered to E^0 mice. The basal oxidative state, measured as lipid peroxides in
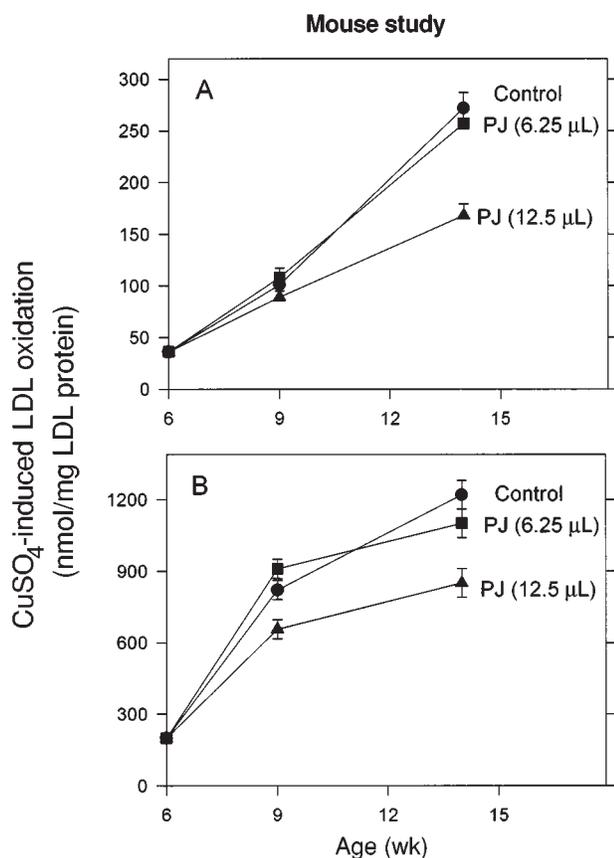
FIGURE 5. Mean (± SD) effect of supplementation of atherosclerotic apolipoprotein E–deficient (E[0]) mice with placebo (control) or 6.25 or 12.5 μL pomegranate juice (PJ)/d (equivalent to 0, 0.175, or 0.350 μmol total polyphenols, respectively) on LDL susceptibility to copper ion–induced oxidation ex vivo. LDL (100 mg protein/L) was isolated at 6, 9, and 14 wk and incubated with 5 μmol CuSO$_4$/L for 2 h at 37 °C. The extent of LDL oxidation was measured by the thiobarbituric acid–reactive substances (A) or lipid peroxides (B) assay. $n = 3$.

and 50 ± 6 min after 1 and 2 wk, respectively; **Figure 4**A). A representative kinetic analysis of copper ion–induced LDL oxidation from this study is shown in Figure 4B.

PJ consumption also reduced the susceptibility of LDL from E[0] mice to copper ion–induced oxidation. In E[0] mice that consumed 6.25 or 12.5 μL PJ/d for 2 mo, LDL oxidation was delayed by 100 or 120 min, respectively, compared with the lag time before PJ administration (data not shown). The progressive increase with age in the susceptibility of LDL to oxidation was significantly attenuated by PJ consumption, in a dose-dependent manner, as shown for both TBARS and lipid peroxides (**Figure 5**).

LDL aggregation. The atherogenicity of LDL is attributed not only to oxidative modification, but also to aggregation (4). It was shown previously that oxidation of LDL leads to its subsequent aggregation (21) and we reported that polyphenols from red wine can reduce LDL aggregation in vitro and in vivo (23). The addition of increasing concentrations of PJ to LDL lowered its susceptibility to aggregation (by vortex mixing) in a dose-dependent manner (**Figure 6**A). When we analyzed the susceptibility to aggregation of LDL isolated from individual subjects who had consumed PJ for 1 and 2 wk, a pattern of reduction in

LDL aggregation was observed in 7 of 13 subjects, although the mean value did not change significantly (Figure 6B).

LDL retention. Extracellular matrix proteoglycans can bind LDL through their glycosaminoglycan moieties. Such interaction can lead to the entrapment of LDL in the arterial wall, a phenomenon called LDL retention (5). Addition of increasing amounts of PJ to LDL induced a substantial dose-dependent reduction in the capacity of LDL to bind chondroitin sulfate. LDL binding to chondroitin sulfate decreased by up to 75% after incubation with 3.5 μmol PJ polyphenols/L (**Figure 7**A).

The capacity of LDLs obtained from subjects supplemented with PJ to bind chondroitin sulfate was also determined as an indication of LDL retention. PJ supplementation for 1 and 2 wk affected LDL retention in only some of the volunteers; there was no significant effect on the mean value for all study participants (Figure 7B). A decrease in the capacity of LDL to bind chondroitin sulfate was observed in 69% and 54% of subjects after 1 and 2 wk of PJ supplementation, respectively.
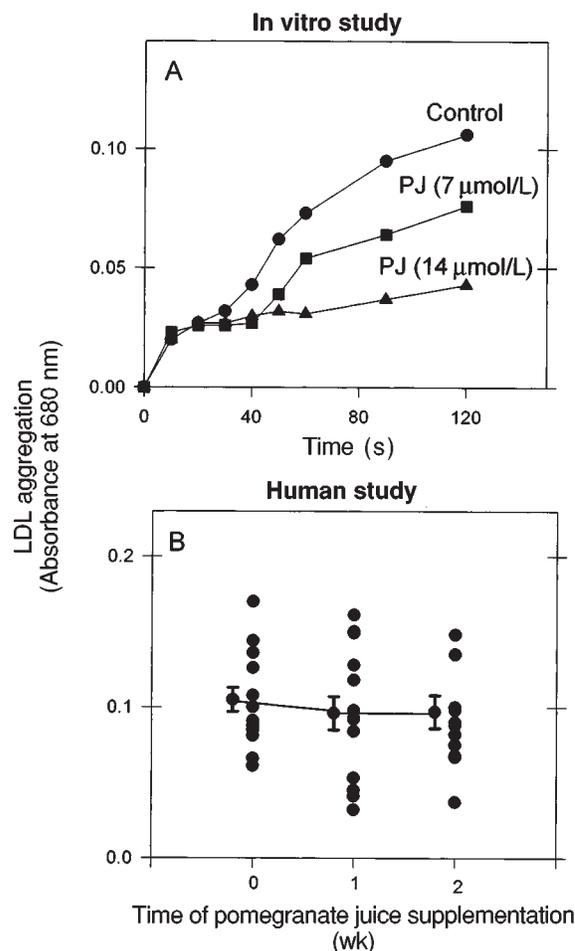

