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Dietary Antioxidants and Paraoxonases Against LDL Oxidation and Atherosclerosis Development

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Abstract Oxidative modification of low-density lipoprotein (LDL) in the arterial wall plays a key role in the pathogenesis of atherosclerosis. Under oxidative stress LDL is exposed to oxidative modifications by arterial wall cells including macrophages. Oxidative stress also induces cellular-lipid peroxidation, resulting in the formation of 'oxidized macrophages', which demonstrate increased capacity to oxidize LDL and increased uptake of oxidized LDL.

Macrophage-mediated oxidation of LDL depends on the balance between pro-oxidants and antioxidants in the lipoprotein and in the cells. LDL is protected from oxidation by antioxidants, as well as by a second line of defense—paraoxonase 1 (PON1), which is a high-density lipoprotein-associated esterase that can hydrolyze and reduce lipid peroxides in lipoproteins and in arterial cells. Cellular paraoxonases (PON2 and PON3) may also play an important protective role against oxidative stress at the cellular level. Many epidemiological studies have indicated a protective role for a diet rich in fruits and vegetables against the development and progression of cardiovascular disease. A large number of studies provide data suggesting that consumption of dietary antioxidants is associated with reduced risk for cardiovascular diseases. Basic research provides plausible mechanisms by which dietary antioxidants might reduce the development of atherosclerosis. These mechanisms include inhibition of LDL oxidation, inhibition of cellular lipid peroxidation and consequently attenuation of cell-mediated oxidation of LDL. An additional possible mechanism is preservation/increment of paraoxonases activity by dietary antioxidants. This review chapter presents recent data on the anti-atherosclerotic effects and mechanism of action of three major groups of dietary antioxidants—vitamin E, carotenoids and polyphenolic flavonoids.

Keywords Antioxidants · LDL · Oxidized-LDL · Paraoxonase · Flavonoids · Vitamin E · Carotenoids · Atherosclerosis

1 LDL Oxidation and Atherosclerosis

The 'oxidative modification of lipoproteins' hypothesis of atherosclerosis proposes that the oxidation of low-density lipoprotein (LDL) plays a pivotal role in early atherogenesis (Albertini et al. 2002; Aviram 1995, 1996, 2000; Berliner and Heinecke 1996; Glass and Witztum 2001; Hayek et al. 2005; Kaplan and Aviram 1999; Jialal and Devaraj 1996; Parthasarathy and Rankin 1992; Parthasarathy et al. 1998; Steinberg 1997; Witztum and Steinberg 1991). This hypothesis is supported by evidence that LDL oxidation occurs in vivo (Herttuala 1998) and contributes to the clinical manifestation of atherosclerosis. The early atherosclerotic lesion is characterized by the accumulation of arterial foam cells, which are initially derived mainly from cholesterol-loaded macrophages (Gerrity 1981; Schaffner et al. 1980). Most of the accumulated cholesterol in foam cells originates from plasma LDL. However, LDL has to undergo oxidative modification in order to be taken up by macrophages at an enhanced rate via the macrophage scavenger receptors pathway [scavenger receptors type A (SRA), CD-36], which, unlike the LDL receptor, are not subjected to downregulation by the cellular cholesterol content (Aviram 1993; Goldstein and Brown 1990; Steinberg et al. 1989). The uptake of oxidized LDL (Ox-LDL) via scavenger receptors promotes cholesterol accumulation and foam cell formation (Aviram 1991; Aviram and Rosenblat 2001; Steinberg et al. 1989; Parthasarathy and Rankin 1992), the hallmark of early atherosclerosis.

1.1
Oxidation of LDL in a Cell-Free System

Oxidation of LDL involves free radical attack on the lipoprotein components including cholesterol, phospholipids, fatty acids and apolipoprotein B-100. LDL oxidation results first in the consumption of its antioxidants (mainly vitamin E and carotenoids), and in a substantial loss of polyunsaturated fatty acids (PUFA) and of cholesterol, which are converted to oxidized PUFA and oxysterols.

During the oxidation of LDL, apolipoprotein B-100 also undergoes direct and indirect modifications. Direct attack of oxidants oxidizes amino acid side chains and fragments the polypeptide backbone.

1.2
Macrophage-Mediated Oxidation of LDL

Macrophage-mediated oxidation of LDL is considerably affected by the oxidative state in the cells, which depends on the balance between cellular oxidases and macrophage-associated antioxidants (Aviram and Fuhrman 1998a; Szuchmann et al. 2005). Macrophage binding of LDL to the LDL receptor initiates the activation of cellular oxygenases (Aviram and Rosenblat 1994; Aviram et al. 1996). When NADPH oxidase is activated, the cytosolic components of the NADPH oxidase complex, P-47 and P-67, translocate to the plasma membrane, where they form–together with the membrane bound cytochrome b558–the active NADPH oxidase complex. On the other hand, macrophage antioxidants also contribute to the extent of cell-mediated oxidation of LDL. Cellular reduced glutathione (GSH) is a most potent antioxidant (Meister and Anderson 1983; Rosenblat and Aviram 1997), and an inverse relationship has been shown between the extent of macrophage-mediated oxidation of LDL and the cellular GSH content (Rosenblat and Aviram 1997). Macrophage-mediated oxidation of LDL can also result from an initial cellular lipid peroxidation. When cultured macrophages were exposed to oxidants such as ferrous ions or angiotensin II, cellular lipid peroxidation took place (Fuhrman et al. 1994, 1997). These 'oxidized macrophages' could easily oxidize the LDL lipids, even in absence of any added transition metal ions. Furthermore, macrophage capacity to oxidize LDL increased during in vivo monocyte−to−macrophage differentation (Fuhrman et al. 2004a). Figure 1 summarizes our current view of macrophage-mediated oxidation of LDL and atherosclerosis.

2
Dietary Antioxidants and LDL Oxidation

For a compound to be defined as an antioxidant it must satisfy at least two basic conditions:

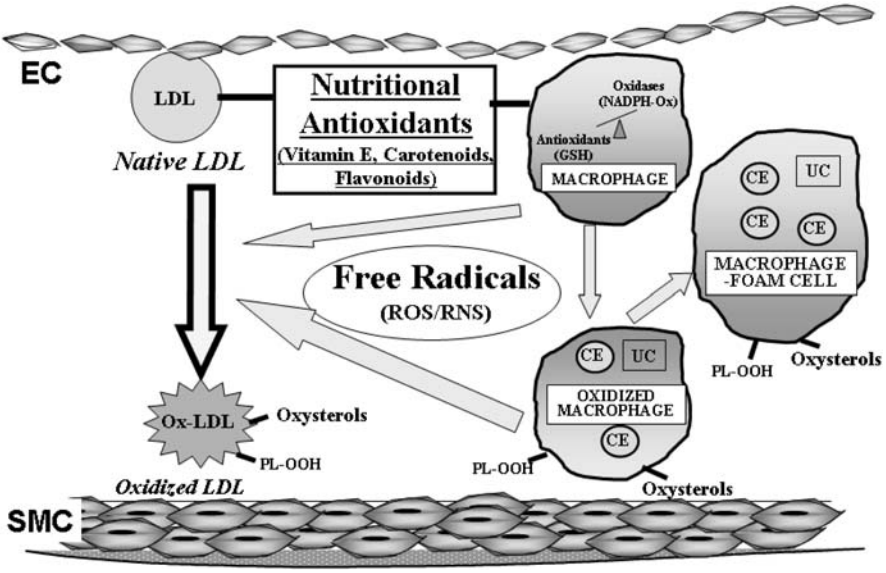

Fig. 1 Nutritional antioxidants and macrophage-foam cell formation. Nutritional antioxidants (vitamin E, carotenoids, flavonoids) can associate directly with LDL, resulting in the inhibition of LDL oxidation. Nutritional antioxidants can also associate with arterial cells such as macrophages, resulting in the inhibition of cellular oxygenases such as NADPH oxidase (*NADPH-Ox*), or in the activation of cellular antioxidants such as the glutathione (*GSH*) system. Reduction in the formation and the release of reactive oxygen/nitrogen species (*ROS/RNS*, respectively) in macrophages by antioxidants thus inhibits the formation of 'oxidized macrophages' and hence reduces cell-mediated oxidation of LDL. Altogether, these effects lead to a reduced formation of macrophage-foam cells, and thus attenuate the development of atherosclerotic lesion

- When present in low concentration relative to the substrate to be oxidized, it can delay, retard, or prevent auto-oxidation or free radical-mediated oxidation.
- The resulting radical formed after scavenging must be stable in order to interrupt the oxidation chain reaction.

