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Pomegranate Byproduct Administration to Apolipoprotein E-Deficient Mice Attenuates Atherosclerosis Development as a Result of Decreased Macrophage Oxidative Stress and Reduced Cellular Uptake of Oxidized Low-Density Lipoprotein

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The effects of a pomegranate byproduct (PBP, which includes the whole pomegranate fruit left after juice preparation) on atherosclerosis development in apolipoprotein E-deficient (E°) mice were studied. Consumption of PBP (17 or 51.5 μg of gallic acid equiv/kg/day) by the mice resulted in a significant reduction in atherosclerotic lesion size by up to 57%. PBP consumption significantly reduced oxidative stress in the mice peritoneal macrophages (MPM): Cellular lipid peroxide content decreased by up to 42%, the reduced glutathione levels increased by up to 53%, and paraoxonase 2 lactonase activity increased by up to 50%, as compared to MPM from E° mice that consumed only water. Furthermore, oxidized low-density lipoprotein (Ox-LDL) uptake by the MPM was reduced by up to 19%. Similar results were observed also in vitro. Treatment of J774A.1 macrophages with PBP (10 or 50 μmol/L of total polyphenols) significantly decreased both cellular total peroxide content and Ox-LDL uptake. It was thus concluded that PBP significantly attenuates atherosclerosis development by its antioxidant properties.

KEYWORDS: Pomegranate; macrophages; oxidative stress; apolipoprotein E-deficient mice; atherosclerosis

INTRODUCTION

Macrophage cholesterol accumulation leading to foam cell formation is the hallmark of early atherosclerosis (*1*−*3*). Cholesterol accumulation in macrophages can result from increased uptake of atherogenic lipoproteins such as oxidized low-density lipoproteins (Ox-LDL; *4*, *5*). Enhanced uptake of Ox-LDL by macrophages via scavenger receptors (*6*, *7*) leads to the formation of lipid-laden foam cells and accelerated development of atherosclerotic lesions. Oxidative stress has been shown to affect lipids not only in the LDL but also in the macrophage (*3*, *8*). We have previously demonstrated that in vitro lipid-peroxidized macrophages, or oxidized lipid-rich peritoneal macrophages derived from the atherosclerotic apolipoprotein E deficient (E°) mice, exhibit increased ability to take up Ox-LDL (*8*, *9*). Because oxidative stress plays an important role in atherogenesis, its inhibition by nutritional antioxidants should retard the progression of the disease. Indeed, we have shown that red wine and licorice polyphenolic flavonoids protected LDL against lipid peroxidation and attenuated the development of atherosclerosis (*10*−*12*). Pomegranate juice (PJ) also possesses impressive antioxidant properties due to its polyphenolics, tannins, and anthocyanins (*13*, *14*). PJ supplementation to E° mice significantly reduced oxidative stress in their serum and macrophages and inhibited the progression of atherosclerotic lesion development (*15*, *16*). Similarly, consumption of PJ by patients with carotid artery stenosis for 3 years decreased oxidative stress in their blood and the size of their atherosclerotic lesions (*17*). Recently, it was also shown that daily consumption of PJ improved stress-induced myocardial ischemia in patients with coronary artery disease (*18*). PJ consumption by diabetic patients for 3 months did not aggravate their diabetic condition, and resulted in a significant reduction in the oxidative status of their serum and monocyte-derived macrophages, as well as in Ox-LDL uptake by these cells (*19*). These cardioprotective effects of PJ could be attributed to its potent polyphenols and also to its unique sugars (*20*).

We have previously shown that aqueous solutions of the pomegranate inner and outer peels contain powerful antioxi-

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Table 1.

pomegranate component	pomegranate juice (65 °Brix), μg/mL	pomegranate byproduct (70 °Brix), μg/mL
phenolics	2369	18100
hydrolyzable tannins	2000	15300
ellagitannins	251	1920
gallotannins	1789	4300
punicalagin	1487	16300
ellagic acid	115	400
anthocyanins and their glycosides	369	387
delphindin	56	59
cyanidin	94	98
pelargonidin	4	6

dants, which were even more potent than the PJ antioxidants (*15*). Thus, in the present study we examined the effects of consuming pomegranate byproduct (PBP; prepared from the pomegranate husks after extraction of the juice) by atherosclerotic E° mice on aortic arch atherosclerotic lesion progression and the antiatherogenic mechanisms involved.

MATERIALS AND METHODS

Reagents. 2′,7′-Dichlorofluorescin diacetate (DCFH) was purchased from Sigma (St. Louis, MO). FITC-conjugated antibody was purchased from Serotec IQ Products (Zerinkepark, The Netherlands). PBS, DMEM, RPMI-1640 medium, FCS (heat-inactivated at 56 °C for 30 min), penicillin, streptomycin, nystatin, L-glutamine, and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel).