FIGURE 6. Effect of pomegranate juice (PJ) on the susceptibility of LDL to aggregation in vitro and ex vivo. (A) LDL (100 mg protein/L) was incubated without (control) or with 7 or 14 μmol polyphenols/L for 10 min at room temperature. LDL aggregation (by vortex mixing) was monitored kinetically at 680 nm. A representative experiment from 3 similar studies is given. (B) The extent of LDL aggregation measured in 13 healthy volunteers before (0) and after 1 or 2 wk of PJ supplementation. Results are given for each individual as well as the mean ± SD.
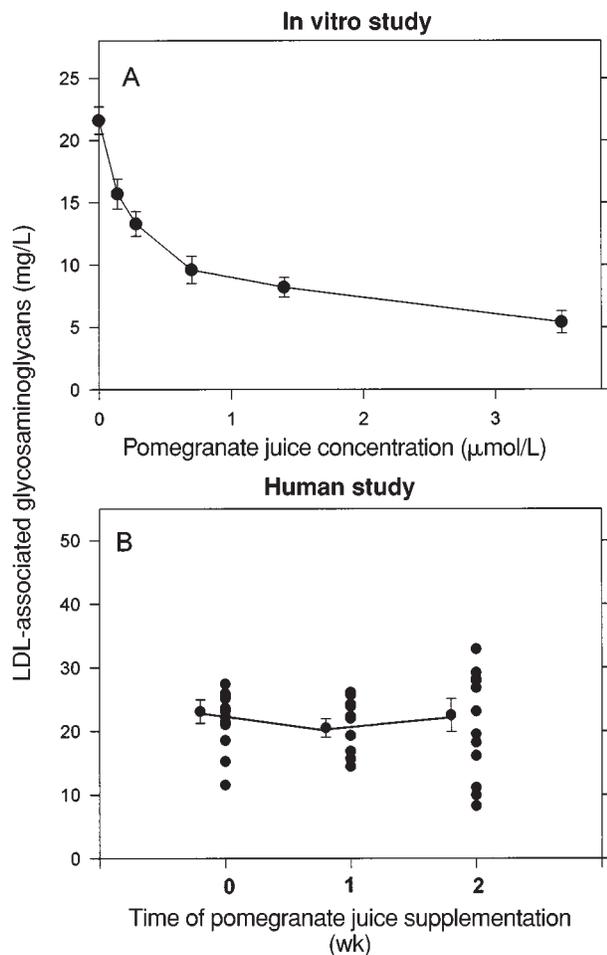
FIGURE 7. Effect of supplementation with pomegranate juice (PJ) on LDL retention as analyzed by the capacity of LDL to bind chondroitin sulfate. Mean (±SD) LDL-associated glycosaminoglycan content was analyzed by using the 1,9-dimethylmethylene blue assay described in Methods. For the in vitro studies (A), LDL (200 mg lipoprotein protein/L) was incubated with increasing concentrations of PJ (containing 0–3.5 μmol polyphenols/L). For the ex vivo studies (B), LDL (200 mg lipoprotein protein/L) was isolated from the plasma of subjects before and after 1 or 2 wk of PJ consumption. Shown in B are both the mean values and the individual results.

In vitro studies

As shown in **Figure 8**, PJ inhibited AAPH-induced plasma lipid peroxidation in vitro in a dose-dependent manner. TBARS formation was inhibited by 46% and lipid peroxide formation by 21% with 0.17 mL PJ/L, which was equivalent to 0.5 μmol total polyphenols/L. The susceptibility of isolated LDL to oxidation induced by copper ions was also substantially inhibited by PJ in a dose-dependent manner, as shown by a reduction in TBARS formation (**Figure 9**A) and a prolongation of the lag time required for the initiation of LDL oxidation by 40 min with the use of 0.24 mL PJ/L (equivalent to 0.7 μmol polyphenols/L) (Figure 9B). With 3.5 mL PJ/L (equivalent to 1 μmol polyphenols/L), LDL oxidation was not initiated even after 180 min. Similarly, PJ dose-dependently inhibited LDL oxidation induced by either AAPH or J-774 A.1 macrophages (Figure 9, C and D).

To study the mechanism responsible for the antioxidative capacity of PJ in vitro, we analyzed the potency of PJ when scavenging free radicals, chelating transition metal ions, or increasing serum paraoxonase activity. The addition of 4.9 mL PJ/L (14 μmol polyphenols/L) to a DPPH solution induced a dose-dependent decrease in absorbance at 517 nm, which reached a plateau within 7 min of incubation in a pattern similar to that obtained with 50 μmol vitamin E/L, which is a potent free radical scavenger (**Figure 10**A). To examine whether PJ inhibited LDL oxidation by chelation of metal ions, we incubated LDL with 0.2 mL PJ/L (0.56 μmol polyphenols/L) in the presence of increasing concentrations of copper ions. Incubation of LDL with 25 μmol Na_2EDTA/L served as a positive control because EDTA is a potent chelator of metal ions. As shown in Figure 10B, 25 μmol EDTA/L inhibited copper ion–induced LDL oxidation with up to 40 μmol $CuSO_4$/L. At higher copper ion concentrations,

