

Brief communication

Pomegranate juice reduces oxidized low-density lipoprotein downregulation of endothelial nitric oxide synthase in human coronary endothelial cells

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Abstract

We examined the hypothesis that pomegranate juice (PJ) can revert the potent downregulation of the expression of endothelial nitric-oxide synthase (NOSIII) induced by oxidized low-density lipoprotein (oxLDL) in human coronary endothelial cells. Western blot and Northern blot analyses showed a significant decrease of NOSIII expression after a 24-h treatment with oxLDL. Accordingly, we observed a significant dose-dependent reduction in nitric oxide bioactivity represented by both basal and bradykinin-stimulated cellular cGMP accumulation. These phenomena were corrected significantly by the concomitant treatment with PJ. Our data suggest that PJ can exert beneficial effects on the evolution of clinical vascular complications, coronary heart disease, and atherogenesis in humans by enhancing the NOSIII bioactivity.

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Pomegranate juice (PJ) is rich in antioxidants of the polyphenolic flavonoid class, which includes tannins and anthocyanins. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10 and alpha-lipoic acid. The antioxidant level in PJ was found to be higher than that in other natural juices, and red wine [1].

Antioxidants in general possess numerous important biological properties including protection against cholesterol oxidation, protection against atherogenesis, anti-inflammatory effects, anti-aging effects, and protection against Alzheimer's disease and diabetes. Atherosclerosis is responsible for myocardial infarctions and ischemic strokes and is characterized by a long lag-time between onset and

clinical manifestation. Work by a number of investigators over the last 10–15 years has demonstrated that modifications of lipoproteins, resulting from oxidative stress and other processes may play an important pathogenic role in atherosclerosis [2,3]. Conditions known as “classical risk factors” such as hypertension, diabetes, and menopause all increase in frequency with advancing age and may contribute both directly and indirectly to lipoprotein modification and vascular injury [3–5]. Indeed, one important consequence of this may be enhanced susceptibility of lipoproteins to oxidation. Moreover, a plethora of additional detrimental oxidation-sensitive mechanisms can increase during hypercholesterolemia [5,6].

Biochemical modifications of low-density lipoprotein (LDL) affecting its composition can greatly enhance the injurious potential of LDL and its involvement in the pathogenesis of various disease states [2,3,5,6]. Indeed, oxidized

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LDL (oxLDL) is prevalent in early and advanced human atherosclerotic lesions [2,3,5–10] and has been implicated in the cascade of events responsible for lesion formation and plaque rupture [2,3,5–10]. Cell-culture experiments demonstrated that oxLDL altered bioactivity of endothelial nitric oxide synthase (eNOS) [2,3,11,12]. Oxidation of LDL, can induce sustained vascular dysfunction and coronary heart disease through alterations of eNOS expression in the arterial wall [2,3,11,12]. Pomegranate juice, because of its robust content of polyphenolic flavonoid antioxidants, could be expected to enhance the biological actions of eNOS in vivo. Thus, we will test whether PJ can exert beneficial effects against oxidized LDL-induced downregulation of eNOS in human coronary cells.

The objective of the proposed research is to determine whether PJ can exert beneficial effects against oxLDL-induced downregulation of eNOS in human coronary cells. There is good reason to believe that such may be the case because PJ is a rich source of potent antioxidants. That is, antioxidants are well known to enhance the biological actions of nitric oxide (NO) by virtue of their capacity to improve eNOS efficiency. In addition, antioxidants interact with and destroy reactive oxygen species and, thereby, prevent the oxidative destruction of NO.

Methods

Preparation of LDL and markers of oxidative modification

LDL was isolated from the plasma of healthy volunteers (2 mmol/l ethylenediamine-tetraacetic acid [EDTA] and 1000 UI/100 ml aprotinin) by ultracentrifugation in a KBr gradient, as previously described [12]. Proteins were assayed by the classical method proposed by Lowry et al. The native LDL, n-LDL (300 µg/ml) was incubated for 12 h at 37 °C with 1 µM copper sulfate [12]. For this in vitro study, oxLDL are far exceeding physiologically relevant ranges. The malondialdehyde concentration was measured by the thiobarbituric acid-reactive substances (TBARS) assay [12]. Relative agarose mobility and trinitrobenzenesulfonic acid (TNBSA) reactivity will serve as markers of oxidatively modified proteins [12]. Oxidized LDL was then incubated for 24 h with endothelial cells at 37 °C, under 95% air and 5% CO₂ [22].

Cell culture

Human coronary endothelial cells were cultured using standard procedures, as described [12]. The incubation medium (delipidated Dulbecco's medium essential medium) was supplemented with 10 ng/ml human epidermal growth factor, penicillin/streptomycin, amphotericin B, and glutamine [12]. Since NOSIII in human endothelial cells decreases with passage number, thus all experiments comparing the oxLDL and juice were carried out with the same passage number (2nd passage).