Pomegranate Byproduct. After the juice had been expelled from the pomegranates, the husks were collected and milled to a fine puree. The puree was enzymatically treated to break down the colloidal structure of the husks and to solubilize sugars, minerals, acids, and polyphenol compounds into the byproduct juice. The obtained juice was passed through an evaporator for preconcentration, pasteurized, and filtered. The final product had a 70 °Brix concentration and was stored at 4 °C.

Pomegranate Juice Preparation. Pomegranates were picked by hand, washed, and stored in tanks. The fruit was crushed and squeezed. The juice was filtered, pasteurized, concentrated, and stored at −18 °C. The concentrated PJ (65 °Brix) was diluted 1:5 (v/v) with water to obtain a single-strength PJ to be used in the study. **Table 1** provides the phenolics composition of PBP in comparison to PJ. It demonstrates a major increased content of the hydrolyzable tannin, punicalagin, in PBP versus PJ, with no significant difference in anthocyanins.

Total Polyphenols in PBP. The total polyphenol concentrations of PBP and PJ were determined spectrophotometrically with phosphomolybdic−phosphotungstic acid reagent and gallic acid as a standard (*21*).

Free Radical Scavenging Capacity (DPPH Assay). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a radical-generating substance widely used to monitor the free radical scavenging abilities of various antioxidants (*22*). To analyze the free radical scavenging capacity of PBP in comparison to PJ, similar volumes (0.2 μL/mL), or similar polyphenol concentrations (10 μmol/L), were mixed with 1 mL of 0.1 mmol DPPH/L in ethanol. The time course for the change in optical density at 517 nm was monitored kinetically.

Mice Studies. The E° mice were generously provided by Jan Breslow of Rockefeller University, New York. These mice are on the background of C57BL/6J × C57BBAJ and develop significant atherosclerosis at the age of 4 months under chow diet. To analyze the effect of PBP on the development of atherosclerotic lesions, 30 male E° mice (6 weeks old) were divided into three groups consisting of 10 mice each. The placebo group received only water. The other two groups received PBP diluted in water for a 3 month period. One group received 17 μg of gallic acid equiv/kg/day and the second group, 51.5 μg of gallic acid equiv/kg/day. These PBP dosages were chosen on the basis of our previous studies with PJ in these mice (*15*, *16*). During the study the mice received chow diet. At the end of the study, mouse peritoneal macrophages (MPM) and aortas were retrieved for analysis. This protocol was approved by the Committee for the Supervision of Animal Experiments and complied with the Guide for Care and Use of Laboratory Animals, the Technion-Israel Institute of Technology, Haifa, Israel.

Cells. *MPM.* MPM were harvested from the peritoneal fluid of the E° mice, 4 days after intraperitoneal injection into each mouse of 3 mL of thioglycolate (40 g/L) in saline. The cells ($10-20 \times 10^6$/mouse) were washed and centrifuged three times with phosphate-buffered saline (PBS) at 1000*g* for 10 min and then resuspended at 10^9/L in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% FCS, 1×10^5 units of penicillin/L, 100 mg of streptomycin/L, and 2 mmol/L glutamine. The dishes were incubated in a humidified incubator (5% CO_2, 95% air) for 2 h and washed with DMEM to remove nonadherent cells, and the analyses to measure cellular oxidative stress were immediately performed.

J774A.1 Macrophages. J774A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown in DMEM containing 5% FCS.

Cellular Oxidative Status. *Assay of Cellular Total Peroxides (DCFH-DA Assay).* Intracellular oxidative stress was assayed by the oxidation of DCFH-DA (*23*), as monitored by flow cytometry (FACS) (*24*). J774A.1 macrophages (1×10^6) that were incubated without or with PBP for 18 h at 37 °C were washed (one time) with PBS and then incubated with 10 μmol/L of DCFH-DA for 30 min at 37 °C. Adherent cells were detached by gentle scraping, and all cells were washed (two times) with PBS. Measurements of cellular fluorescence determined by FACS were performed at 510−540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).

Basal Lipid Peroxides in Macrophages. MPM derived from the mice after 3 months of PBP consumption or J774A.1 macrophages (2×10^6) that were incubated without or with PBP for 18 h at 37 °C were washed and lipid extracted with hexane/2-propanol (3:2, v/v). The hexane phase was evaporated under nitrogen, and the levels of lipid peroxides were determined according to the El-Saadani method (*25*). The cellular protein was determined by using the Lowry assay (*26*), after the addition of 0.1 mol/L NaOH.

Macrophage Susceptibility to Oxidation. J774A.1 macrophages (2×10^6) were incubated for 5 h at 37 °C with 500 μmol/L of iron ascorbate or with 5 mmol/L of the free radical generator 2,2′-azobis-(2-amidinopropane hydrochloride) (AAPH; Wako Chemical Industries, Ltd., Osaka, Japan). The cells were then washed and the levels of cellular lipid peroxides determined as described above.