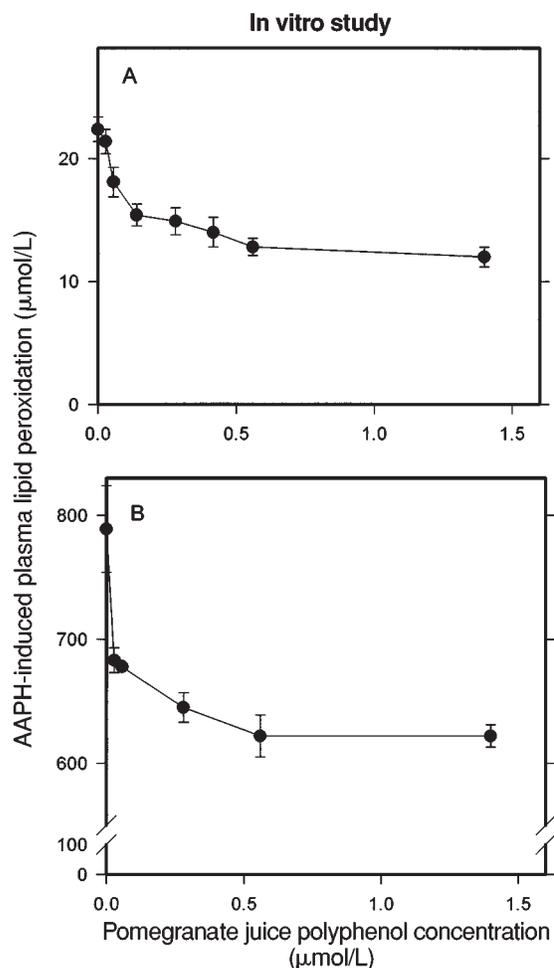**FIGURE 8.** Mean (±SD) effect of pomegranate juice (PJ) on 2,2′-azobis-2-amidinopropane hydrochloride (AAPH)-induced plasma lipid peroxidation. Normal human plasma was incubated with increasing concentrations of PJ (containing 0–1.5 μmol polyphenols/L), in the absence or presence of 100 mmol AAPH/L for 2 h at 37 °C. The extent of plasma lipid peroxidation was measured by the thiobarbituric acid–reactive substances (A) and lipid peroxides (B) assays. Values obtained in the absence of AAPH were subtracted from those obtained in the presence of AAPH. $n = 3$.
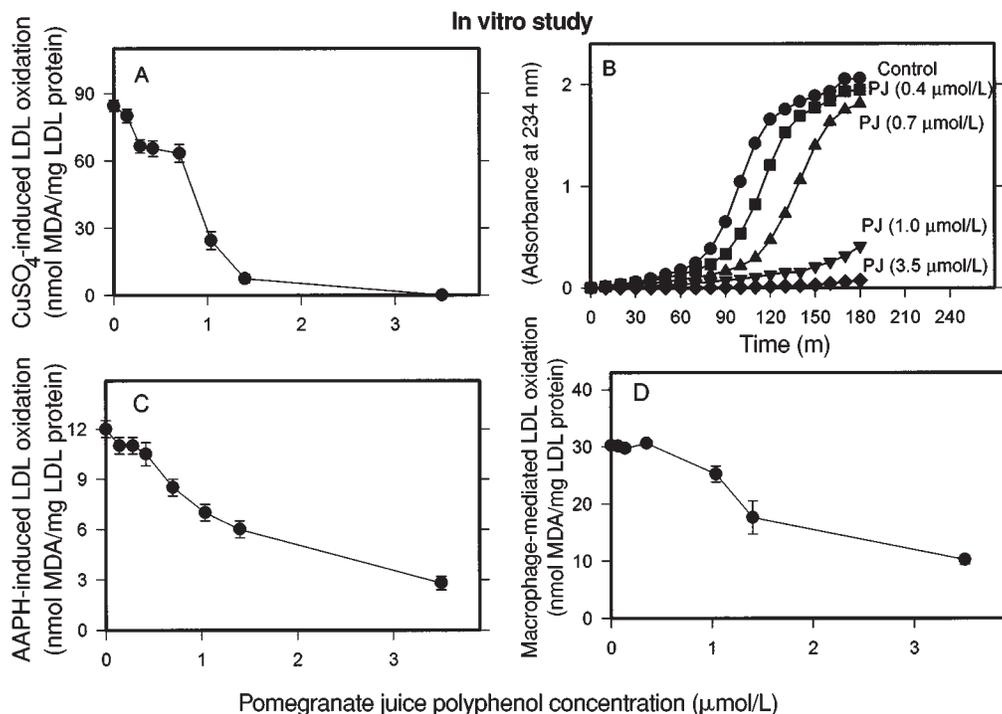
FIGURE 9. Mean (±SD) effect of pomegranate juice (PJ) on LDL susceptibility to oxidation. LDL (100 mg protein/L) was incubated with increasing concentrations of PJ (containing 0–3.5 μmol polyphenols/L). LDL oxidation was induced by incubation with 5 μmol CuSO₄/L and was measured as thiobarbituric acid–reactive substances (TBARS) formation after 2 h of incubation (A) or as conjugated dienes formation, monitored kinetically at 234 nm (B). LDL oxidation was also induced by 5 mmol 2,2′-azobis-2-amidinopropane hydrochloride (AAPH)/L (C) or by J-774 A.1 macrophages in the presence of 2 μmol CuSO₄/L (D) and measured as TBARS formation. $n = 3$. MDA, malondialdehyde.

EDTA could no longer overcome the prooxidative effect of $CuSO_4$. In contrast, 0.2 mL PJ/L inhibited $CuSO_4$-induced LDL oxidation even at a $CuSO_4$ concentration as high as 80 μmol/L, suggesting that PJ does not chelate copper ions.

HDL-associated paraoxonase activity in serum is related to protection of LDL against oxidation. On incubation of human serum with increasing concentrations of PJ for 10 min at 37 °C, PJ dose-dependently increased serum paraoxonase activity by up to 33% (Figure 10C). These results suggest that PJ inhibits plasma LDL lipid peroxidation in vitro and that this effect is associated with its capacity to scavenge free radicals as well as to increase serum paraoxonase activity.