Western blot analysis

Whole-cell extracts were prepared by a modification of the standard procedure of Western blot analysis [12]. Western blot analysis was performed with antibodies (1:500) against NOSIII (N-20, Santa Cruz, San Diego, California), with an epitope corresponding to an amino acid sequence mapped at the aminoterminal of NOSIII of human origin. The protein content of the extract was normalized with a polyclonal antibody against gamma-tubulin protein (Sigma–Aldrich, Milan, Italy). Semiquantitative densitometry of blots were performed using a Scan LKB (Pharmacia–Sweden, Bromma, Sweden) [12].

Northern blot analysis

Total ribonucleic acid (RNA) was isolated using spin columns (RNeasy, Qiagen, Valencia, California). Agarose/formaldehyde gels were loaded with 10 µg RNA per lane, and RNA resolved by electrophoresis. The RNA was transferred to a nylon membrane (Hybond-N, Amersham, Piscataway, New Jersey) and cross-linked by ultraviolet radiation. The membrane was hybridized with radiolabeled alpha-³²P deoxycytidine triphosphate (random prime kit, Boehringer, Munich, Germany), with the eNOS fragment spanning between exon 3 and 4 (from nucleotides 1982 to 2703 of the human complementary deoxyribonucleic acid [cDNA] sequence), and glyceraldehyde-3-phosphate dehydrogenase (GeneBank, National Library of Medicine/NIH) cDNA in a hybridization solution (0.25 M Na₂PO₄, pH 7.2, 1 mM EDTA, 20% sodium dodecyl sulfate). Hybridization was performed overnight at 65 °C using 2 × 10⁶ cpm/ml for each probe.

Electrophoretic mobility shift assay (EMSA)

A double-stranded probe (–1393 to –1374) containing the putative sterol-responsive element (SRE) present in the eNOS promoter was used as a probe in EMSA analysis. Nuclear extracts (10 µg) were used for the shift assay, as described [12].

Determination of cyclic guanosine monophosphate (cGMP)

Endothelial cells (10⁶ cells) were incubated with 0.5 mmol/l isobutyl-1-methylxanthine at the time of oxLDL addition and incubated with LDL for 24 h. During the last 3 min, bradykinin (Sigma) at a final concentration of 3 nM was added, followed by extraction with trichloroacetic acid solution [12]. The content of cGMP was measured using a specific immunoreactivity kit (Amersham), according to the manufacturer's recommendations.

Pomegranate juice

Pomegranate juice concentrate (Wonderful variety, POM Wonderful, LLC, Los Angeles, CA) was used in this

study. Pomegranates 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 juice and byproducts, which included the inner and outer peels and the seeds, as described [1,13]. Pectinase hydrolyzes α -1,4-galacturonide bonds in pectin, improving extraction and filtration, and prevents formation of pectin gels. Flavonoids constitute 40% (anthocyanins, catechins, and phenols) of total polyphenols in pomegranate juice [1,13]. More complex polyphenols are also present in the juice [1,13]. The pomegranate juice was filtered, pasteurized, concentrated, and stored at -20°C until use. More details on PJ preparation should be addressed to www.pomwonderful.com.

Statistical analysis

Data are expressed as means \pm SD. The difference among groups was evaluated by a 1- or 2-factor ANOVA by two independent investigators in a blinded fashion. Statistical significance was accepted at $p < 0.05$.

Results

Effects of oxidation on LDL and PJ-induced protection

The purpose of this set of experiments is to learn whether PJ is capable of reversing the deleterious effects of oxidizing agents on LDL. Table 1 shows the effects of oxidation on LDL. Hence, MDA (an index which increases during peroxidation) and REM (an index which increases during lipoprotein oxidation) were increased and TNBS decreased (an index which decreases during oxidation) in oxLDL more than in n-LDL. PJ significantly reverted such indexes of LDL oxidation (Table 1). Our results indicate that PJ induced prevention of LDL oxidation.

cGMP activity and PJ

We evaluated the effects of oxLDL on cGMP levels, a NO-related signal transduction event. As shown in Table 2,

Table 1
Effects of oxidation on isolated LDL: protection by pomegranate juice (PJ)

	MDA (nmoles/mg of protein)	REM (cm)	TNBS % Decrease
n-LDL	2.0 \pm 0.6	1.2 \pm 0.2	—
oxLDL	28.9 \pm 4.2***	2.3 \pm 0.3***	29.6 \pm 5.6
oxLDL+ 7 μL PJ	23.8 \pm 5.5*	2.0 \pm 0.2	24.8 \pm 5.2*
oxLDL+14 μL PJ	15.6 \pm 5.9**	1.6 \pm 0.5*	18.2 \pm 4.5**

Note. n-LDL: native-LDL; oxLDL: oxidized-LDL; MDA: malondialdehyde; REM: relative electrophoresis mobility on agarose gel (cm from the baseline); TNBS: trinitrobenzenesulphonic acid. Decrease of TNBS reactivity in 18 h oxLDL vs. respective controls in the absence of oxidant; means \pm SD of four different experiments.