Macrophage Reduced Glutathione (GSH) Content. All of the preparation steps were carried out on ice. MPM derived from the mice from triplicate dishes (2×10^6 per dish) were washed, scraped from the dish, and sonicated in an ultrasonic processor (3×20 s at 80 W). The protein content was measured by using the Lowry method (*26*) and reduced glutathione content by the DTNB-GSSG reductase recycling assay (*27*).

Macrophage Paraoxonase 2 (PON2) Lactonase Activity. MPM derived from the mice (2×10^6) were incubated with 1 mmol/L of dihydrocoumarin in Tris buffer for 10 min. As a control, dihydrocoumarin was also incubated with no cells. PON2 activity was determined spectrophotometrically at 270 nm and expressed as units per milligram of cell protein (*28*).

Ox-LDL Uptake by Macrophages. LDL was separated from the plasma of normal healthy volunteers by discontinuous density-gradient ultracentrifugation (*29*) and dialyzed against saline with EDTA (1 mmol/L). LDL protein concentration was determined according to the Lowry method (*26*). Before oxidation, LDL was diluted in PBS to 1 mg/mL and dialyzed overnight against PBS at 4 °C to remove the EDTA. Oxidation of LDL was carried out at 37 °C under air in a shaking water bath. LDL (1 mg/mL) was incubated for 18 h at 37 °C with 5 μmol/L freshly prepared $CuSO_4$. Oxidation was terminated by refrigeration at 4 °C. The extent of LDL oxidation was determined by using the TBARS assay (*30*). Ox-LDL was conjugated to fluoroisothiocyanate

(FITC) for cellular uptake studies. MPM were incubated at 37 °C for 3 h with FITC-conjugated Ox-LDL at a final concentration of 25 μg of protein/mL. The uptake of the lipoprotein was determined by flow cytometry. Measurements of cellular fluorescence determined by FACS were done at 510−540 nm after excitation of the cells at 488 nm with an argon ion laser. Cellular fluorescence was measured in terms of mean fluorescence intensity (MFI).

Macrophage Cholesterol Mass. Lipids from the MPM (2×10^6) were extracted with hexane/2-propanol (3:2, v/v), and the hexane phase was evaporated under nitrogen. The amount of cellular cholesterol was determined using a kit (CHOL, Roche Diagnostics GMbH, Mannheim, Germany).

Histopathology of Aortic Atherosclerotic Lesions. After PBP consumption by E° mice for 3 months, the heart and entire aorta were rapidly dissected out and immersion-fixed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer with 0.01% calcium chloride, pH 7.4, at room temperature. The procedure of the lesion sections preparation for histomorphometrically analyses was previously described (*15*). 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° mice, and the areas are well defined with a clear starting point (aortic valves). Histopathology determinations of the lesion size were performed by using an Olympus Cue-2 image analysis system with appropriate morphometry software (Olympus Corp., 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, Japan) with the video image seen on a 14-in. color monitor (Sony, Tokyo, Japan) and an IBM-compatible PC. Measurements were made in standardized "windows" (fields) with an area of 176758 μm^2. Our method of using epon-embedded thin sections stained with osmium provides detailed information regarding lesion morphology and very good resolution for image analysis. The boundaries of the lesions are clearly shown due to the black staining with the osmium of the lipid components. A further advantage of our method is the ability to cut very thin (1 μm) sections for a greatly improved histopathology (as seen in our illustrations, **Figure 2**), rather than those of standard wax or frozen sections. Indeed, it does not provide a measure of the overall extent of atherosclerosis, as this would be possible only if many serial sections were taken. Nonetheless, our sampling methodology (number of animals per group and image analysis technique) truly reflects the changes in lesion size between the various groups and gives a true measure for comparative purposes.

Statistics. Statistical analysis was performed using Student's *t* test for comparison of the mean of two groups. Results are given as mean ± standard deviation (SD).

RESULTS

Antioxidative Properties of PBP in Comparison to PJ. The antioxidative properties of PBP were determined and compared to those of PJ (*13*, *14*). The concentration of total polyphenols in PBP was 8-fold higher than that found in PJ (**Figure 1A**). The free radical scavenging capacity of PBP in comparison to that of PJ was next analyzed. As a result of the higher polyphenols content in PBP versus PJ, upon the addition of a similar volume (0.2 μL/mL) of juices, the absorbance of DPPH at 517 nm (which indicates antioxidant capacity) decreased after 5 min of incubation by 86% on using PBP, in comparison to only a 36% reduction obtained by PJ (**Figure 1B**). We next compared free radical scavenging capacities of PBP and PJ on a similar polyphenolic concentration (10 μmol/L) basis. Whereas PJ reduced the absorbance at 517 nm by 60%, PBP reduced it by as much as 77% (**Figure 1C**), demonstrating that the polyphenols present in PBP are more potent in their ability to scavenge free radicals than those present in PJ.