The oxidation rate of LDL was shown to be reduced by dietary antioxidants intervention. Dietary antioxidants can inhibit LDL oxidation by several means:

- By scavenging free radicals, chelation of transition metal ions, or protection of the intrinsic antioxidants in the LDL particle (vitamin E and carotenoids) from oxidation.
- By protecting cells in the arterial wall against oxidative damage, and–as a result–inhibition of cell-mediated oxidation of LDL.
- By preservation of serum paraoxonase activity, and–as a result–promotion of the hydrolysis of LDL and arterial cell-associated lipid peroxides.

The beneficial health effects, attributed to the consumption of fruits and vegetables, are related at least in part, to their antioxidant activity.

Vitamin E (α-tocopherol) has been proposed to be the most important lipid-soluble radical-scavenging antioxidant in cellular and subcellular membranes and also in plasma lipoproteins (Burton et al. 1983). Vitamin E is synthesized by plants, and it is found primarily in plant products. Rich sources of vitamin E are vegetable oils, margarine, nuts, seeds and cereal grains. LDL is the major carrier of vitamin E in the circulation. It is estimated that for individuals who are not receiving any supplement, the LDL particle contains six molecules of vitamin E.

Carotenoids are natural pigments with lipophylic properties, widely distributed in fruits and vegetables, and possess some antioxidant characteristics (Krinsky 2001; Sies and Stahl 1995; Stahl and Sies 1997). β-Carotene and lycopene are the major carotenoids in human plasma. Lycopene is the open chain analog of β-carotene, and it is an acyclic carotenoid that contains 11 conjugated double bonds arranged linearly in the all-*trans* form. Carotenoids are transported in human blood complexed to plasma lipoproteins and mainly to the LDL particle. Lycopene, which lacks hydrophilic substituents, is extremely hydrophobic, is located within the hydrophobic core of LDL and thus, its free radical-scavenging ability is limited mostly to the interior of the lipoprotein.

Polyphenols constitute one of the largest category of phytochemicals, most widely distributed among plants, and are an integral part of the human diet. Flavonoids compose the largest and most studied group of plant polyphenols, and over 4,000 different flavonoids have been identified to date. Flavonoids are powerful antioxidants against LDL oxidation, and their activity is related to their localization in the LDL particle, as well as to their chemical structure (Rice-Evans et al. 1996). The free radical scavenging capability of flavonoids stems from the fact that their reducing potential is lower than that of the alkyl peroxyl radical and the superoxide radical and hence it results in free radicals inactivation. Flavonoids are effective scavengers of hydroxyl and peroxyl radicals, as well as superoxide anion (Yuting et al. 1999; Morel et al. 1993), and some of them act as antioxidants due to their potent chelation capacity to transition metal ions.

2.1 Vitamin E

Enrichment of LDL with vitamin E was reported to protect LDL against ex vivo oxidative modification (Dieber et al. 1991; Jialal et al. 1995; Reaven et al. 1993; Wen et al. 1999). However, recent mechanistic studies of the early stage of lipoprotein lipid peroxidation show that the role of vitamin E in this process is not simply that of a classical antioxidant (Stocker 1999a). It was demonstrated that vitamin E can display neutral, anti-, or even pro-oxidant activity under certain conditions (Noguchi and Niki 1998; Upston et al. 1999), depending on

the fate of α-tocopheroxyl radical (α-TO˙) formed during the oxidation process. Unless the α-TO˙ is eliminated, it can replace the lipid peroxyl radical (LOO˙) as the peroxidation-chain carrying species. Effective protection of LDL lipids requires the presence of vitamin E plus a suitable reducing agent, which can reduce α-TO˙ and eliminate the resulting radical from interaction with the LDL particle. Vitamin E is regenerated by the water-soluble vitamin C (Sharma and Buttner 1993), and also by other co-antioxidants, including ubiquinol-10 or α-tocopheryl hydroquinone, which is obtained from the diet. Thus, the benefits of vitamin E supplementation together with other antioxidants that work in concert may explain why dietary vitamin E might be more beneficial against cardiovascular diseases than vitamin E supplements. Furthermore, we have recently demonstrated that vitamin E can act synergistically with lycopene, with β-carotene or with flavonoids, providing a better protection of LDL against oxidation (Fuhrman et al. 2000).

As our understanding of the antioxidant effect of vitamin E evolved, it became clear that vitamin E can also affect inhibition of cellular oxidative responses such as cell-mediated LDL oxidation. In vitro, cell supplementation with vitamin E did not influence their ability to oxidize LDL (Baoutina et al. 1998), whereas dietary supplementation of vitamin E to apolipoprotein E deficient (E^0) mice for a period of 6 weeks resulted in reduced capacity of their harvested macrophages to oxidize LDL (Rosenblat et al. 2002). This effect was associated with reduced cellular content of oxysterols, and inhibition of superoxide production by impairing the assembly of the NADPH–oxidase complex (Cachia et al. 1998; Rosenblat et al. 2002). One common mechanism to account for these effects is the inhibition of protein kinase C activation by vitamin E, which in turn maintains normal vascular homeostasis (Keany et al. 1999).

2.2 Carotenoids

We have previously demonstrated that LDL supplementation with β-carotene or with lycopene increases its resistance to oxidation, in some, but not all, LDL samples that were studied (Fuhrman et al. 1997a). This effect was further potentiated when the carotenoids were present in combination with vitamin E. When lycopene was supplemented as tomato oleoresin, which besides lycopene contains several other micronutrient antioxidants, including vitamin E, the inhibition of LDL oxidation was significantly greater than that of lycopene alone. Dietary antioxidants exist in nature in combination, and combinations of different antioxidants may act additively and even synergistically. Our study presented the first evidence that lycopene can indeed act as an effective antioxidant against LDL oxidation in synergism with several natural antioxidants, including vitamin E, the isoflavan glabridin and also the phenolics rosmarinic and carnosic acids (but not with tocotrienols) (Fuhrman et al. 2000a).

Enrichment of LDL with a mixture of vitamin E, β-carotene lycopene, anthaxanthin and lutein following a single oral supplementation resulted in the protection of LDL PUFA and of its cholesterol moieties against oxidative modification (Linseisen et al. 1998).

Lycopene was shown to react also with peroxynitrite, and to protect LDL against peroxynitrite-induced oxidation (Panasenko et al. 2000), suggesting that this carotenoid scavenges peroxynitrite in vivo. We have extended our studies to analyze the effect of lycopene on the susceptibility of LDL to oxidation in E^0 mice, as their LDL is highly susceptible to oxidation. Dietary supplementation of lycopene (50μg/mouse/week) for 6 weeks to E^0 mice resulted in a significant reduction in the susceptibility of their LDL to copper ion-induced oxidation. However, when lycopene was administered as Lycomato (the tomato's lipid extract where lycopene is present in combination with vitamin E, β-carotene and phytofluene), its antioxidative effect was substantially potentiated (Fuhrman et al. 1997a).

Dietary supplementation of β-carotene (180 mg/day) for 2 weeks to healthy volunteers resulted in enrichment of the subjects' LDL, as well as their monocyte derived macrophages (MDM) with β-carotene. However, β-carotene enrichment of MDM did not affect the capacity of the cells to oxidize LDL (Levy et al. 1996).