To analyze the antioxidant properties of pomegranate constituents other than the juice, we prepared aqueous solutions of the inner and outer peels and the crushed seeds. The aqueous solutions of the concentrated PJ, the inner peel, the outer peel, and the seeds contained 22 830, 10 320, 6314, and 630 μmol total polyphenols/L, respectively. When we compared the inhibitory effects of these pomegranate constituents, based on an equal polyphenol concentration, we found that the aqueous extracts of the inner and outer peels were more powerful antioxidants than the juice, suggesting that the inner and outer peels may contain more potent antioxidant polyphenols. The concentrations of polyphenols required to inhibit LDL oxidation by 50% (IC_{50}) were 0.57 and 0.66 μmol/L for the inner and outer peel, respectively, compared with 1.00 μmol/L for the juice (**Figure 11**A). The aqueous extract obtained from the crushed seeds was found to be a weak antioxidant against LDL oxidation.

Because it is possible that substances other than polyphenols contributed to the antioxidant activity of the pomegranate constituents, we also analyzed the effect of increasing weight concentrations of the constituents (rather than increasing total polyphenol content as above) on copper ion–induced LDL oxidation (Figure 11B). In this experiment, the inner and outer peels contained potent antioxidants whereas the crushed seeds and PJ showed no inhibitory effects at the concentrations used. Per 1 mg, the inner and outer peels contain 20–30-fold more polyphenols than do the aqueous fractions of the seeds and PJ (566 and 739 nmol polyphenols/mg compared with 22 and 25 nmol polyphenols/mg, respectively). The ineffectiveness of PJ at inhibiting LDL oxidation in this experiment (Figure 11B), in contrast with its potency in the previous experiment (Figure 11A), may be related to the much lower total polyphenol concentration of the aqueous extract used (about 900-fold lower than that used for the experiment shown in Figure 11A).

Studies of macrophage atherogenicity in E^0 mice

We showed previously that macrophages can undergo lipid peroxidation under oxidative stress and that, subsequently, these cells can oxidize LDL (52, 53). LDL oxidation by macrophages is considered to be a major event in early atherogenesis and is associated with cellular uptake of the modified lipoprotein, leading to macrophage cholesterol accumulation and foam cell formation (1). We thus studied the effect of PJ supplementation of E^0 mice on macrophage lipid peroxidation and, subsequently, on macrophage activities related to foam
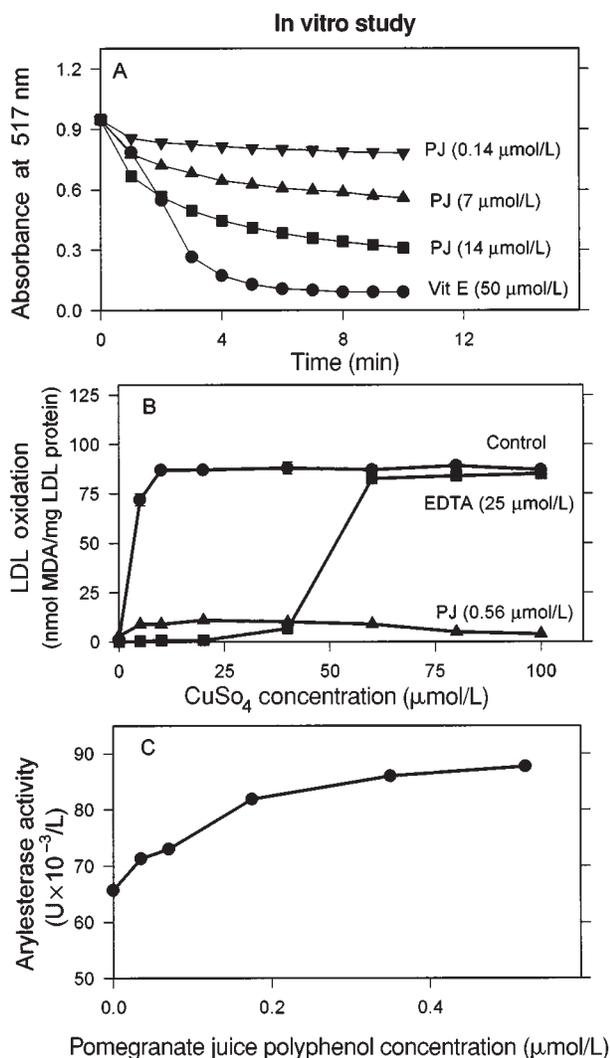
FIGURE 10. Mechanisms for pomegranate juice (PJ) protection against LDL oxidation. (A) Free radical scavenging capacity of PJ. 1,1-Diphenyl- 2-picrylhydrazyl (DPPH) ethanolic solution at a final concentration of 100 µmol/L was mixed with increasing concentrations of PJ (containing 0–14 µmol polyphenols/L) or with 50 µmol vitamin (Vit) E/L. The time course of the changes in absorbance was monitored continuously at 517 nm. (B) Capacity of PJ to chelate copper ions. LDL (100 mg protein/L) was incubated with increasing concentrations of $CuSO_4$ (0–100 µmol/L) in the absence (control) or presence of 25 µmol Na_2EDTA/L (EDTA) and 0.56 µmol polyphenols/L (PJ) for 2 h at 37 °C. The extent of LDL oxidation was measured by the thiobarbituric acid–reactive substances assay. (C) Effect of PJ on serum paraoxonase activity. Increasing concentrations of PJ (containing 0–0.5 µmol of polyphenols/L) were added to human serum (obtained from normolipidemic volunteers) and the samples were incubated for 10 min at 37 °C before arylesterase activity was measured. MDA, malondialdehyde.

cell formation, including cell-mediated oxidation of LDL and cellular uptake of lipoproteins.