Effect of PBP Administration to E° Mice on Their Atherosclerotic Lesion Size. Consumption of PBP (17 or 51.5 μg of gallic acid equiv/kg/day) by the E° mice for 3 months

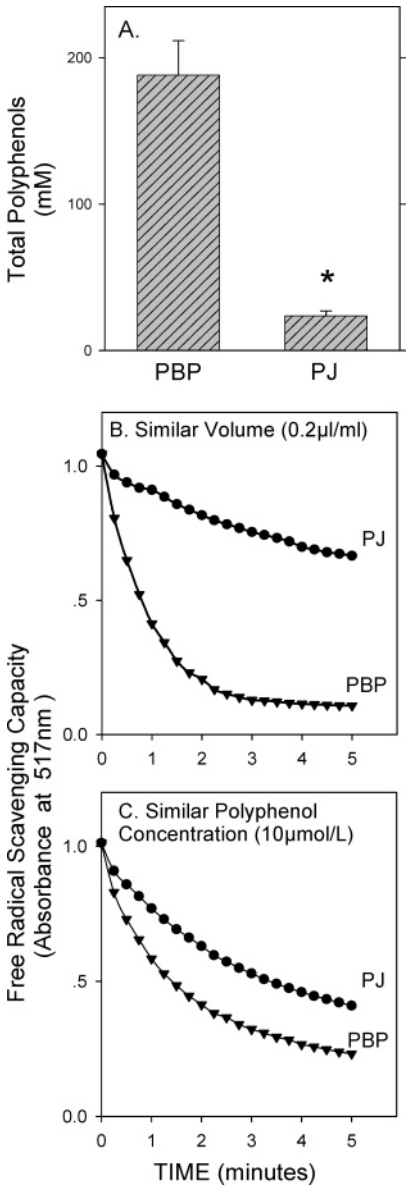

Figure 1. Antioxidative properties of PBP in comparison to PJ: (**A**) total content of polyphenols in PBP and in PJ; (**B**, **C**) free radical scavenging capacity of PBP and PJ determined by the DPPH assay as described under Materials and Methods [(**B**) similar volumes (0.2 μL/mL) of PBP and PJ were used; (**C**) similar polyphenolic concentrations (10 μmol/L) of PBP and PJ were used]. Results are expressed as mean ± SD of three different experiments. *, $p < 0.01$ versus PBP.

resulted in significant reductions in their aortic arch atherosclerotic lesion size by 38 and 57%, respectively, in comparison to lesion size in the placebo group (**Figure 2A**). Photomicrographs of typical atherosclerotic lesions in E° mice that consumed water (placebo) or PBP are shown in **Figure 2B**−**D**. The lesions from the placebo mice were larger and more developed with more lipid-laden macrophage foam cells in comparison to PBP-treated mice (**Figure 2B** vs **Figure 2C**,**D**).

Effect of PBP on Macrophage Oxidative Status: Ex Vivo and In Vitro Studies. The effect of PBP consumption by E° mice on oxidative status parameters of their MPM was studied. Consumption of PBP (17 or 51.5 μg of gallic acid equiv/kg/day) for 3 months significantly decreased the total macrophage peroxide levels by 8 and 27%, respectively (**Figure 3A**) and the cellular lipid peroxide content by up to 42% (**Figure 3B**). Reduced glutathione (GSH) is a major cellular antioxidant, and