The impact of LDL carotenoid content on its oxidation by human aortic endothelial cells was also studied (Dugas et al. 1998, 1999) and the results showed that enrichment of LDL with β-carotene, but not with lycopene, or with lutein, in vivo or in vitro, protected it from oxidation by endothelial cells.

2.3 Flavonoids

The antioxidant capacity against LDL oxidation of a respective flavonoid or flavonoid-rich nutrient is determined by its quantity and its quality. This is evidenced by the observation that white wine, which is very poor in flavonoids in comparison to red wine, exhibits very limited antioxidant protection against LDL oxidation when studied in vitro, as well as in vivo (Fuhrman and Aviram 1996; Fuhrman et al. 2001; Tubaro et al. 1999; Vinson and Hontz 1995). However, enrichment of white wine with flavonoids (by incubation of whole squeezed grapes for 18 h with 18% alcohol) increased significantly the wine antioxidant capacity (inhibition by 87% of copper ion-induced LDL oxidation), almost similar to the antioxidant capacity of red wine, although the flavonoid content was still fourfold less than that found in red wine (Fuhrman et al. 2001). These results suggest that not only is the quantity of flavonoids an important determinant of the wine antioxidant capacity, but also that the diversity of flavonoid types plays an important role in this protective effect against LDL oxidation. This was further evidenced by the discrepancies in the results obtained in human intervention studies with red wine (Howard et al. 2002), which could be

related to the variations in flavonoid composition of the various wines used. Different groups of flavonoids exhibit different extents of inhibitory activity against LDL oxidation at a similar concentration. Among the different groups of flavonoids, the flavonols, flavanols and isoflavans are most potent protectors of LDL against copper ion-induced oxidation. Furthermore, within each group of flavonoids, there are differences in the antioxidant capacity of the individual flavonoids, which is a function of their structure. Structure–function studies on the inhibitory effect of the licorice derived isoflavan glabridin on LDL oxidation, revealed that the antioxidant effect of glabridin on LDL oxidation resides mainly in the 2′-hydroxyl group of the isoflavan B ring. The hydrophobic moiety of the isoflavan was also essential to obtain the inhibitory effect of glabridin on LDL oxidation, and the position of the hydroxyl groups at the B ring significantly affected the ability of glabridin to inhibit LDL oxidation (Belinky et al. 1998a).

Flavonoids can also protect LDL from oxidation by their ability to spare LDL-associated antioxidants. We have demonstrated that enrichment of LDL with glabridin prevented the consumption of β-carotene and that of lycopene by 41% and 50%, respectively, after 1 h of LDL oxidation in the presence of the free radical generator AAPH, but failed to protect vitamin E, the major LDL-associated antioxidant, from oxidation (Belinky et al. 1998b). On the contrary, other flavonoids (quercetin glycosides, as well as its aglycone form) were shown to inhibit the consumption of LDL-associated vitamin E during its oxidation (De-Whalley et al. 1990).

The beneficial effects of flavonoid consumption on LDL oxidation were studied in humans and in animal models. A substantial increase in the resistance of LDL to oxidation was obtained following red wine consumption by humans (Aviram and Fuhrman 1998; Fuhrman et al. 1995; Nigdikar et al. 1998) or by the atherosclerotic E^0 mice (Hayek et al. 1997). Flavonoids from red wine were shown to be absorbed following red wine ingestion, and to bind to the LDL particle, thus protecting it from oxidation. On the contrary, a recent study showed that although red wine consumption increased plasma phenols concentration, this increase was insufficient to protect LDL from oxidation (Caccetta et al. 2000). Red wine consumption increased the resistance of LDL to oxidation also in the postprandial state (Miyagi et al. 1997). Studies on the effect of the nonalcoholic components of red wine resulted in contradictory results. Dealcoholized red wine did not affect the susceptibility of LDL to copper ion-induced oxidation (de Rijke et al. 1996), whereas ingestion of purple grape juice was shown to reduce LDL susceptibility to oxidation in patients with coronary artery disease (CAD) (Stein et al. 1999). Following administration of pro-anthocyanidins-rich extract from grape seeds, these flavonoids protected LDL from oxidation, although they could be detected only in plasma but not in LDL (Yamakoshi et al. 1999). Potent antioxidant activities against LDL oxidation were also obtained following consumption of other groups of flavonoids. Consumption by humans or by E^0 mice of licorice extract or its

major polyphenol glabridin, resulted in increased resistance of LDL to oxidation and to aggregation (Aviram et al. 2004b; Fuhrman et al. 1997; Belinky et al. 1998). This effect could also be related to the absorption of glabridin and it's binding to plasma LDL. Consumption by human volunteers of soy bars containing genistein and daidzein resulted in a marked increase in plasma flavonoid's level, and an increase in the resistance of the LDL to oxidation although LDL-associated flavonoids were not increased (Tikkanen et al. 1998). Tea flavonoids also inhibited LDL oxidation in some (Ishikawa et al. 1997; Serafini et al. 1996), but not in all studies (McAnlis et al. 1998; Princen et al. 1998; Van het Hof et al. 1997). Remarkable inhibition of LDL oxidation was observed following consumption of pomegranate juice (PJ) (Aviram et al. 2000a), ginger extract (Fuhrman et al. 2000b), or olive oil (Aviram and Eias 1993).

Consumption of PJ resulted in the inhibition of cellular lipid peroxidation and the formation of 'oxidized macrophages' (Aviram et al. 2000a). Consumption of nutrients rich in flavonoids such as PJ (Aviram et al. 2000), or red wine (Hayek et al. 1997), or the use of purified flavonoids such as glabridin, catechin or quercetin, by E^0 mice, resulted in a reduced capacity of the mice-harvested macrophages to oxidize LDL (Aviram and Fuhrman 1998b).

In an attempt to explore the mechanism by which flavonoids inhibit macrophage-mediated oxidation of LDL, cells were incubated in vitro with different flavonoids. Upon incubation of macrophages with the isoflavan glabridin, with the flavanol catechin, or with the flavonol quercetin, all of these flavonoids accumulated in the cells in a time- and dose-dependent manner, and this phenomenon was accompanied by a substantial reduction in the capacity of the flavonoid-enriched cells to oxidize LDL. We have shown that glabridin, which accumulated in the macrophages, inhibited cell-mediated oxidation of LDL via the inhibition of superoxide anions release due to the inhibition of the macrophage NADPH oxidase machinery (Rosenblat et al. 1999). Glabridin inhibited the activation of NADPH oxidase, secondary to its inhibitory effect on the translocation of the cytosolic component P-47 to the plasma membrane, and this effect was related to inhibition of the macrophage protein kinase C.

3
Dietary Antioxidants and Atherosclerosis Development

Epidemiological studies have demonstrated an association between increased intake of antioxidant vitamins and reduced morbidity and mortality from CAD (Chan 1998; Hertog et al. 1993; Kaul et al. 2001; Mayne 1996; Muldoon and Kritchevsky 1996). The beneficial health effects, attributed to the consumption of fruits and vegetables are related, at least in part, to their antioxidant activities (Frei 1999; Halliwel 1994; Sies and Sthal 1995; Stahl and Sies 1997; Stocker 1999b). Animal studies have shown that dietary antioxidant supplementation inhibits the progression of atherosclerosis development (Fuhrman

and Aviram 2001a; Fuhrman et al. 2005a; Kaplan and Aviram 2004; Maor et al. 1997; Pratico et al. 1998). However, the results of randomized trials in human with antioxidants demonstrated inconsistent results (Chopra and Thurnham 1999; Futterman and Lemberg 1999; Jialal and Devaraj 2003; Paolisso et al. 1999; Rao 2002; Ursini et al. 1999; Visioli et al. 2002). There are several major issues that should be addressed when performing dietary antioxidant studies as illustrated in Table 1. For a dietary antioxidant to act against LDL oxidation in the arterial wall, it should be absorbed and reach the appropriate tissue, cell and subcellular localization, it should reach appropriate levels, and it also should be active and being able to be reactivated.