Macrophage-mediated oxidation of LDL

MPMs were isolated from the peritoneal cavity of control E^0 mice and from E^0 mice that consumed 12.5 µL PJ/d (equivalent

to 0.350 µmol total polyphenols/d) for 2 mo. Lipid peroxidation of MPMs isolated from E^0 mice after consumption of PJ was 53% less than lipid peroxidation of MPMs isolated from control mice (**Figure 12**A). Incubation of these cells with LDL for 18 h under oxidative stress showed that PJ consumption resulted in an 82% inhibition of macrophage-mediated LDL oxidation, as measured by the TBARS assay (Figure 12B).

Macrophage-mediated LDL oxidation was shown to involve activation of NADPH oxidase and superoxide anion release (12) and to depend on the balance between cellular oxidants and antioxidants, including the glutathione system (13, 47). Also shown in
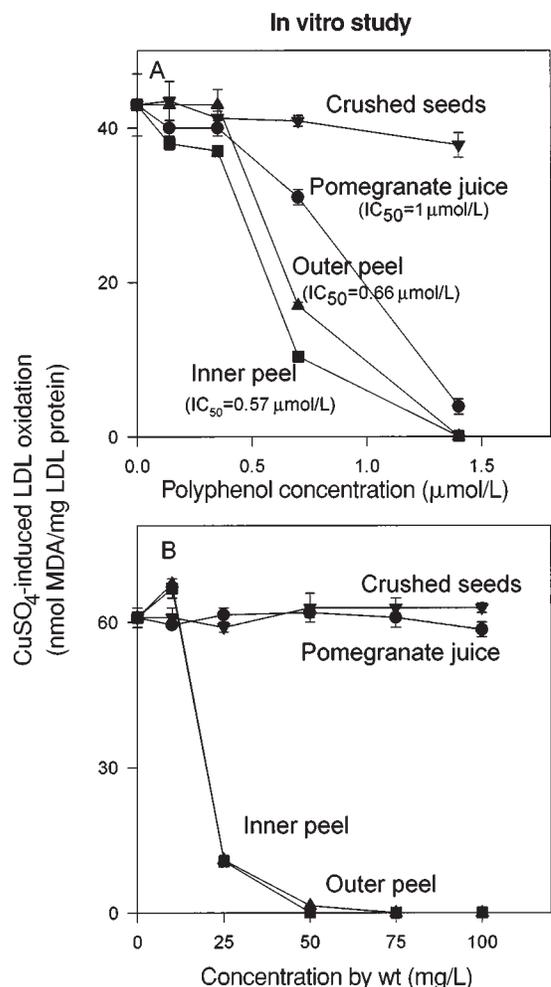

FIGURE 11. Mean (±SD) capacity of pomegranate constituents (juice, peels, and seeds) to inhibit copper ion–induced LDL oxidation. (A) Aqueous extracts of PJ, crushed seeds, and inner and outer peels were prepared and the polyphenol content was determined as described in Methods. LDL (100 mg protein/L) was incubated with increasing concentrations of polyphenols (0–1.5 µmol/L) for 2 h at 37 °C in the presence of 5 µmol $CuSO_4$/L. (B) The PJ and the aqueous extracts of crushed seeds and inner and outer peels were lyophilized and their dry weights measured. All samples were dissolved in water and diluted to 1 g wt/L. LDL (100 mg protein/L) was then incubated with increasing concentrations of the pomegranate fractions (0–100 mg weight/L) for 2 h at 37 °C in the presence of 5 µmol $CuSO_4$/L. LDL oxidation was analyzed by the thiobarbituric acid–reactive substances assay. $n = 3$. MDA, malondialdehyde.
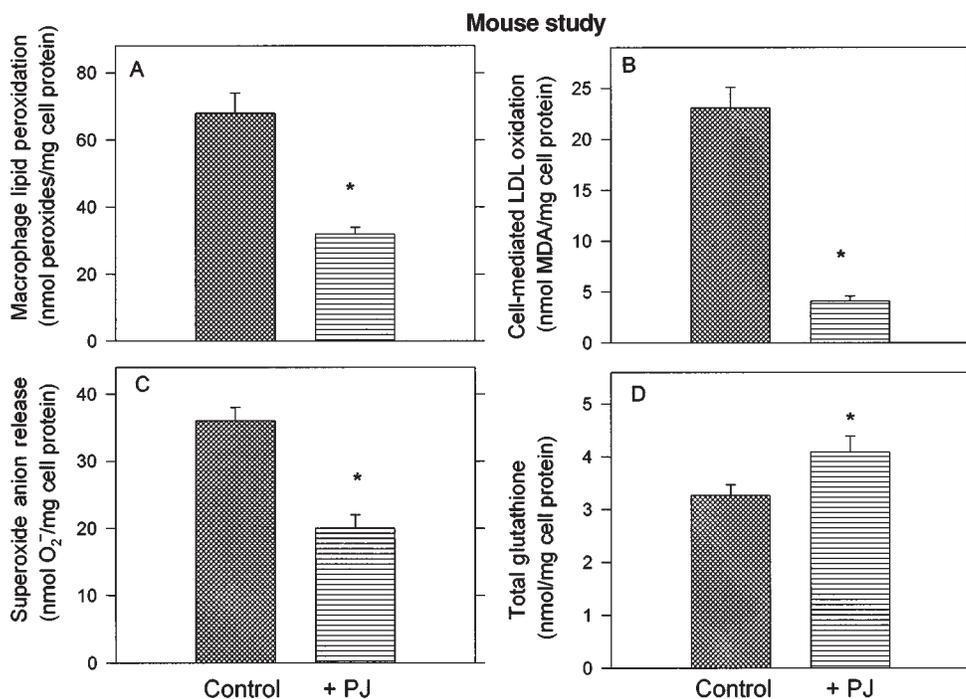
FIGURE 12. Mean ($\pm$SD) effect of pomegranate juice (PJ) consumption by atherosclerotic apolipoprotein E–deficient (E^0) mice on peritoneal macrophage lipid peroxidation (A), macrophage-mediated oxidation of LDL (B), macrophage superoxide anion release (C), and macrophage total glutathione content (D). Mouse peritoneal macrophages (MPMs) were isolated from the peritoneal fluid of control E^0 mice (consumed no PJ) and of E^0 mice that consumed 12.5 μL juice/d for 2 mo. In A, lipid peroxide content was assayed in cell sonicates of MPMs. In B, MPMs were incubated for 6 h at 37°C with LDL (100 mg protein/L) in the presence of 2 μmol $CuSO_4$/L. LDL oxidation was measured directly by the thiobarbituric acid–reactive substances assay. In C, cells were stimulated by the addition of LDL (100 mg protein/L) and $CuSO_4$ (5 μmol/L) for 1 h at 37°C. Superoxide anion release was measured as described in Methods. In D, the cell sonicate was used as described in Methods. $n = 3$. MDA, malondialdehyde. *Significantly different from control, $P < 0.01$.