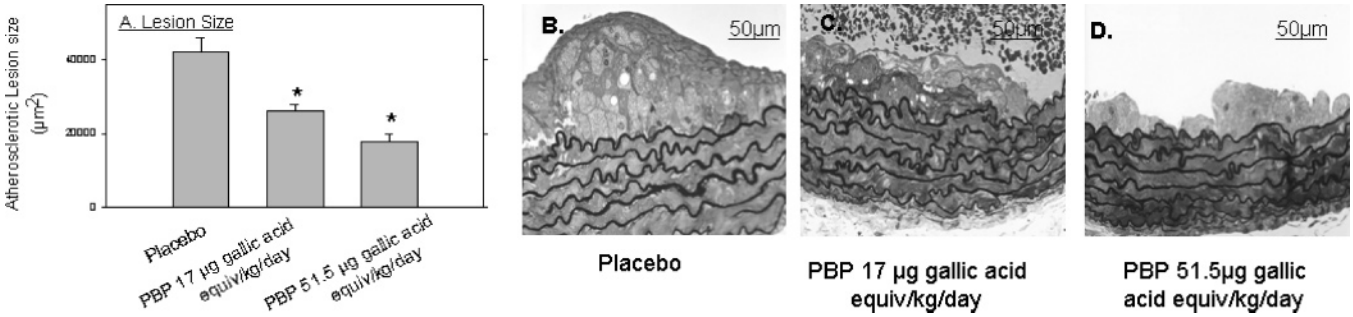

Figure 2. Effect of PBP consumption by E° mice on the size of their atherosclerotic lesions and on foam cell morphology. E° mice consumed PBP (17 or 51.5 μg of gallic acid equiv/kg/day) for 3 months. Control mice received only water (placebo). (**A**) Atherosclerotic lesion size. Results are expressed as mean ± SD of 10 mice in each group. *, $p < 0.01$ versus placebo. (**B**–**D**) Photomicrographs of typical foam cells: (**B**) placebo group; (**C**) E° mice that consumed PBP, 17 μg of gallic acid equiv/kg/day; (**D**) E° mice that consumed PBP, 51.5 μg of gallic acid equiv/kg/day.

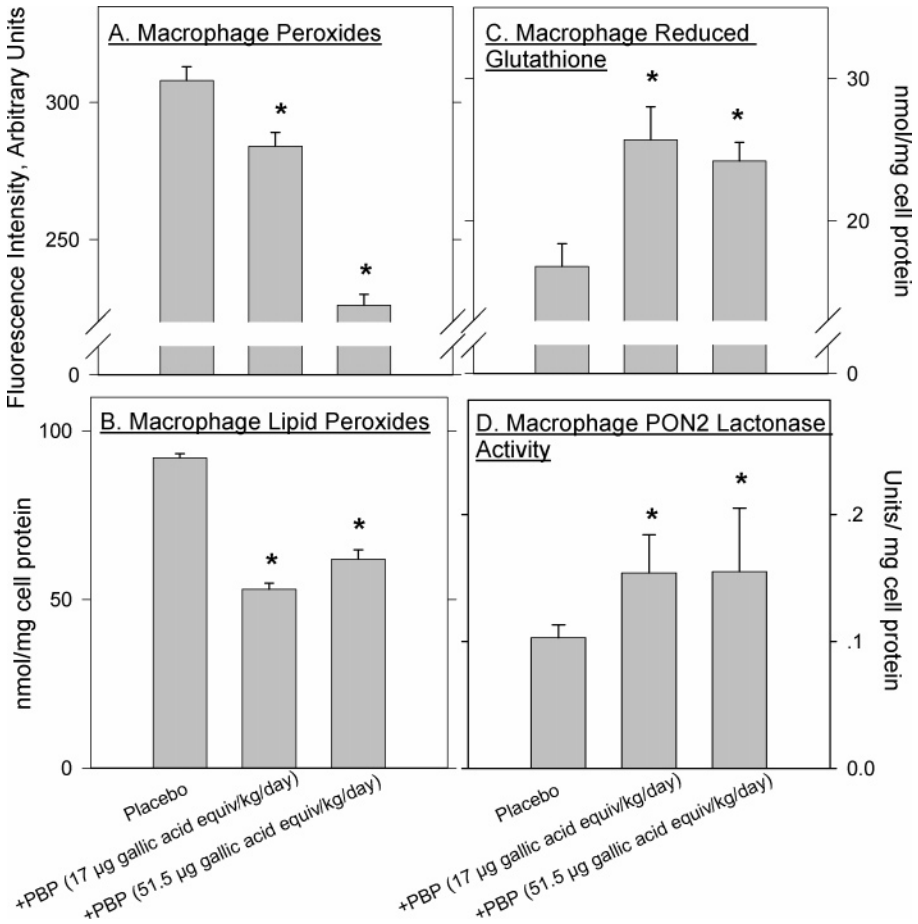

Figure 3. Effect of PBP consumption by E° mice on their MPM oxidative status. E° mice consumed PBP (17 or 51.5 μg of gallic acid equiv/kg/day) for 3 months. The control mice received only water (placebo). (**A**) Total cellular peroxide levels were determined by the DCFH-DA assay; (**B**) cells (2×10^6) were extracted with hexane/2-propanol (3:2, v/v), and lipid peroxide content was determined in the dried hexane phase; (**C**) cellular GSH content in MPM was measured by the DTNB recycling assay; (**D**) for MPM PON2 lactonase activity measurement, MPM (2×10^6) were incubated with 1 mmol/L dihydrocoumarin in Tris buffer, and the hydrolysis rate was determined after 10 min of incubation at 25 °C. Results are expressed as mean ± SD of three different determinations ($n = 3$). *, $p < 0.01$ versus placebo.

under oxidative stress, as present in E° MPM, its levels were shown to be depleted (*31*, *32*). Significant increments in cellular GSH levels by up to 53% were noted upon consumption of PBP, in comparison to the GSH levels in macrophages from the placebo-treated mice (**Figure 3C**). Paraoxonase 2 (PON2), which is a cellular esterase/lactonase expressed in macrophages, acts also as a cellular antioxidant and was shown to reduce cellular oxidative stress (*28*, *33*). PBP consumption (17 or 51.5 μg of gallic acid equiv/kg/day) by the E° mice significantly increased macrophage PON2 lactonase activity by 50% (**Figure 3D**). These results demonstrate that PBP consumption by E° mice resulted in a significant reduction in MPM oxidative status.