Table 1 Factors that determine the antioxidative capacity of an antioxidant

1. Biological absorption
2. Concentration
3. Rate constants for radical reactions
4. Location—aqueous or lipid domains (or in both phases)
5. Mobility in hydrophobic domains
6. Lifetime
7. Rate of regeneration or recycling activity
8. Metal scavenging (chelating, binding) activity
9. The presence of an additional (different) antioxidant
10. The extent of oxidative stress in the studied system

3.1 Vitamin E

3.1.1 Human Studies: Epidemiology

The role of vitamin E in the prevention of cardiovascular disease (CVD) is controversial and the subject of active debate (Chan 1998; Emmert and Kirchner 1999; Jialal and Devaraj 2003; Pryor 2000; Susukawa et al. 1998; Swain and Kaplan 1999; Visioli et al. 2002). Although, contradictory findings were reported in the literature regarding vitamin E supplementation, most of the studies demonstrated that populations using vitamin E supplementation are protected against CVD. Epidemiological data suggest that dietary consumption of vitamin E reduces the incidence of CVD. A substantial reduction in mortality generally correlates with elevated levels of vitamin E in plasma. A cross-sectional study of 16 European populations, the MONICA study, showed a significant inverse correlation between α-tocopherol concentrations and mortality from CAD (Gey et al. 1991). A 40% higher plasma vitamin E concentration was shown to be associated with an 84% lower mortality rate (Gey et al. 1991). Moreover, one longitudinal study involving 5133 Finnish men and women reported an inverse association between dietary α-tocopherol intake and coronary mor-

tality with a 32% risk reduction (Knekt et al. 1994). Three large prospective epidemiologic studies that included the Nurses Health Study (Stampfer et al. 1993 which investigated 87,245 nurses), the Health Professionals Follow-Up Study (Rimm et al. 1993; which investigated 39,910 male health professionals), and an Elderly US population study (Losonczy 1996; composed of 11,178 elderly individuals), found that α-tocopherol supplementation reduced the risk of CAD. In the US Nurses' Health Study (Stampfer et al. 1993) vitamin E supplement, rather than dietary vitamin E, reduced the risk for CHD. Both the Nurses and the Health Professionals studies found that subjects in the highest quintile of α-tocopherol intake had about 40% reduction in CVD (Rimm et al. 1993; Stampfer et al. 1993). In the Elderly population study α-tocopherol supplement use was associated with a 41% reduction in CAD mortality and a 37% reduction in total mortality (Losonczy 1996). A Canadian study (of 2226 men) also reported a significant risk reduction for subjects using α-tocopherol (Meyer et al. 1994). Retrospective evaluation of clinical trials like the Cholesterol-Lowering Atherosclerosis Study (CLAS), a randomized placebo controlled study, provided additional support that coronary artery lesion progression was lowered with α-tocopherol (>100 IU/day) (Hodis et al.1995).

However, in the Iowa Womens' Health Study, Kushi et al. (1996) reported a risk reduction of coronary mortality (21,809 women) from food-derived α-tocopherol intake (>9.64 IU/day) but not from supplements.

3.1.2 Human Studies: Intervention

Prospective large-scale, randomized control clinical trials have been inconclusive regarding the protective role of vitamin E against atherosclerosis (Kaul et al. 2001).

Results from the Cambridge Heart Antioxidant Study (CHAOS) showed that vitamin E therapy (400–800 IU/day for 510 days) significantly reduced nonfatal myocardial infarction by 77% in 2002 patients with angiographically proven CAD (Stephen et al. 1996). Also, Steiner et al. (1995) showed in a double-blind randomized study in 100 patients with transient ischemic attacks, that the group receiving α-tocopherol (400 IU/day) in addition to aspirin, had significantly decreased platelet adhesion and lower incidence of recurrent transient ischemic attacks and ischemic strokes, than patients receiving aspirin alone.

The secondary prevention of CVD in end-stage renal disease (SPACE) with antioxidants, reported a significant reduction in composite CVD endpoints and myocardial infarction with vitamin E supplementation in patients with pre-existing CVD (Boaz et al. 2000). In this study only those patients with increased oxidative stress showed cardiovascular manifestation (Aviram 2003a; Boaz et al. 2003). It thus might be important to use vitamin E or an antioxidant treatment in general, only in those patients with enhanced oxidative stress (Aviram 2003a).

Two trials (ABTC and GSSI) demonstrated benefit for vitamin E supplementation on certain endpoints, despite the primary endpoint not being significant. The ATBC trial, which used α-tocopherol alone or in combination with β-carotene, failed to show any effect on coronary heart disease (CHD) (Rapola 1997). However, the ABTC trial did show that vitamin E supplementation significantly reduced cerebral infarction and onset of angina (Rapola 1997). Furthermore, the GISSI trial showed that in 2,830 patients who had prior myocardial infarction and were on a Mediterranean diet (which is enriched with antioxidants) supplementation with all rac-α-tocopherol (272 IU/day for 3.5 years) had no effect on the composite endpoint of death, nonfatal myocardial infarction and stroke (Investigators 1999). However, when a more appropriate four-way analysis was undertaken, the following significant effects were observed: 20% reduction in cardiovascular deaths, 23% reduction in cardiac death, 25% reduction in coronary death and 35% reduction in sudden death (Investigators 1999).

In the Heart Outcomes Prevention Evaluation (HOPE) study, 2,545 women and 6,696 men 55 years or older who were at high risk for CAD events, were enrolled. They were randomized to receive 400 IU of α-tocopherol from natural sources or placebo and either an angiotensin converting enzyme inhibitor or a matching placebo for a mean of 4.5 years (Hope Investigators 2000). Primary outcome was a composite of myocardial infarction, stroke, and CAD death. There were no significant differences in primary or secondary outcome variables in the subjects taking α-tocopherol. However, this study was undertaken in many countries where dietary intakes of antioxidants and objective measures of supplementation (e.g., plasma levels of vitamin E), were not reported.

The effects of vitamin E on regression of atherosclerotic lesions are also inconsistent. There is evidence to suggest that people on vitamin E supplements (>100 IU/day) demonstrate less atherosclerotic lesion progression in comparison to those who do not consume supplements (Hodis et al. 1995). Moreover, supplementation with antioxidant vitamins C and E retards the early progression of transplant-associated coronary arteriosclerosis given as average intimal index (plaque area divided by vessel area) and measured by intravascular ultrasonography (Fang et al. 2002). In the Antioxidant Supplementation in Atherosclerosis Prevention Study (ASAP) (Salonen et al. 2003), after 6 years of vitamin E supplementation (136 IU) twice daily plus 250 mg of slow-release vitamin C to 520 hypercholesterolemic subjects, a slowed progression of carotid atherosclerosis was found in men but not in women, thus confirming previous findings published after 3 years of supplementation (Salonen et al. 2000).

The vitamin E atherosclerosis prevention study (VEAPS) has shown that vitamin E supplementation (400 IU/day) had no effect on the progression of the common carotid artery far-wall intima-media thickness (IMT) assessed by computer image-processed B-mode ultrasonograms in healthy men and women at low risk for CVD (Hodis et al. 2002). Table 2 summarizes the results of human interventional trials with vitamin E supplementation.

