Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages
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Abstract
Macrophage cholesterol accumulation and foam cell formation are the hallmarks of early atherogenesis. Pomegranate juice (PJ) was shown to inhibit macrophage foam cell formation and development of atherosclerotic lesions. The aim of this study was to elucidate possible mechanisms by which PJ reduces cholesterol accumulation in macrophages. J774.A1 macrophages were preincubated with PJ followed by analysis of cholesterol influx [evaluated as LDL or as oxidized LDL (Ox-LDL) cellular degradation], cholesterol efflux and cholesterol biosynthesis. Preincubation of macrophages with PJ resulted in a significant reduction (P<.01) in Ox-LDL degradation by 40%. On the contrary, PJ had no effect on macrophage degradation of native LDL or on macrophage cholesterol efflux. Macrophage cholesterol biosynthesis was inhibited by 50% (P<.01) after cell incubation with PJ. This inhibition, however, was not mediated at the 3-hydroxy-3 methylglutaryl coenzyme A reductase level along the biosynthetic pathway. We conclude that PJ-mediated suppression of Ox-LDL degradation and of cholesterol biosynthesis in macrophages can lead to reduced cellular cholesterol accumulation and foam cell formation. © 2005 Elsevier Inc. All rights reserved.

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1. Introduction
Macrophage cholesterol accumulation and foam cell formation are the hallmarks of early atherogenesis [1]. Cholesterol accumulation in macrophages can result from an impaired balance between external and internal cholesterol sources. LDL, which undergoes oxidative modification, is the main external source for the macrophage-accumulated cholesterol. Oxidized LDL (Ox-LDL) is taken up by macrophages at an enhanced rate via scavenger receptors [2,3], which, unlike LDL receptors, are not down-regulated by intracellular cholesterol content [4]. The macrophage scavenger receptors include the type A scavenger receptor (SR-A) [5,6] and the CD-36, which was defined as a type B scavenger receptor [7]. The macrophage CD36 receptor was shown to be involved in the formation of foam cells and is highly expressed in atherosclerotic lesions [8]. The interaction of Ox-LDL with the CD36 macrophage scavenger receptor plays an important role in cellular cholesterol accumulation as a 50% decrement in Ox-LDL binding to human macrophages was shown in CD36-deficient macrophages [9]. Macrophage cholesterol from internal sources originates from the biosynthesis of cholesterol. The enzyme 3-hydroxy-3 methylglutaryl coenzyme A reductase (HMGCoA) reductase catalyzes the rate-limiting step in the mevalonate biosynthetic pathway [10] and is subjected to a negative feedback regulation by the cellular cholesterol level. In addition to cellular uptake of lipoproteins and to cholesterol biosynthesis, macrophage cholesterol accumulation can also result from a decreased cholesterol efflux from the cells [11].

Oxidative stress has been implicated in the pathogenesis of atherosclerosis [12,13] as it has been shown to considerably attack lipids in LDL as well as in arterial macrophages [14,15]. We have previously shown that “lipid-peroxidized macrophages” exhibit an increased ability to oxidize LDL and take up Ox-LDL [16], thus leading to cellular accumulation of cholesterol and oxidized lipids, which characterize early atherogenesis. Inhibition of oxidative stress in LDL and in arterial cells by some nutritional antioxidants feeding in animal models attenuated the
progression of atherosclerosis [17–19]. Pomegranate juice (PJ), which is rich in some specific flavonoids (unique tannins such as punicalagin and anthocyanins), was recently shown to possess antiatherogenic properties secondary to its very potent antioxidative characteristics [20–22]. Recently, we demonstrated that supplementation of PJ to atherosclerotic apolipoprotein E-deficient (E0) mice, which already exhibited advanced atherosclerotic lesions, reduced macrophage lipid peroxides along with a reduction in macrophage cholesterol accumulation and foam cell formation [23]. This effect resulted from reduced cholesterol influx and reduced cholesterol esterification rates as well as from enhanced macrophage cholesterol efflux. However, these results in the mice study do not indicate whether PJ exhibits a direct effect on the macrophages or whether the effects shown in mice were secondary to PJ effects on other tissues and on the circulation constituents, which can possibly only then affect macrophage atherogenicity, leading to foam cell formation and atherosclerosis development. Therefore, the aim of the present study was to determine the direct cellular effect of PJ on atherogenic processes that lead to cholesterol accumulation in macrophages, including cellular oxidative stress, as well as macrophage cholesterol influx, cholesterol efflux and cholesterol biosynthesis.