Similar results were noted in vitro upon incubation of cultured macrophages (J774A.1) with PBP for 18 h at 37 °C. Upon cell incubation with PBP (10 or 50 μmol/L of total polyphenols), basal levels of total peroxides in the cells were significantly reduced by 56 and by 76%, respectively (**Figure 4A**). We next examined the effect of PBP on the susceptibility of the cells to oxidation induced by ferric ascorbate (Fe-Asc) or by the free radical generator AAPH. Preincubation of J774A.1 macrophages

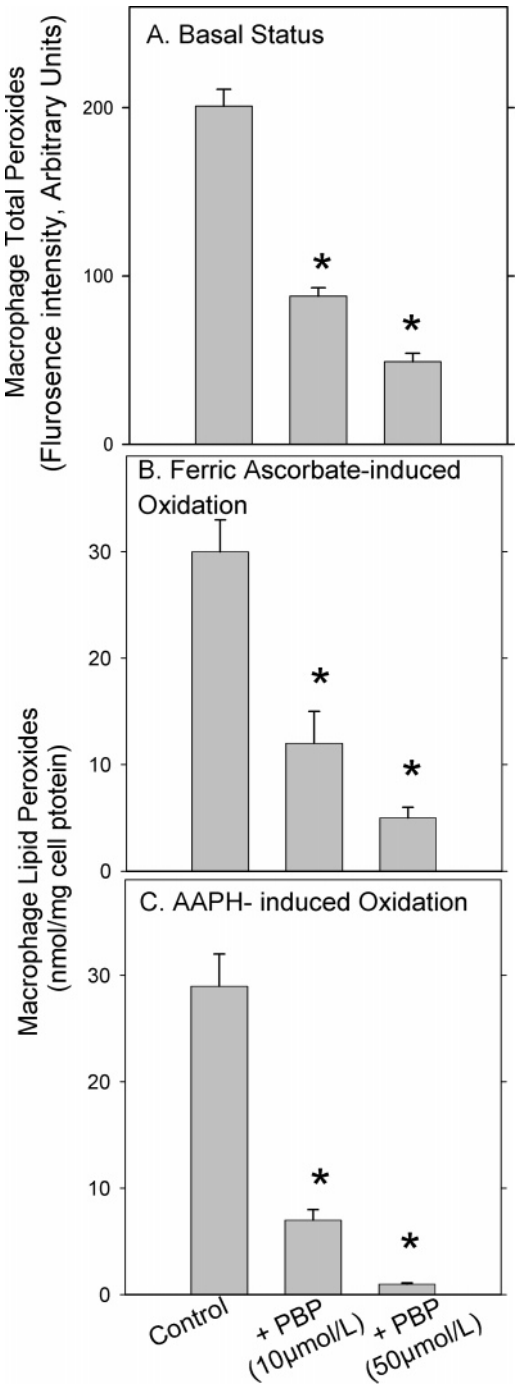

Figure 4. Effect of PBP on J774A.1 macrophage oxidative stress: in vitro study. J774A.1 macrophages were incubated for 18 h at 37 °C with no addition (control) or with PBP (10 or 50 μmol/L of total polyphenols). (**A**) Cells were washed and total cellular peroxide content was determined by the DCFH-DA assay. Cells were washed and further incubated with (**B**) 500 μmol/L of ferric ascorbate or (**C**) 5 mmol/L of the free radical generator AAPH, for 5 h at 37 °C. Cells were washed and their lipids extracted with hexane/2-propanol (3:2, v/v). Lipid peroxide content was determined in the dried hexane phase. Results are expressed as mean ± SD of three different experiments. *, $p < 0.01$ versus control.

with PBP (10 or 50 μmol/L of total polyphenols) followed by a 5 h incubation with 500 μmol/L Fe-Asc resulted in significant reductions, by 60 and 83%, respectively, in the cellular lipid peroxide content, in comparison to the lipid peroxide levels observed in control cells that were pretreated without PBP (**Figure 4B**). Similar results were noted after oxidation of the