Table 2 Human intervention trials with vitamin E or with carotenoid supplements

Study	Dose and duration	Population	Primary endpoint	Other endpoints	Reference
A. Vitamin E					
CHAOS	400 or 800 IU vit E/day for 510 days	CAD patients	↓ 77% in non fatal infarction		Stephens et al. 1996
SPACE	800 IU vit E/day for 519 days	Hemodialysis patients	↓ Myocardial infarction		Boaz et al. 2000
Transplant associated arteriosclerosis	800 IU vit E/day + 1,000 IU vit C/day for 1 year	Patients after cardiac transplantation (0–2 years)	↓ Coronary arteriosclerosis		Fang et al, 2002
Vitamin E administration plus aspirin	400 IU/day vit E with aspirin for 1 year	Patients with ischemic attacks	↓ Incidence of ischemic events		Steiner et al, 1995
ABTC	50 IU vit E/day for up to 8 years	Male smokers	↔ Lung cancer	↓ Cerebral infarction	Rapola 1997
GSSI	272 IU vit E (all rac)/day for up to 5 years	Patients with prior myocardial infarction	↔ Death, myocardial infarction	↓ Cardiovascular death	Investigators 1999
HOPE	400 IU vit E/day for up to 6 years	Patients with high risk for CAD	↔ Myocardial infarction, Stroke, cardiovascular death	↔ Death from any cause	Hope Investigators 2000
VEAPS	400 IU vit E/day for 3 years	Healthy men and women	↔ IMT thickness		Hodis et al, 2002
ASAP	272 IU/day vit E+ 500 mg vit C/day for 6 years	Hypercholesterolemic patients	↓ IMT thickness		Salonen et al. 2000, 2003
B. Carotenoids					
CARET	30 mg β-Carotene and 25,000 IU Vit A/day for *1 year*	High risk for lung cancer	↑ 46% lung cancer	↑ 26% cardiovascular deaths	Redlich et al. 1999
Beta carotene prevention	50 mg β-carotene/day for 4.3 years	Patients enrolled for prevention of nonmelanoma skin cancer	↔ Death from all causes	↔ Cardiovascular death	Hennekens et al. 1996
Randomized trial of α-tocopherol and β-carotene	50 mg vit E and 20 mg β-carotene/day for 5.3 years	Smokers	↑ Death from coronary disease		Rapola 1997

vit, Vitamin.

3.1.3 Animal Studies

Animal studies allow a more direct investigation of the effect of vitamin E supplements on atherosclerosis. Although the earliest animal studies yielded ambivalent results (Kaul et al. 2001), most of the later studies described here have supported slow progression and prevention of atherosclerosis following vitamin E supplementation. We were the first to demonstrate that consumption of vitamin E by E^0 mice resulted in a 35% reduction in their aortic lesion area (Maor et al. 1997). Pratico et al. (1998) also reported that E^0 mice supplementation with vitamin E (2,000 IU/kg chow) significantly reduced aortic lesion. Cholesterol-fed macaques that were supplemented with vitamin E exhibited a 35% inhibition of atherosclerotic lesion formation as assessed by carotid Doppler studies over a 3-year period (Verlangieri and Buxh 1992). Moreover, in cholesterol-fed rabbits, a 70% inhibition of atherosclerotic lesion formation with 40 mg/kg/day vitamin E was reported (Prasad 1980). Reduced restenosis after angioplasty in rabbits with established experimental atherosclerosis was seen following vitamin E supplementation (Lafont et al. 1995). In another study (Williams et al. 1992), dietary vitamin E administered to modified Watanabe rabbits, led to reduced cholesterol levels and reduced LDL oxidation associated with the inhibition of early aortic lesion development. Also, chickens fed high doses of vitamin E had reduced concentrations of plasma peroxides and less aortic intimal thickening compared with controls (Smith and Kummerow 1989). Recently, administration of vitamin E-supplemented diet to LDL-deficient mice (Cyrus et al. 2003) as well as administration of a vitamin E water soluble to atherosclerotic rabbits (Yoshida et al. 2002), was also shown to reduce the progression of atherosclerosis. Table 3 summarizes the animal's trials with vitamin E supplementation.

At present, there is no conclusive evidence in epidemiological studies that vitamin E supplements provide the benefits observed for vitamin E rich nutrients.

The inconsistent results may be due to the various biological functions of vitamin E, including its role in protection of LDL against oxidation, as well as its other activities on cells of the vascular wall. The dosages of vitamin E administrated in the various studies varied considerably, and it seems that there might be a threshold dose of vitamin E (>800 IU/day) that is effective (Jialal and Devaraj 2003).

Most importantly, the problem of patient selection in human intervention trials has been highlighted recently (Jialal et al. 2001; Visioli et al. 2002). Several parameters must be taken into consideration when planning antioxidants clinical trials. These include the use of analyses, detailed information on the patient's dietary intake, reliable biological markers for oxidative stress and the selection of a population that is suitable for antioxidant treatment (Aviram 2003a).

Table 3 Animals supplementation with vitamin E, carotenoids or flavonoids and atherosclerotic lesions development

Study	Dose and duration	Animal population	Endpoint
A. Vitamin E			
Maor et al.	50 mg vit E/kg/day for 3 months	Apolipoprotein E deficient mice	↓ 33% Aortic lesion area
Prasad et al.	40 mg/kg/day vit E for 2 months	Hypercholesterolemic rabbits	↓ 70% Aortic lesion area
Lafont et al.	5 g/kg diet for 19 days	Hypercholesterolemic rabbits after angioplasty	↓ 43% Restenosis
Williams et al.	0.5% vit E for 12 weeks	Watanabe heritable hyperlipidemic rabbits	↓ 32% Aortic arch lesion area
Pratico et al.	2,000 IU/kg chow vit E for 16 weeks	Apolipoprotein E deficient mice	↓ 67% Aortic lesion area
Smith et al.	1,000 IU vit E /kg diet for 2 months	Hyperlipidemic hens	↓ 38% Intimal thickness
Cyrus et al.	2,000 IU vit E/kg diet for 3 months	LDL-receptor deficient mice	↓ 50% Aortic lesion area
Yoshida	0.8% water soluble vitamin E for 12 weeks	Watanabe heritable hyperlipidemic rabbits	↓ 20% Aortic lesion area
B. Carotenoids			
Shaish et al.	0.01% all trans β-carotene for 11 weeks	Hypercholesterolemic rabbits	↓ 38% Aortic arch lesion area
Dwyer et al.	0.2% Synthetic β-carotene /weight for 8 weeks	Apolipoprotein E deficient mice	↓ 43% Aortic lesion area
Sun et al.	Intravenous injection β-carotene (215 mg/kg/twice weekly)+0.5% vit E for 8 weeks	Hypercholesterolemic rabbits	↓ Atherosclerotic lesion area, intimal thickness
Crawford et al.	1,000 mg β-carotene/kg /day +200 mg vit E/kg /d+100 mg vit C/kg/day for 8weeks	LDL-receptor deficient mice	↓ 60% Aortic lesion area
Shaish A et al.	0.05% all-*trans* β-carotene +0.05% vit E for 16 weeks	Apolipoprotein E deficient mice	↔ Aortic sinus lesion ↔ aortic lesion area
C. Flavonoids			
Hayek et al.	0.5 ml Red wine/mouse /day for 12 weeks	Apolipoprotein E deficient mice	↓ 48% Atherosclerotic lesion area,
Aviram et al.	31 μl Pomegranate juice /mouse/day for 12 weeks	Apolipoprotein E deficient mice	↓ 44% Atherosclerotic lesion area
Fuhrman et al.	200 μg Licorice/mouse/day for 12 weeks	Apolipoprotein E deficient mice	↓ Atherosclerotic lesion area
Fuhrman et al.	250 μg ginger/mouse/day for 12 weeks	Apolipoprotein E deficient mice	↓ 44% Atherosclerotic lesion area

vit, Vitamin.

Table 3 continued

Study	Dose and duration	Animal population	Endpoint
Lee et al.	0.1% naringin or 0.05% naringenin for 8 weeks	New Zealand rabbits	↓ 18% Aortic fatty streak area
Vinson et al.	100 mg/kg/day grape seed proanthocyanidin extract for 10 weeks	Hypercholesterolemic hamsters	↓ 63% Atherosclerosis development
Yamakashi et al.	0.1% grape seed proanthocyanidin-rich extract for 8 weeks	Hypercholesterolemic rabbits	↓24% Aortic arch lesion
Wakabayashi et al.	7 ml red wine/kg/day for 14 months	Watanabe heritable hyperlypidemic rabbits	↔ Aortic and coronary atherosclerotic lesion size

vit, Vitamin.