2. Methods

2.1. Cells

A J774.A1 macrophage-like cell line was obtained from the American Tissue Culture Collection (Rockville, MD, USA) and maintained in DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated (56°C for 30 min) FCS.

2.2. Pomegranate processing

Pomegranates were washed, chilled at 32°C and stored in tanks. Then, the fruits were crushed and squeezed to yield PJ. The juice was filtered, concentrated and stored at −18°C.

2.3. Polyphenol determination

Total polyphenols were determined spectrophotometrically by the method of Singleton and Rossi [24] modified for small volumes [25]. Quercetin stock solution was prepared in ethanol at a concentration of 1 mmol/L and served as a standard. Volumes of 10, 20, 40 and 60 μL diluted in 1 ml solution (equivalent to 3, 6, 13 and 19 μg, respectively) were used for the standard curve.

2.4. LDL preparation

LDL was separated from the plasma of normal healthy volunteers by discontinuous density-gradient ultracentrifugation [26] and dialyzed against saline with EDTA (1 mmol/L). Before the oxidation study, LDL was diluted in phosphate-buffered saline (PBS) to 1 g protein/L and dialyzed overnight against PBS at 4°C to remove the EDTA. LDL protein concentration was determined with the Folin phenol reagent [27]. LDL was radioiodinated by the iodine monochloride method as modified for lipoproteins [28].

2.5. Ox-LDL preparation

Oxidation of LDL was carried out at 37°C under air in a shaking water bath. LDL (1 g protein/L) was incubated for 18 h at 37°C with freshly prepared CuSO4 (5 μmol/L; Sigma, St. Louis, MO, USA). Oxidation was terminated by refrigeration at 4°C. The extent of Ox-LDL oxidation was determined by the TBARS assay [29,30]. Radioiodinated Ox-LDL (125I-Ox-LDL) was prepared by oxidation of 125I-LDL.

2.6. Macrophage uptake of lipoproteins by macrophages

125I-Ox-LDL or native LDL (125I-LDL) at a concentration of 10 mg protein/L was incubated with the cells at 37°C for 5 h. The uptake of the lipoproteins was determined as lipoprotein degradation and as lipoprotein cell association. Lipoprotein cellular degradation was measured in the collected medium as the trichloroacetic acid-soluble, non-lipid radioactivity, which was not due to free iodide [31]. Lipoprotein degradation in a cell-free system that was measured under identical conditions was minimal (<10%) and was subtracted from the total degradation. The remaining cells were washed three times with cold PBS dissolved in 0.1 mol/L NaOH and used both for protein and for cell-associated lipoprotein determination.

2.7. Cellular cholesterol efflux

J774.A1 macrophages were incubated with [3H]labeled cholesterol for 18 h at 37°C followed by cell wash in ice-cold PBS (3×) and a further incubation in the presence of 100 mg HDL protein/L for 3 h at 37°C. Cellular and medium [3H] labels were quantitated and HDL-mediated cholesterol efflux was calculated as the ratio of [3H] label in the medium to [3H] label in the medium plus [3H] label in cells [32].

2.8. Cellular cholesterol biosynthesis

Cellular cholesterol biosynthesis was assayed by incubation of the cells with [3H]acetate (1 mCi/L) or with [14C] (1 mCi/L) for 18 h, after which cellular lipids were extracted in hexane/isopropanol (3:2 vol/vol), separated by thin-layer chromatography on silica gel plates and developed in hexane/ether/acetic acid (80:20:1.5 vol/vol). Unesterified cholesterol spots were visualized by iodine vapor (using appropriate standard) scraped into scintillation vials and counted in a β-counter.