Figure 12 is that superoxide anion release from macrophages activated by incubation with LDL in the presence of copper ions was significantly lower (by 49%) for supplemented mice than for control mice (Figure 12C). In parallel, the cellular content of glutathione was 25% higher in macrophages from supplemented mice than in macrophages from control mice (Figure 12D).

Macrophage uptake of oxidized LDL and native LDL

We next questioned the effect of PJ consumption on macrophage uptake of oxidized LDL and native LDL. Cellular lipoprotein binding, cell association, and degradation were 16%, 22%, and 15% lower, respectively, in MPMs collected from E^0 mice that consumed 12.5 μL PJ/d for 2 mo and incubated with ^{125}I-labeled oxidized LDL (10 mg protein/L) than in MPMs from control E^0 mice (**Figure 13**). Similarly, macrophage binding, cell association, and degradation of native LDL was 31%, 19%, and 27% lower, respectively, in MPMs from supplemented mice than in MPMs from control mice.

Atherosclerotic lesion formation in E^0 mice

Shown in **Figure 14** are photomicrographs of typical atherosclerotic lesions in E^0 mice that consumed water (control) or PJ. The lesions in control mice were larger than those in PJ-treated mice and consisted of many more lipid-laden macrophage foam cells. Moreover, the lesion areas in PJ-treated mice were significantly smaller, by 44%, than the lesion areas in aortas from control mice.

Platelet aggregation

Circulating human platelets play an important role in the development of atherosclerosis, and increased platelet aggregation is associated with enhanced atherogenicity (6–8). To study whether PJ could inhibit platelet aggregation, we incubated platelet-rich plasma with increasing concentrations of PJ and then induced aggregation by adding collagen. PJ inhibited collagen-induced platelet aggregation, by up to 90%, in a dose-dependent manner (**Figure 15**A). Analysis of platelet-rich plasma aggregation was also studied ex vivo. After 2 wk of PJ consumption, collagen-induced platelet aggregation was significantly reduced by 11% ($P < 0.02$) compared with platelet aggregation before PJ consumption (Figure 15B).

DISCUSSION

The present study showed for the first time the antiatherogenic properties of PJ as related to its inhibitory effect on lipid peroxidation in plasma, in lipoproteins, and in macrophages. Most importantly, PJ treatment significantly and substantially inhibited the progression of atherosclerotic lesions. PJ inhibited atherogenic modifications of LDL, including its retention, oxidation, and aggregation. Finally, the antiatherogenicity of PJ may be related to its ability to attenuate platelet activation, an additional important risk factor for atherosclerosis.

The lipid peroxidation hypothesis of atherosclerosis (1–3) is supported by evidence of oxidized lipoproteins in atherosclerotic
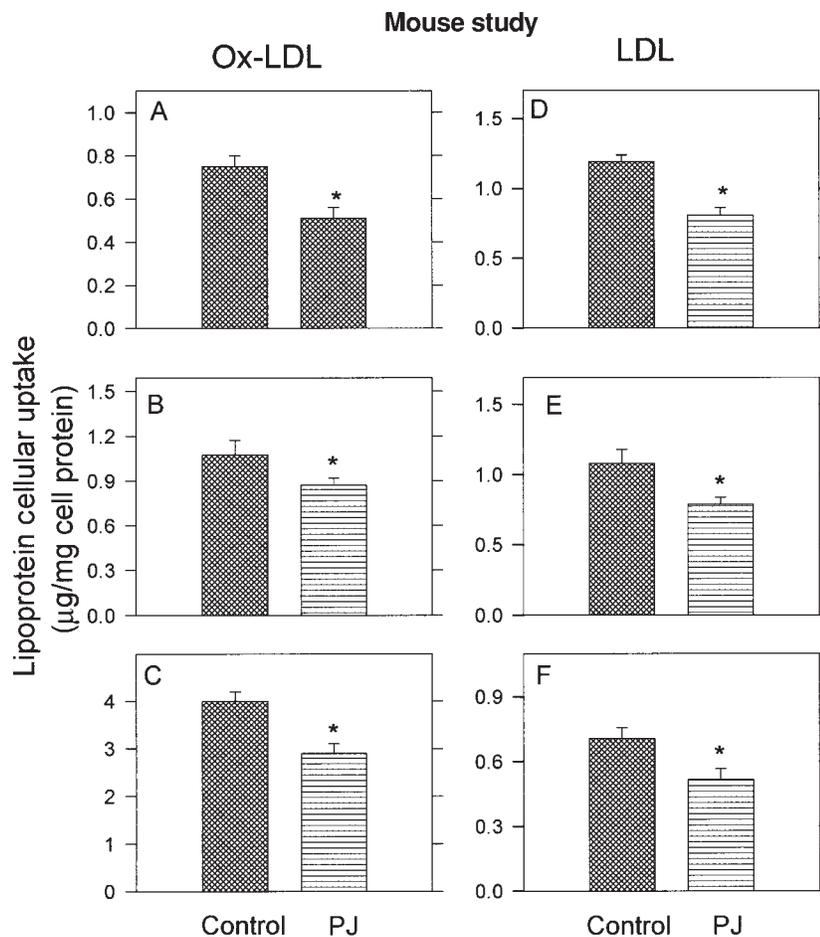
FIGURE 13. Mean ($\pm$SD) effect of pomegranate juice (PJ) consumption by atherosclerotic apolipoprotein E–deficient (E^0) mice on macrophage uptake of native or oxidized LDL. Mouse peritoneal macrophages (MPMs) were isolated from the peritoneal fluid of control E^0 mice and E^0 mice that consumed 12.5 µL PJ for 2 mo. MPMs were incubated with [^{125}I]oxidized LDL (Ox-LDL) or [^{125}I]LDL (10 mg protein/L) at 4 °C for 2 h for determination of lipoprotein binding (A, D) or at 37 °C for 5 h for determination of lipoprotein cell association (B, E) and lipoprotein degradation (C, F). $n = 3$. *Significantly different from control, $P < 0.01$.