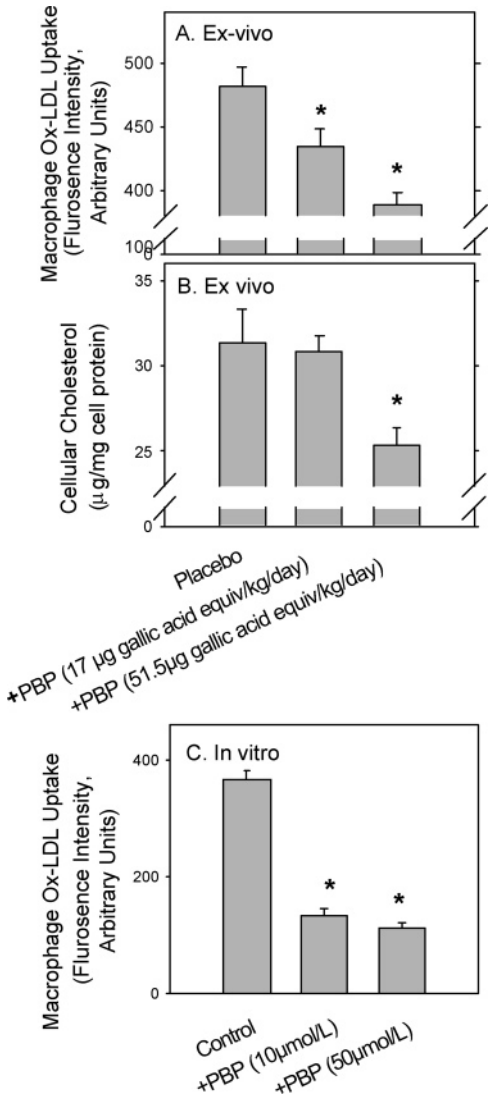

Figure 5. Effect of PBP consumption by E° mice on Ox-LDL uptake by the MPM. E° mice consumed PBP (17 or 51.5 μg of gallic acid equiv/kg/day) for 3 months. Control mice received only water (placebo). (**A**) Extent of Ox-LDL (25 μg of protein/mL, labeled with FITC) cellular uptake (by 1×10^6 cells) was determined by flow cytometry. (**B**) MPM were washed and lipid extracted with hexane/2-propanol (3:2, v/v). Cellular cholesterol content was determined in the dried hexane phase. (**C**) In vitro study. J774A.1 macrophages were incubated for 18 h at 37 °C with no addition (control) or with PBP (10 or 50 μmol/L of total polyphenols). The cells were then washed, and the extent of Ox-LDL (25 μg of protein/mL labeled with FITC) cellular uptake was determined by flow cytometry. Results are expressed as mean ± SD of three different determinations ($n = 3$).

cells with 5 mmol/L of AAPH, with 76 and 97% reductions, respectively, in macrophage lipid peroxide content (**Figure 4C**). These results demonstrate that PBP not only in vivo, but also directly in vitro, substantially attenuated macrophage oxidative stress in a dose-dependent manner.

Effect of PBP on Ox-LDL Uptake by Macrophages: Ex Vivo and In Vitro Studies. Ox-LDL is taken up by macrophages via their scavenger receptors, leading to cellular cholesterol accumulation and foam cell formation (*1*−*7*). We have previously shown an enhanced rate of Ox-LDL uptake by "oxidized macrophages" obtained from E° mice (*9*). As we demonstrated that PBP reduces the macrophage oxidative state, we next analyzed the effects of PBP consumption by atherosclerotic E°

Table 2. Summary of Studies in Mice and Humans Demonstrating That Pomegranate Juice and Byproduct Consumption Attenuated Atherosclerosis Development

researcher	Aviram et al.	Kaplan et al.	Aviram et al.	Nigris et al.		Rosenblat et al.	
study year	2000	2001	2004	2005		2005	
journal published	*Am. J. Clin. Nutr.*	*J. Nutr.*	*Clin. Nutr.*	*Proc. Natl. Acad. Sci. U.S.A.*		*J. Agric. Food Chem.*	
product	juice	juice	juice	juice		byproduct	
polyphenol concn (μg of gallic acid equiv/g/day)	11.9	29.7	11.9	29.7		17.0	51.5
type of animal model[a]	E° mice	E° mice	CAS patients	LDLR−/−mice		E° mice	
no. of animals	10	10	9	12	12	10	
animal age	6 weeks	4 months	50–60 years	3 months	6 months	6 weeks	
duration of study	3 months	2 months	1 year	6 months		2 months	
diet	chow	chow	Mediterenian	high cholesterol		chow	
% reduction in lesion size	44	17	30	21	19	38	57

[a] E° mice, apolipoprotein E-deficient mice; CAS patients, patients with carotid artery stenosis.

mice on the extent of Ox-LDL uptake by their MPM and on macrophage cholesterol content. PBP (17 or 51.5 μg of gallic acid equiv/kg/day) consumption by the E° mice significantly decreased the extent of Ox-LDL (25 μg of protein/mL) uptake by their peritoneal macrophages by 10 and 19%, respectively (**Figure 5A**) as compared to the extent of Ox-LDL uptake by MPM derived from the placebo mice. Furthermore, PBP (51.5 μg of gallic acid equiv/kg/day) consumption by the mice resulted in a significant 19% decrement in their MPM cholesterol content (**Figure 5B**).