3.2 Carotenoids

3.2.1 Human Studies: Epidemiology

Dietary carotenoid consumption was shown in epidemiological studies to be associated with reduced cardiovascular mortality (Kohlmeier and Hasting 1995; Pavia and Russell 1999). However, intervention trials with carotenoid supplements demonstrated no effect or even the opposite effects (Tavani and La Vecchia 1999). A Mediterranean diet rich in tomatoes, tomato products, lycopene, and other carotenoids is associated with the low incidence of atherosclerosis and CHD (Rao 2002). Low serum levels of carotenoids were associated with an increased risk of subsequent myocardial infarction among smokers (Street et al. 1994).

The cross-sectional association between intake of carotenoids with provitamin A activity and carotid artery plaques, as examined in 12,773 participants in the Atherosclerosis Risk in Communities Study, suggests that carotenoids may exert their influence later, rather than earlier, in the atherosclerotic process. It thus supports the hypothesis that carotenoids may play a role in preventing arterial plaque formation (Kritchevsky et al 1998). In a cross-sectional study comparing Lithuanian and Swedish populations showing diverging mortality rates from CHD, lower blood lycopene levels were associated with increased risk and mortality from CHD (Kritenson et al. 1997). In another study, a comparison of determinants for coronary heart disease was made among Czech, Bavarian and Israeli men (Bobak et al. 1999). The mortality rates, as well as the prevalence of CHD, were highest in Czech, intermediate in Bavarian and low in Israelis, and these observations correlated with the lycopene concentrations in plasma.

An inverse association between carotid IMT and lycopene was found in patients with essential hypertension and peripheral vascular disease (Gianetti et al. 2002). Low blood levels of lycopene were found to be associated with atherosclerosis risk in middle-aged men from eastern Finland. In women, however, the protective effect was weaker (Tiina et al. 2002).

The strongest population-based evidence comes from a multicenter case–control study (EURAMIC) that evaluated the relationship between adipose tissue antioxidant status and acute myocardial infarction (Kohlmeier et al. 1997). In subjects (662 cases and 717 controls) from ten European countries adipose tissue levels of α- and β-carotenes, lycopene, and α-tocopherol were measured shortly after myocardial infarction. After adjusting for age, body mass index, socioeconomic status, smoking, hypertension, and maternal and paternal history of the disease, only lycopene levels, but not β-carotene, were found to be protective.

3.2.2
Human Studies: Intervention

Several interventional trials (Greenberg et al.1996; Omenn et al. 1996; Rapola 1997; Redlich et al. 1999) using high dose of β-carotene supplements, showed an increase in CVD mortality in the supplemented groups, ranged from 12% to 26%, whereas in a trial among 11,036 healthy physicians, 12 years' supplementation with β-carotene (50 mg on alternate days) produced neither benefit not harm in terms of CVD or death from all causes (Hennekens et al. 1996). Rather, the high-risk population (smokers and asbestos workers) in these interventional trials showed an increase in cancer and angina cases. It appears that carotenoids (including β-carotene) can promote health when taken in small dosages, but may have adverse effects when taken in high dose by subjects who smoke or who have been exposed to asbestos (Pavia and Russell 1999). The Carotene and Retinol Efficacy Lung Cancer Chemoprevention Trial (CARET) (Redlich et al. 1999) ended prematurely due to the unexpected findings that the active treatment group on the combination of 30 mg β-carotene and 25,000 IU retinyl palmitate had a 46% increased lung cancer mortality and a 26% increased cardiovascular mortality compared with the placebo group (Redlich et al. 1999). Table 2 summarizes the results of human interventional trials with carotenoids.

3.2.3
Animals Studies

Supplementation of synthetic β-carotene (Shaish et al. 1995), or synthetic lutein (Dwyer et al. 2001) to atherosclerotic animals showed a protective effect on the progression of atherosclerosis . However, administration of dietary α-tocopherol in combination with β-carotene significantly inhibited the de-

velopment of atherosclerotic lesion in some (Crawford et al. 1998; Sun et al. 1997) but not all studies (Shaish et al. 1999). Table 3 summarizes the results of animal trials with carotenoids.

The epidemiologic evidence is generally supportive of the notion that a diet rich in carotenoids is associated with a reduced risk for CHD. The clinical trials however show that supplementation of β-carotene does not prevent CHD, although the benefits of other carotenoids, such as lycopene, have not been ruled out.

It might be that serum β-carotene levels are confounded by one or more unmeasured factors that may correlate with reduced β-carotene levels and predict the risk of CHD risk (Kritchevsky 1999). Moreover, no trials have been conducted with lycopene, although its numerous antioxidative activities, mainly in combination with other antioxidants, were demonstrated (Fuhrman et al. 2000; Krinsky 2001; Levy et al. 1996).

3.3 Flavonoids

3.3.1 Human Studies

Consumption of flavonoids in the diet was shown to be inversely associated with morbidity and mortality from CHD (Hertog et al. 1995). Moreover, an inverse association between flavonoid intake and subsequent occurrence of ischemic heart disease, or cerebrovascular disease was shown (Knekt et al. 1996, 2002). Reduced risk for ischemic heart disease mortality was shown in individuals with high intake of apples and onions, which are rich with the flavonols quercetin and kaempferol (Knekt et al. 2002).

In most countries, a high intake of saturated fats is strongly correlated with high mortality from CHD, but this is not the case in some regions of France, the so-called 'French paradox' (Renaud and de Lorgeril 1992). This anomaly has been attributed to the regular intake of red wine (Fuhrman et al. 2001).

We investigated the effects of PJ consumption by patients with carotid artery stenosis (CAS) on carotid lesion development in association with changes in oxidative stress (Aviram et al. 2004a). Ten patients were supplemented with PJ for up to 1 year, and nine other patients that did not consume PJ served as a control group. Blood samples were collected before treatment and after 3, 6, 9 and 12 months of PJ consumption. Patients' carotid IMT was compared between the PJ group and the control group. While in the control group IMT increased by 10% during 1 year, PJ consumption resulted in a significant IMT reduction by up to 43%. Our results clearly demonstrate that PJ consumption by patients with CAS decrease lesion size and systolic blood pressure, and these effects could be related to the potent antioxidant characteristics of PJ.

3.3.2 Animal Studies

Dietary consumption of flavonoid-rich nutrients, as well as pure flavonoids, was shown to attenuate the progression of atherosclerosis in animals (Aviram 1996, 1999a, 2000a; Aviram and Fuhrman 1998b, 2003; Fuhrman and Aviram 2001b, 2001c). Reduced development of atherosclerotic lesion areas in the atherosclerotic E^0 mice was demonstrated following consumption of PJ (Aviram et al. 2000a; Kaplan et al. 2001), red wine (Hayek et al. 1997), grape powder (Fuhrman et al. 2005a), licorice root extract (Fuhrman et al. 1997), or ginger extract (Fuhrman et al. 2000b). Consumption of the flavonol quercetin or the isoflavan glabridin also showed a remarkable attenuation of lesion size in E^0 mice (Hayek et al. 1997).

In New Zealand white rabbits supplemented with 0.1% naringin or 0.05% naringenin, aortic fatty streak areas were significantly lower by 20% in comparison to the control group (Lee et al. 2001). In hamsters fed with hypercholesterolemic diet that was supplemented with grape seed proanthocyanidin extract (100 mg/kg/day), the atherosclerotic lesion size was reduced by up to 63% (Vinson et al. 2002). Ingestion of proanthocyanidin-rich extract from grape seeds also reduced severe atherosclerosis in the aorta of cholesterol-fed rabbits (Yamakoshi et al. 1999). In the Watanabe heritable hyperlipidemic rabbits however, administration of red wine reduced the susceptibility of LDL to oxidation, but it failed to prevent the progression of atherosclerotic lesion development (Wakabayashi 1999). Table 2 summarizes the results of the animal's trials with flavonoids.