2.9. mRNA expression of CD36, LDL receptor and HMGCoA reductase

mRNA expression of CD36, LDL receptor and HMGCoA reductase was analyzed by reverse transcriptase (RT) PCR. Total RNA was extracted from cells with Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH,
USA). cDNAs were generated from 1 μg of total RNA using RT (Boehringer-Mannheim, Mannheim, Germany). Products of the RT reaction were diluted 1:10 or 1:2.5 and subjected to PCR amplifications using specific primers as listed below:

CD36: sense, 5'-TGC GAA CTG TGG GCT CAT TG-3'; antisense, 5'-CCT CGG GTT CCT GAG TTA TAT TTT C-3' (58°C, 32 cycles, 314 bp);

LDL receptor: sense, 5'-GAC TGC AAG GAC ATG AGC GA-3'; antisense, 5'-CGG TTT GGT GGT TCA GTT AG-3' (58°C, 32 cycles, 414 bp);

HMGCoA reductase: sense, 5'-GGG ACG GTG ACA CTT ACG ATC TGT ATG ATG-3'; antisense, 5'-ATC ATC TTT GAG AGA TAA AAC TGC CA-3' (58°C, 35 cycles, 882 bp);

GAPDH: sense, 5'-CTG CCA TTT GCA GTG GCA AAG TGG-3'; antisense, 5'-TTG TCA TGG ATG ACC TTG GCC AGG-3' (58°C, 30 cycles, 439 bp).

2.10. CD36 protein expression

Protein expression of CD36 was quantified by FACS (Becton-Dickinson, Ontario, Canada). Briefly, 2 × 10⁶ cells were scraped into 100 μl PBS and incubated for 30 min at room temperature with 10 μl of FITC-conjugated anti-CD36 antibodies or the corresponding FITC-conjugated isotype-matched control (Serotec IQ Products, Zerinepark, The Netherlands). Fluorescence was determined by FACS using a 488-nm argon laser and data relating to the gated cell population were collected. Measurement of mean fluorescence intensity (MFI) and analysis of data were done using Cell Quest Software.

2.11. Macrophage oxidative stress

Intracellular oxidative stress was assayed through the oxidation of 2',7'-dichlorofluorescin diacetate (DCFH-DA) [33] and monitored by flow cytometry [34,35]. The cells were washed (once) with PBS and incubated with 10 μmol/L DCFH-DA, respectively, in medium for 30 min at 37°C. Reaction was stopped by rinsing adherent cells with PBS. Adherent cells were detached by gentle scraping, and all cells were washed (2 ×) with PBS. Measurements of cellular fluorescence determined by FACS were done at 510 to 540 after excitation of the cells at 488 nm with an argon ion laser. Ten thousand events were registered for each experiment. Cellular fluorescence was quantitated by MFI.

2.12. Statistical analysis

Student’s t test was performed for all statistical analyses.

3. Results

We questioned whether cell incubation with PJ reduces oxidative stress in macrophages. Incubation of J774.A1 macrophages with increasing concentrations of PJ for 90 min gradually reduced, in a concentration-dependent manner, cellular oxidative stress by up to 40% using 75 mmol total polyphenols/L (Fig. 1). Since 75 mmol total polyphenols/L of PJ induced a maximal effect, we used this concentration in the succeeding experiments.