* $p < 0.05$ vs. ox-LDL.

** $p < 0.01$ vs. ox-LDL.

*** $p < 0.001$ vs. n-LDL.

Table 2
cGMP levels in human endothelial cells after a 24-h exposure to LDL and PJ

	cGMP (mol/ μg)	cGMP and 3 nM bradykinin (mol/ μg)
n-LDL	6.0 \pm 0.3	92 \pm 5
oxLDL (100 $\mu\text{g}/\text{ml}$)	4.5 \pm 0.2***	38 \pm 4***
oxLDL+ 7 μL PJ	4.9 \pm 0.2	43 \pm 4
oxLDL+14 μL PJ	5.2 \pm 0.3*	56 \pm 5**

Note. n-LDL: native-LDL; oxLDL: oxidized-LDL; means \pm SD of four different experiments. During the last 3 min, bradykinin at a final concentration of 3 nM was added, followed by extraction with trichloroacetic acid solution [see Methods].

* $p < 0.05$ vs. ox-LDL.

** $p < 0.01$ vs. ox-LDL.

*** $p < 0.001$ vs. n-LDL.

we detected a marked reduction of cGMP levels in both resting and bradykinin-stimulated cells after exposure to oxLDL for 24 h. Cells treated with oxLDL in the presence of 14 μL PJ had increased levels of cGMP (Table 2).

Effects of PJ on oxLDL-induced reduction of NOSIII expression

Western blot analysis performed on whole protein extracts showed that the amount of NOSIII protein significantly decreased after exposure of endothelial cells to oxLDL (Table 3 and Fig. 1). This was consistent with the downregulation of NOSIII mRNA expression observed using Northern blot (Table 3 and Fig. 1). Densitometric analysis showed a significant increase in NOSIII expression induced by concomitant treatment with PJ (Table 3). The effects of PJ on eNOS expression and activity result from PJ-induced upregulation of eNOS independently of its effect on LDL oxidation. This point was confirmed also in cells exposed to different doses of PJ (Napoli and Ignarro, personal communication).

Pyrogallol is another chemical oxidizing agent that catalyzes the production of superoxide anion from the oxygen present in the aqueous buffer solution. The addition of pyrogallol to the buffer containing the NO resulted in a rapid and extensive destruction of the NO. On the other hand, the inclusion of PJ in the solution (100 μL of a 30-fold dilution of PJ concentrate added to a reaction volume of 5 ml, just prior to addition of pyrogallol and DEA/NO), markedly protected against the pyrogallol-elicited oxidative destruction of NO (Napoli and Ignarro, personal communication).

Sterol-responsive element

To further explore whether oxLDL interfered with regulatory elements present in the NOSIII promoter, SRE was analyzed. EMSA detects specific DNA–protein complexes between nuclear extracts from cells and the probe containing the SRE sequence. Cells were incubated with oxLDL

Table 3
Effects of pomegranate juice on NOSIII expression

	NOSIII mRNA (densitometric analysis of Northern blots in human coronary endothelial cells)	NOSIII protein (densitometric analysis of Western blots in human coronary endothelial cells)
n-LDL	3.1 ± 0.3	3.2 ± 0.3
oxLDL	2.0 ± 0.2***	2.2 ± 0.3***
oxLDL + 7 μL PJ	2.3 ± 0.3	2.5 ± 0.2*
oxLDL + 14 μL PJ	2.7 ± 0.3*	2.8 ± 0.3**

Note. n-LDL: native LDL; oxLDL: oxidized LDL; NOS III; endothelial nitric oxide synthase; The protein content of the extract was normalized with a polyclonal antibody against gamma-tubulin protein while mRNA by GADPH.

Data are the means ± SD of six separate experiments.

* $p < 0.05$ vs. ox-LDL.