The epidemiologic evidence, along with results observed in animal studies, clearly suggest that a diet rich in flavonoids may possess potent antiatherosclerotic effects also in humans.

Figure 2 summarizes the effect of antioxidant nutrients consumption by the atherosclerotic E^0 mice on their LDL oxidation in a cell- free system (A), on macrophage-mediated LDL oxidation (B), and on atherosclerotic lesion development (C).

4 The Paraoxonase Gene Family

The paraoxonase (PON) gene family includes three members, PON1, PON2, and PON3 (Hegele 1999; Primo-Parmo et al. 1996). These PON genes appear to have arisen by gene duplication of a common evolutionary precursor because they share considerable structural homology and are located adjacently on chromosome 7 in humans, and on chromosome 6 in mice. Within a given species, PON1, PON2, and PON3 share about 70% identity at the nucleotide level.

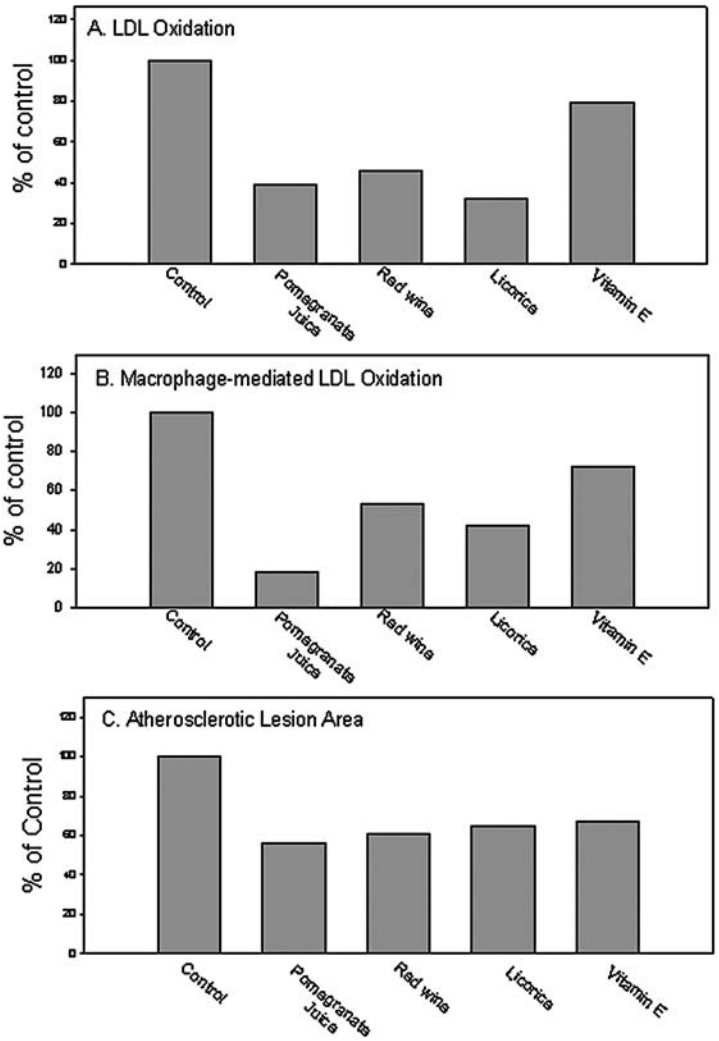

Fig. 2A–C Antioxidants consumption protects LDL from oxidation and attenuates atherosclerotic lesion development. Consumption of nutritional antioxidants (12.5 µl/day pomegranate juice, 0.5 ml/day red wine, 200µg/day licorice extract, or 1 mg/day vitamin E) by E^0 mice inhibits oxidation of LDL (**A**), macrophage-mediated oxidation of LDL (**B**), and the development of atherosclerotic lesion (**C**)

4.1 Serum Paraoxonase 1

Human serum paraoxonase (PON1) is an esterase of 354 amino acids and a molecular mass of 43 kDa (La Du et al. 1993; Mackness et al. 1996), which is physically associated with HDL (Mackness et al. 1996), and is also distributed

in tissues such as liver, kidney and intestine (Rodrigo et al. 2001). PON1 is bound to the HDL phospholipids via its retained N-terminal leader sequence, and HDL-associated apolipoprotein A-I stabilized PON1 activity (Sorenson et al. 1999). PON1 was also present in postprandial chylomicrons (Fuhrman et al. 2005b).

4.1.1 PON1 and Atherosclerosis

Human serum PON1 activity was shown to be inversely related to the risk of CVD (Aviram 1999b; Aviram 2004; Durrington et al. 2001; Mackness et al. 2001), as shown in atherosclerotic, hypercholesterolemic and diabetic patients (Abbott et al. 1995; Boemi et al. 2001; Garin et al. 1997; Letellier et al. 2002; Mackness et al. 1991a), as well as in the atherosclerotic E^0 mice. PON1 activity was also decreased in rabbits fed a pro-atherogenic diet (Mackness et al. 2000a).

PON1/apolipoprotein E dual knockout mice exhibited accelerated atherosclerosis (Shih et al. 2000), and in human PON1 transgenic mice, a decreased lesion formation was shown in comparison to control mice (Rozenberg et al. 2005; Tward et al. 2002). This effect may be related to PON1 ability to enhance HDL-mediated macrphage cholesterol efflux via the ABCA1 transporter (Rosenblat et al. 2005).

4.1.2 PON1 and Oxidative Stress

An inverse relationship between serum PON1 activity and the extent of lipid peroxidation was indeed shown (La Du 1996). In PON1/apolipoprotein E dual knockout mice, increased lipoprotein oxidation was shown (Shih et al. 2000). Furthermore, HDL isolated from human PON1 transgenic mice was more protected from copper ion-induced oxidation than control HDL (Oda et al. 2002). PON1 protects both LDL and HDL against lipid peroxidation (Aviram et al 1998; Mackness et al. 1991b, 1993, 2000b; Navab et al. 1996). Inhibition of HDL oxidation by PON1 was shown to preserve the anti-atherogenic effects of HDL in reverse cholesterol transport. PON1 hydrolyzes oxidized cholesteryl esters, as well as specific oxidized phospholipids in oxidized lipoproteins and also in cells (macrophages) and in atherosclerotic tissue (Ahmed et al. 2001; Aviram et al. 2000b; Navab et al. 1996; Rozenberg et al. 2005). This mode of action resembles that of acetylcholine esterase (AChE), which share with PON1 the AChE-PON1 locus on chromosome 7 (Fuhrman et al. 2004b).

We have recently demonstrated that PON1 deficiency results in increased oxidative stress not only in serum, but also in tissues, as evident in arterial, as well as in peritoneal macrophages. This phenomenon may contribute to the accelerated atherosclerosis seen in PON1 knockout mice (Rozenberg et al. 2003). Incubation of macrophages from $PON1^0/E^0$ mice with purified human PON1 resulted in a reduction in the level of lipid peroxides, in the amount of

superoxide anion release (Rozenberg et al. 2003), and in macrophage-mediated oxidation of LDL. This phenomenon may be related to the ability of PON1 to hydrolyze lipid peroxides in 'oxidized macrophages' obtained from the atherosclerotic E^0 mice (Fig. 3) (Kaplan and Aviram 2004b). While PON1 can hydrolyze lipid peroxides and thus protects against oxidative stress on the one hand, PON1 was shown to be inactivated by oxidative stress on the other hand. Furthermore, injection of oxidized phospholipid (oxidized 1-palmitoyol-2-arachidonyl-*sn*-glycerol-3-phosphoryl choline, Ox-PAPC) into C57BL/6J mice resulted in a marked reduction in PON1 activity.