As cellular oxidative stress influences both scavenger receptor-mediated uptake of Ox-LDL and cellular cholesterol biosynthesis, we tested the direct effect of PJ on cellular cholesterol influx, determined as macrophtage uptake of ¹²⁵I-Ox-LDL and of ¹²⁵I-LDL. J774.A1 macrophages were incubated without (control) or with PJ (75 mmol polyphenols/L) for 90 min at 37°C followed by further incubation with either ¹²⁵I-Ox-LDL or ¹²⁵I-LDL (10 mg lipoprotein protein/L) for 5 h at 37°C. Degradation (Fig. 2A) and cell association (Fig. 2B) of Ox-LDL by macrophages that were pretreated with PJ were both reduced by 40% and 48%, respectively, as compared with control macrophages that were incubated without PJ (Fig. 2A and B). Next, we analyzed by RT-PCR the mRNA expression for the scavenger receptor CD36, which mediates macrophage uptake of Ox-LDL. No significant change was found in CD36 mRNA expression after treatment of the cells with PJ (Fig. 2C), suggesting that the reduction in Ox-LDL uptake induced by PJ was not mediated via down-regulation of CD36 at the gene transcription level. Furthermore, we could not find any changes in CD36 protein expression on the cell surface (87±3 and 82±5 MFI in control cells and in cells preincubated with PJ, respectively).

The cellular uptake of ¹²⁵I-LDL by macrophages was not significantly affected by PJ supplementation (Fig. 2D and E). In accordance to these results, the LDL receptor mRNA expression was also not significantly affected following macrophage incubation with PJ (Fig. 2F).

![Graph showing cellular oxidative stress as a function of Pomegranate Juice Concentration](image-url)

Fig. 1. Effect of PJ on macrophage oxidative status. J774.A1 macrophages were incubated without (control) or with increasing concentrations of PJ (PJ) for 90 min at 37°C. Then, cellular oxidative stress was measured following cell incubation with 10 μmol/L of DCFH-DA for 30 min at 37°C. Cellular fluorescence was determined by a flow cytometry analysis. Results are given as mean±S.D.; n=3; *P<.01 vs. control.
Next, we determined the effect of PJ on macrophage cholesterol efflux. For this purpose, J774.A1 macrophages were first radiolabeled with \[^{3}H\]-cholesterol followed by cell incubation in the presence of HDL and, finally, determination of the amount of labeled cholesterol released from the cells to the medium. No significant change was observed in HDL-mediated cholesterol efflux from macrophages that were preincubated with PJ (2.5% (n=3) cholesterol efflux observed in macrophages that were incubated without PJ vs. 4.4% (n=3) cholesterol efflux observed in PJ-treated macrophages).

Finally, the level of cholesterol in macrophages was also determined by the contribution of cellular cholesterol biosynthesis. Fig. 3A demonstrates a significant decrease \((P<.01)\) by 50% in the rate of macrophage cholesterol synthesis from \[^{3}H\]-acetate in cells that were preincubated with PJ, in comparison with control cells incubated without PJ. In order to examine whether PJ affects the synthesis of cholesterol in macrophages along the pathway from acetyl CoA to mevalonate due to a regulatory effect on the rate-limiting enzyme in cholesterol biosynthesis, HMGCoA reductase, we analyzed HMGCoA reductase mRNA expression in macrophages incubated without PJ (control) and in cells that were treated with PJ. As shown in Fig. 3B, HMGCoA reductase mRNA expression was not significantly affected in cells that were preincubated with PJ in comparison with control cells. Furthermore, we tested the ability of PJ to inhibit cholesterol synthesis from \[^{14}C\] mevalonate. Incubation of J774.A1 macrophages with PJ inhibited cholesterol synthesis from mevalonate by 63% (Fig. 3C), thus further suggesting that PJ regulates macrophage cholesterol synthesis at other points along the biosynthetic pathway, below the mevalonic acid.

4. Discussion

The present study demonstrates that upon in vitro incubation of macrophages with PJ, cellular uptake of Ox-LDL and cellular cholesterol biosynthesis were significantly reduced parallel to a significant reduction in cellular oxidative stress. In a previous study [23] we demonstrated that consumption of PJ by atherosclerotic \(E^0\) mice reduced
the uptake of Ox-LDL by their macrophages and increased cellular cholesterol efflux. These effects were possibly responsible for the observed reduction in foam cell formation and atherosclerotic lesion attenuation by PJ consumption of these mice. In the present study, we extended previous in vivo findings by employing an in vitro system in order to examine whether PJ has direct effects on macrophage oxidative stress and cellular cholesterol metabolism.