Similar results were noted when J774A.1 cultured macrophages were preincubated with PBP in vitro. The uptake of Ox-LDL (25 μg of protein/mL) by cells treated with PBP (10 or 50 μmol/L of total polyphenols) was significantly reduced by 63 and 69%, respectively, as compared to the extent of Ox-LDL uptake by cells that were pretreated without PBP (**Figure 5C**).

DISCUSSION

In the present study we have demonstrated, for the first time, the antiatherogenic properties of a PBP prepared from the pomegranate husks after the extraction of the juice. PBP consumption by the atherosclerotic E° mice resulted in a significant reduction in the mouse macrophage oxidative stress and in the extent of Ox-LDL uptake by the cells, and these effects were associated with a significant attenuation of atherosclerotic lesion development. E° mice are widely used as a model to study atherogenesis because they develop severe hypercholesterolemia, exhibit high oxidative stress in their serum and macrophages, and develop significant atherosclerosis at the age of 3 months (*34*−*36*). The E° mice used in our previous studies and in the current study are all on the background of C57CL/6J × C57BLBBAJ, and they develop significant atherosclerosis even under a chow diet.

The dosages of PBP given to the E° mice, or those used in the in vitro studies, were chosen according to our previous studies performed with PJ (*15*, *16*). PBP consumption by E° mice resulted in a significant dose-dependent decrement in their macrophage oxidative stress, as measured by total cellular peroxide and lipid peroxide levels, cellular content of GSH, and macrophage PON2 activity. However, in this case, the lower PBP dose (17 μg of gallic acid equiv/kg/day) had a stronger protective effect than the higher PBP dose on macrophage lipid peroxides and on GSH levels. PBP has a direct effect on macrophage oxidative state. PBP incubation with J774A.1 macrophages (in vitro studies) reduced cellular levels of basal lipid peroxides and the susceptibility of the cells to oxidation induced by either Fe-Asc or AAPH, a phenomenon that was PBP polyphenols dose-dependent. These effects could be attributed to the free radical scavenging capacity of PBP most potent polyphenols (**Figure 1**).

GSH is a major cellular antioxidant (*31*), and in macrophages from E° mice, its content was found to be decreased (*32*). PBP consumption by the mice significantly increased macrophage GSH content, an increment that was previously shown to be associated with reduced capability of the cells to oxidize LDL (*32*, *37*).

In addition to cellular glutathione, it has been suggested that PON2, which is present in cells including macrophages, but, unlike PON1, not in the circulation, may play a role in protecting cells against oxidative stress (*28*, *33*). PBP consumption by E° mice significantly increased MPM PON2 lactonase activity. This effect could be related to the PBP polyphenols, which bind to the cell's surface and activate plasma membrane PON2. Such a PBP-induced increment in macrophage PON2 activity can be an additional mechanism contributing to the reduction observed in cellular oxidative state. The decrease in macrophage oxidative stress thus may involve a direct effect of PBP on the cells (as shown in the in vitro studies), as well as the result of PBP-induced increment in macrophage GSH levels and/or PON2 activity.

Ox-LDL uptake by macrophages via the scavenger receptors contributes to cellular accumulation of oxidized lipids and cholesterol and to foam cell formation (*1*−*7*). PBP consumption by the E° mice significantly decreased the extent of Ox-LDL uptake by their macrophages. A similar effect was also noted in vitro upon J774A.1 macrophage incubation with PBP. These effects could be secondary to the PBP-induced reduction in macrophage oxidative stress. Indeed, we have previously demonstrated that "oxidized macrophages" take up Ox-LDL at an enhanced rate (*8*, *9*). PBP polyphenols not only reduce oxidative status in cells but may also bind to the macrophage surface and, hence, interfere with Ox-LDL binding to the macrophage scavenger receptors (*38*). Similar PJ effects on Ox-LDL uptake by macrophages were previously shown in the atherosclerotic E° mice that consumed PJ and in cultured J774A.1 macrophages (*15*, *16*, *39*).

The cholesterol content of macrophages from E° mice was significantly reduced after PBP consumption, in correlation with the observed decrement in the extent of Ox-LDL uptake by these cells. This could have also partially resulted from the inhibition in macrophage cholesterol biosynthesis by PJ (*39*).

All of the above antiatherogenic effects of PBP (shown in both the in vitro and in vivo studies) could have contributed to the significant reduction observed in the atherosclerotic lesion

size and in the number of lesion foam cells observed in E° mice that consumed PBP, in comparison to the placebo group.

Table 2 summarizes the studies performed in humans and mice that demonstrate attenuation of atherosclerosis lesion development, by PJ or by PBP (current study) consumption.

We conclude that the PBP, which was prepared from the pomegranate husks after the extraction of the juice, possesses greater antiatherosclerotic characteristics compared to PJ, which could be related to its antioxidative properties and its impressive ability to inhibit macrophage uptake of atherogenic oxidized LDL.

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