lesions (54), by the relatively greater oxidizability of LDL from atherosclerotic patients (55), and by the antiatherogenicity of some antioxidants against LDL oxidation (13, 56). The impressive ability of PJ to inhibit in vitro and ex vivo lipid peroxidation in plasma, as well as in isolated LDL and HDL, was shown in several different oxidative systems including transition metal ions, free radical generators, and arterial cells. By more than one assay (TBARS, lipid peroxide, and conjugated diene formation) we showed the substantial antioxidative capacity of PJ to scavenge free radicals, a major mechanism of action of some potent natural antioxidants, including vitamin E and flavonoids (57, 58). The inhibitory effect of PJ against LDL oxidation was also shared by aqueous extracts of the outer and inner peel of pomegranates. When compared per total polyphenol content (or per weight), the peels were more potent antioxidants than the juice. These fractions may contain different flavonoids from those present in PJ that are more potent antioxidants.

Paraoxonase, an HDL-associated esterase, was shown to protect both HDL and LDL from oxidation. This protection is probably the result of the ability of paraoxonase to hydrolyze specific oxidized lipids in oxidized lipoproteins and human atherosclerotic lesions

(59–61). Paraoxonase is inactivated by lipid peroxides (62), and we showed that red wine flavonoids (23) and licorice-derived glabridin (62) can preserve paraoxonase activity during lipoprotein oxidation. However, we showed not only a preservation of activity, but also an enhancement of activity of PJ. These results further strengthen the inverse association between serum paraoxonase activity and lipid peroxidation (59).

PJ supplementation of E^0 mice, which are under oxidative stress (63), resulted in substantially lower plasma lipid peroxidation than in control mice, as well as a lower susceptibility of LDL to copper ion–induced oxidation. These inhibitory effects were greater in the atherosclerotic mice than in healthy human volunteers. This phenomenon may be related to the high initial oxidative stress in the E^0 mice.

Atherosclerosis is a multifactorial disease and factors other than LDL oxidation can accelerate atherogenesis independently or in association with lipid peroxidation. Such factors include LDL retention (5) and LDL aggregation (3). LDL oxidation is thought to occur in the arterial wall after lipoprotein binding to extracellular matrix proteoglycans. Although an impressive inhibitory effect of PJ on LDL retention was observed in vitro, in the ex vivo study
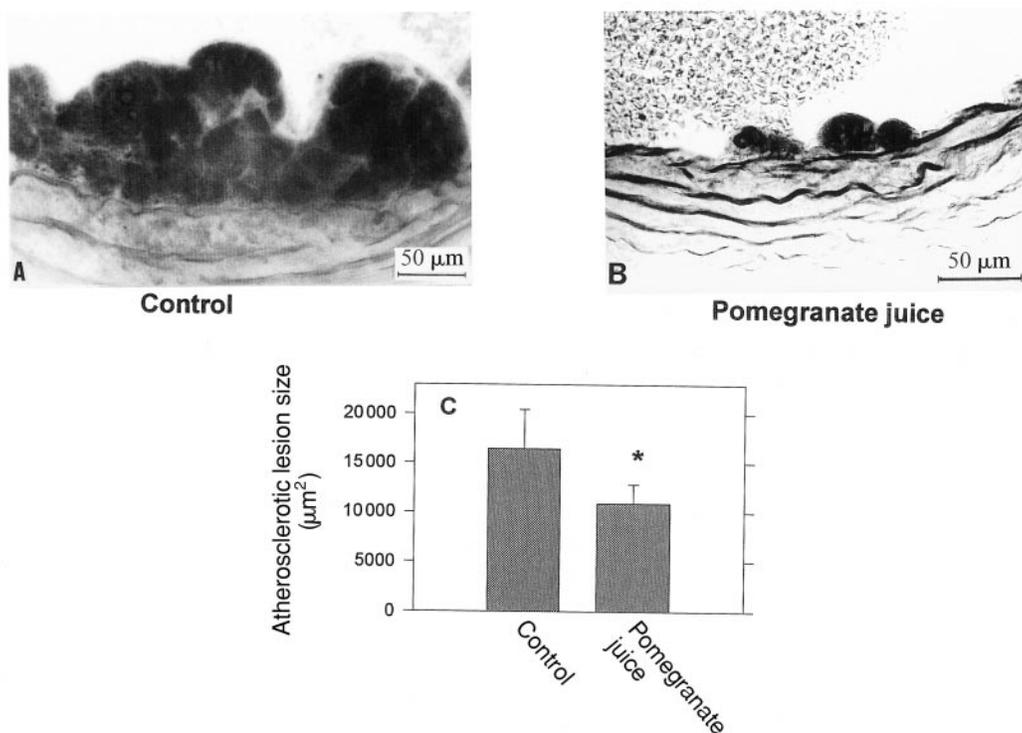
FIGURE 14. Effect of pomegranate juice (PJ) consumption by atherosclerotic apolipoprotein E–deficient (E^0) mice on foam cell formation and on the size of atherosclerotic lesions. Photomicrographs of typical foam cells from E^0 mice that consumed water (A) or PJ (B) are presented. Lipids in macrophages of the lesions are colored black by osmium tetraoxide. All micrographs are at the same magnification. The mean (±SD) lesion size of atherosclerotic lesions in the aortic arches of E^0 mice that consumed PJ or water (control) for 11 wk was analyzed histomorphometrically (C). *Significantly different from control, $P < 0.01$.