Cell culture imposes a state of oxidative stress on cells [36], and PJ reduced cellular oxidative stress under these conditions, similar to the effect of PJ previously shown in vivo in mice. However, it is not known whether the same compounds that affect the cellular system are those that have an effect in the animal model.

PJ-mediated reduction in cellular uptake of Ox-LDL could not be related to a down-regulation in the expression of the scavenger receptor CD36 mRNA either at the transcription level or at the protein level on cell surface. However, CD36 is not the only surface binding site that mediates the uptake of Ox-LDL by macrophages. Specifically, PJ could interact with other scavenger receptors such as SR-A (SR-AI, SR-AII) or with proteoglycans [37], which were also shown to mediate uptake of Ox-LDL by macrophages [38,39], resulting in interference of the cellular uptake of the lipoprotein. PJ polyphenols could also interfere with the uptake of Ox-LDL by interaction with macrophage surface phospholipids and/or kinases [40,41].

The cellular uptake of 125I-LDL, which occurs via the LDL receptor, was not affected by PJ. This may be related to the inability of 125I-LDL to cause macrophage cholesterol accumulation as the LDL receptor is tightly regulated by cellular cholesterol content.

Macrophage cholesterol efflux was not significantly changed after cell incubation with PJ, in contrast to the in vivo findings observed in E0 mice, where PJ was shown to stimulate macrophage cholesterol efflux [23]. This could be because exposure of mice to specific compounds in the juice is known to be modified during absorption and metabolism of these parent compounds in the animal system and these modified compounds may exert an effect in vivo, which may not be reproduced in vitro by the whole juice.

This discrepancy implies that PJ does not directly stimulate macrophage efflux of cholesterol. Thus, the stimulation of cholesterol efflux from macrophages observed in the mice study is probably secondary to pleiotropic effects exerted by PJ on other cells including arterial and blood cells as well as on body fluids, which in turn affect macrophages.

Finally, the PJ-mediated inhibition of macrophage cholesterol biosynthesis was shown in the present study for the first time and was found to be independent of the HMGCoA reductase regulation. It is thus possible that PJ interferes with the macrophage cholesterol synthesis at other metabolic points along the synthetic pathway, beyond the HMGCoA reductase step and beyond the mevalonic acid-processing step. Furthermore, it is possible that PJ impairs the specific enzyme-substrate reactions, thus leading to a reduction in the rate of the cholesterol biosynthesis.

The PJ-mediated reduction of cellular uptake of Ox-LDL and cellular cholesterol biosynthesis were associated with a significant reduction in cellular oxidative stress. PJ contains a significant amount of polyphenols, which are known for their antioxidant capacity [19]. However, we demonstrated that despite an identical content of poly-
phenols, PJ exhibited more efficient antioxidant activity against LDL oxidation in comparison with other polyphenol-rich juices and beverages including red wine, cranberry juice and blueberry juice [22]. This effect can be related to specific polyphenols in PJ such as punicalagin, which was shown to remarkably reduce the development of atherosclerotic lesions in E0 mice by 54% [23]. In this study, we incubated the cells with 75 μmol/L of PJ polyphenols, which is in the range of physiological concentration in human plasma [42]. The total intake of dietary polyphenols is ~1 g/day, and polyphenol concentrations in plasma may range between 10 and 100 μmol/L after ingestion of a single phenolic compound. However, there is a wide variability in the bioavailability of different polyphenols [43]. Incubation of macrophages with PJ was performed for 90 min only, thus assuring the stability of the juice under cell culture conditions.

In conclusion, PJ exerts a direct effect on macrophage cholesterol metabolism by reducing cellular uptake of Ox-LDL and inhibiting cellular cholesterol biosynthesis. Both of these processes eventually lead to a reduction in macrophage cholesterol accumulation and foam cell formation and attenuation of atherosclerosis development, as indeed shown in atherosclerotic mice after PJ consumption [20,23].

References