** $p < 0.01$ vs. ox-LDL.

*** $p < 0.001$ vs. n-LDL.

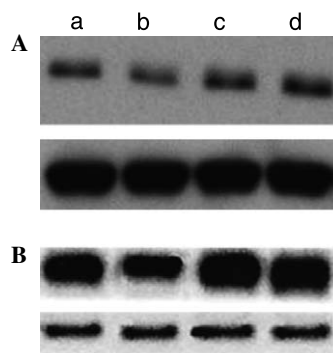


Fig. 1. Representative image of Western blot (A) and Northern blot (B) of endothelial nitric oxide synthase. The protein content of the extract in the Western blot was normalized with a polyclonal antibody against gamma-tubulin protein (lower lane) while in the Northern blot by GADPH (lower lane). Lane a, native LDL; lane b, oxidized LDL; lane c, oxidized LDL + 7 μL; and lane d, oxidized LDL + 14 μL PJ.

with or without PJ (14 μL) but this did not induce a significant reduction of the retarded band (not shown).

Discussion

This study demonstrates, for the first time, that pomegranate juice reduces the potent downregulation of NOSIII induced by oxidation of LDL in human coronary endothelial cells. We have found that exposure of cells to oxLDL for 24 h resulted in a decrease of both NOSIII mRNA and protein, this effect was restored by the treatment with PJ. Therefore, this protection can restore eNOS bioactivity. Although cell-culture studies may indeed bear only partial relevance to pathophysiologic mechanisms activated in humans, oxidation and NO-related cascades of events influence the evolution of atherogenesis, coronary heart disease, ischemic stroke, peripheral arterial disease and other vascular complications in hypercholesterolemic patients. Moreover, the relevance of the cellular damage induced by in vitro oxLDL concentrations would be higher to what is seen in physiological or pathophysiological in vivo models represented by hypercholesterolemic or normocholesterolemic mice (reviewed in: [2,3,5–7]). Since NO is the body's most important molecule in affording protection against

cardiovascular disease, PJ is likely to produce similar beneficial and therapeutic actions by virtue of its capacity to enhance NO production and action. Deficiencies in NO and NOSIII bioactivity lead to the development of vascular damage. Overall, the clinical meaning of these studies are relevant in the clinical setting of cardiovascular diseases.

OxLDL reduced NOSIII expression, decreasing its protein and mRNA levels in endothelial cells from human saphenous veins [14]. We also show that SRE, which is present in the 5'-flanking region of the human NOSIII [15], is not involved in the regulation of NOSIII transcription. Indeed, the EMSA experiments (that detected specific DNA–protein complexes between cell nuclear extracts and the probe containing the SRE) did not reveal a decrease in the retarded band after a 24-h exposure to oxLDL in the presence or in the absence of PJ. The reduction of NOSIII mRNA by oxLDL required a 24-h incubation, which is a long lag-time for simple downregulation at the transcriptional level due to a reduction at the binding-site of the NOSIII promoter. For this reason, we cannot exclude the possibility that a more complex mechanism might be involved in transcriptional regulation of NOSIII by oxLDL in vivo. Moreover, it is highly likely that another mechanism such as increase of mRNA degradation may be playing a role. Furthermore, we show that these effects of oxLDL on NOSIII, and the protection afforded by PJ, were functionally significant, because exposure of cells to oxLDL for 24 h resulted in dose-dependent decrease in cGMP levels that was reverted by PJ.

We already demonstrated that prolonged supplementation with PJ can largely correct the perturbed shear stress-induced proatherogenic disequilibrium by increasing eNOS activity and decreasing redox-sensitive transcription factors both in vitro in cultured human coronary artery endothelial cells and in vivo in hypercholesterolemic mice [13]. Polyphenols from red wine can reduce LDL aggregation in vitro and in vivo [16–18], and PJ administered to hypertensive patients causes also a significant decline in systemic blood pressure [19]. Consistently, a recent study shows that pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduced common carotid intima-media thickness, blood pressure and LDL oxidation [20].

Accordingly, an early study showed that tea-pigment (and possibly polyphenols) exerted some anti-atherosclerotic effects [21]. More recently, it was shown that short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary heart disease [22]. Similarly, the ingestion of polyphenols contained in purple grape juice had beneficial effects on endothelial function in patients with coronary heart disease [23]. Taken together, these data suggest that polyphenols can protect arteries from vascular damage via antioxidant effects and NO restoration.

By using PJ therapeutic intervention, we demonstrate that it is possible to attenuate the proatherogenic scenario associated to decreased NOSIII bioactivity. Antioxidant protection elicits decreased cellular production and release of oxygen radicals in the vascular wall, inhibits endothelial activation of oxidation-sensitive genes, and improves the biologic activity of NO through a cell- or tissue-specific antioxidant action [24].

In summary, treatment with antioxidant polyphenols contained in pomegranate juice may promote a sustained correction of the NOSIII downregulation induced by oxLDL in human coronary endothelial cells. Although cell culture studies may indeed bear only partial relevance to pathophysiological mechanisms activated *in vivo*, these findings may have important implications for the prevention of atherosclerosis and its clinical sequelae.

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