LDL retention was inhibited in only 50–60% of the subjects. Even though the inhibitory response in LDL retention was not significant when all volunteers were studied together, it seems that there were responders and nonresponders in this respect. This may be related to additional factors that affect LDL retention in vivo, such as LDL density, charge, and sialic acid content (64, 65).

LDL retention can predispose the lipoprotein to oxidation, and LDL oxidation can lead to an additional atherogenic modification: lipoprotein aggregation (21). Aggregated LDL is taken up by macrophages at an enhanced rate, leading to cellular cholesterol accumulation and foam cell formation (4). Macrophages can also cause LDL aggregation, independently of its oxidation, after the secretion of proteoglycans from the cells under certain conditions (66, 67). The present study showed that LDL aggregation was also inhibited in vitro by PJ; this inhibition may be related to hydrophobic interactions between constituents of PJ and the lipoprotein (68). Arterial wall macrophages play a major role in early atherogenesis. We showed that under oxidative stress, lipid peroxidation affects not only lipoproteins but also cellular lipids (52). Furthermore, cell-mediated oxidation of LDL can be achieved after lipid-peroxidized macrophages are incubated with LDL, even in the absence of a transition metal ion (52, 53). Macrophage-mediated oxidation of LDL is associated with activation of cellular NADPH oxidase, which produces superoxide anions (12). Superoxide ions can be converted under certain conditions into a more potent reactive oxygen species (69), which can then convert native LDL to atherogenic oxidized LDL. Macrophage-mediated oxidation of LDL is substantially

increased in glutathione-depleted cells and cellular lipid peroxides are formed under these conditions (13, 47). The present study clearly showed that macrophage-mediated oxidation of LDL was substantially lower for macrophages derived from E^0 mice after PJ consumption than for control mice. This antiatherogenic effect was associated with higher cellular glutathione content, lower macrophage superoxide anion release, and lower macrophage lipid peroxidation. These observations further support a key role for cellular lipid peroxidation in macrophage-mediated oxidation of LDL (52, 53).

Polyphenolic flavonoids not only affect cellular oxygenases but also cause conformational changes in plasma membrane constituents, such as cellular receptors for lipoproteins. We thus analyzed the uptake of both oxidized LDL and native LDL by peritoneal macrophages from E^0 mice after PJ consumption. We showed that cellular degradation, cell association, and cellular binding of both lipoproteins were lower in cells from mice fed PJ than in cells from control mice. Consumption of PJ by the atherosclerotic E^0 mice thus reduced oxidative stress in the cells (which was associated with reduced cell-mediated oxidation of LDL) and also reduced the uptake of oxidized LDL. Both of these processes contribute to attenuation of macrophage cholesterol accumulation and foam cell formation. Most importantly, our finding of reductions in the atherosclerotic lesions in PJ-treated mice clearly strengthens the correlation between the antioxidative effect and the antiatherogenic properties of PJ.

Finally, platelet activation, an additional risk factor for atherosclerosis (6) that is also associated with oxidative stress (7), was
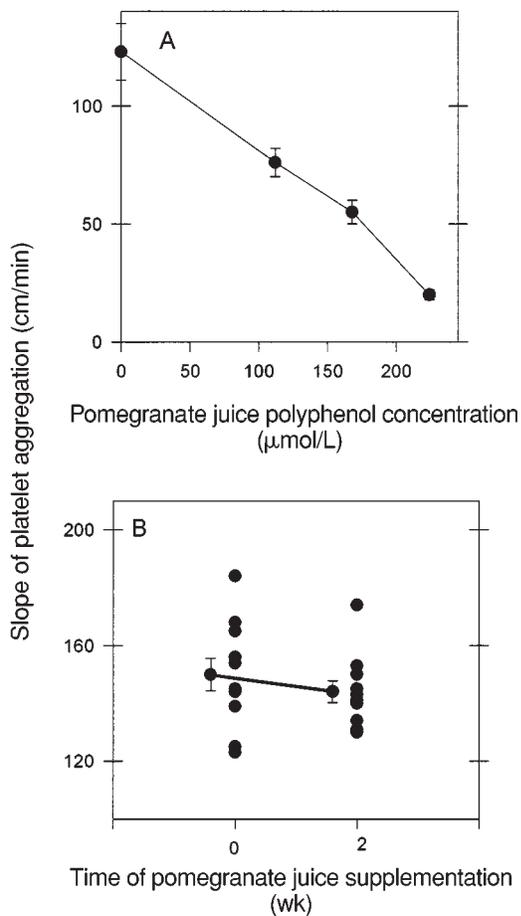
FIGURE 15. The effect of pomegranate juice (PJ) on human platelet aggregation in vitro and ex vivo. (A) Platelet-rich plasma (PRP) was incubated with increasing concentrations of PJ (containing 0–220 μmol polyphenols/L) for 10 min at 37 °C before analysis of platelet aggregation in response to 2 mg collagen/L. $\bar{x} \pm SD$. (B) PRP was also prepared from 11 healthy volunteers before (0) or after 2 wk of PJ supplementation. Results are given for each individual and also as the mean ± SD ($n = 11$).

also inhibited by PJ consumption. This effect may be related to an interaction of PJ constituents with the platelet surface binding sites for collagen or by their ability to scavenge free radicals and hence to attenuate platelet activation induced by oxidative stress (7).

In conclusion, we showed the antiatherogenic capabilities of PJ in 3 related components of atherosclerosis: plasma lipoproteins, arterial macrophages, and blood platelets. The potent antioxidative capacity of PJ against lipid peroxidation may be the central link for the antiatherogenic effects of PJ on lipoproteins, macrophages, and platelets.

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