4.1.3 Dietary Antioxidants and PON1

Consumption of antioxidant-rich nutrients, such as PJ or red wine, by healthy subjects or by atherosclerotic patients, as well as by the atherosclerotic E^0 mice was shown to preserve PON1 activity, probably by reducing the oxidative stress, thereby contributing to PON1 hydrolytic activity on lipid peroxides in oxidized lipoproteins and in atherosclerotic lesions (Aviram 2003b, 2004, Aviram and Rosenblat 2004, Aviram et al. 2000b, 2004, Fuhrman and Aviram 2002; Kaplan et al. 2001; Rosenblat and Aviram 2005). In a recent study it was demonstrated that vitamin C and vitamin E intake by patients with CAS was associated with increased PON1 activity (Jarvik et al. 2002).

We have shown that PJ consumption by patients with CAS significantly reduced their LDL oxidation rate, and that this was paralleled by a substantial increment in serum PON1 activity (Aviram et al. 2004a).

Intake of the monounsaturated fatty acid, oleic acid, by healthy subjects increased serum PON1 activity, especially in patients carrying the PON1-192R allele.

In another study, meals rich in olive oil (oleic acid rich) were associated with increased postprandial serum PON1 activity in middle-aged and older diabetic women (Tomas et al. 2001), whereas a diet rich in safflower oil had no such effect (Wallace et al. 2001).

In contrast, a high intake of vegetables, berries, and apples combined with a high intake of linoleic or oleic acid for 6 weeks only slightly affected markers of lipid peroxidation and paraoxonase activity (Freese et al. 2002), and in some studies even a reduction in PON1 activity was noted (Kleemola et al. 2002; Rantala et al. 2002). In vitro studies (Aviram et al. 1999) suggest that dietary antioxidants and antioxidant enzymes and PON1 showed a co-activity in protecting LDL from oxidation (Sozmen et al. 2001). Antioxidants, such as the flavonoids glabridin (from licorice root), or quercetin (from red wine) when present during LDL oxidation together with PON1, reduced the amount of lipoprotein-associated lipid peroxides and preserved PON1 activities, including its ability to hydrolyze Ox-LDL cholesteryl linoleate hydroperoxides (Aviram et al. 1999).

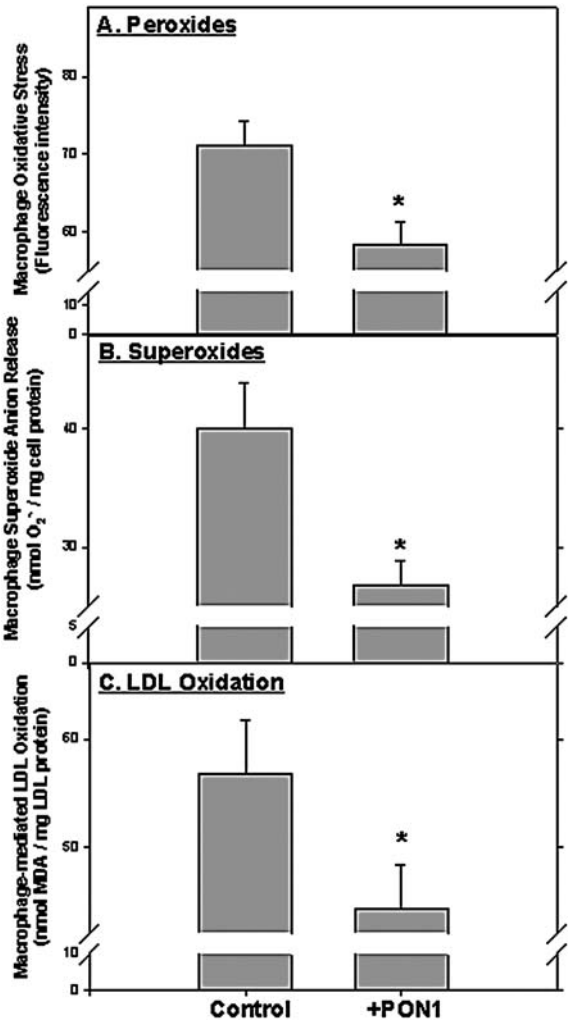

Fig. 3A–C Serum paraoxonase 1 (*PON1*) decreases macrophage oxidative stress. Mouse peritoneal macrophages (*MPM*) were harvested from $PON1^0/E^0$ mice, and incubated for 18 h at 37°C without or with purified PON1 (7.5 arylesterase Units/ml). Macrophage oxidative stress was expressed as cellular peroxide content (**A**), superoxide anions release (**B**) and cell capacity to oxidize LDL (**C**). **A** For cellular peroxide content, cells were incubated for 30 min at 37°C with DCFH-DA and cellular fluorescence was determined by flow cytometry. **B** Superoxide anions release from macrophage was measured after 1 h of cell incubation with cytochrome C at 37°C. **C** LDL oxidation was determined after cell incubation for 6 h at 37°C with LDL (100 mg protein/l), in the presence of 5 µmol/l of $CuSO_4$. At the end of the incubation, LDL oxidation was measured by the TBARS assay. All the results are given as mean percentage±SD (n=5). *P<0.01 (vs. control)

PONs possess lactonase activity, and they are capable of hydrolyzing statins (HMG-CoA reductase inhibitors, which are potent hypocholesterolmic drugs). Statin therapy can reduce the level of oxidized lipids (in the serum of hypercholesterolemic patients) and hence, preserves or even increases PON1 activity. Indeed, atorvastatin or simvastatin therapies increased serum PON1 activity (Fuhrman et al. 2002; Rosenblat et al 2004, Tomas et al. 2000,). In rats, cerivastatin decreased the level of oxidative stress, improved plasma antioxidant defense and also enhanced PON activity (Beltowski et al. 2002).

4.2 PON 2 and PON 3 and Oxidative Stress

By using PON3 specific peptide antibodies, human PON3 was detected as a 40-kDa protein, which, like PON1, is also associated with serum high-density lipoprotein (HDL) (La Du 2001; Reddy et al. 2001). In contrast to PON1, PON3 has very limited arylesterase activity, and no PON activity at all, but it rapidly hydrolyzes lactones such as statin pro-drugs like lovastatin (Draganov et al. 2000). While the mRNA expression of PON1 and PON3 is restricted primarily to the liver, PON2 mRNA is more widely expressed, and is found in a number of tissues, including brain, liver, kidney, testis, and also in white blood cells (Mochizuki et al. 1998).

PON2 overexpression was shown to lower the intracellular oxidative state of cells that were pretreated with either hydrogen peroxide or with Ox-PAPC (Ng et al. 2001). Minimally modified LDL (MM-LDL) that was incubated with cells overexpressing PON2 showed lower levels of lipid hydroperoxides, and was less able to induce monocyte chemotaxis than MM-LDL incubated with control cells (Ng et al. 2001). These data suggest that PON2 may act as a cellular antioxidant, and may thus play an antiatherogenic role by reducing the cellular oxidative stress.

We have recently demonstrated the presence (mRNA, protein, activity) of PON2 and PON3, but not PON1, in murine macrophages, whereas in human macrophages, only PON2 was expressed (Rosenblat et al. 2003). PON2 expression was shown to be upregulated via a NADPH oxidase-dependent mechanism during monocyte differentation into macrophages (Shiner et al. 2004). Like serum PON 1, also macrophage PON3 (but not PON2) was shown to be inactivated, by up to 57%, under oxidative stress. Dietary antioxidants such as vitamin E or PJ significantly increased (23%–40%) macrophage PON3 activity.

5
Perspectives and Future Directions

LDL oxidation by arterial cells, including macrophages, and the uptake of Ox-LDL by arterial macrophages, leading to foam cell formation, the hallmark of early atherogenesis, is a seminal event in atherosclerosis development. Epidemiological studies, randomized clinical trials and basic research studies, support the hypothesis that changes in dietary patterns to increase dietary antioxidant consumption, will decrease the risk of atherosclerosis. However, clinical trials on antioxidants, mainly with vitamin E and β-carotene, were not supportive of the effects observed in epidemiological studies. There are several important issues that must be addressed prior to the use of an antioxidant. Because a combination of antioxidants can provide a wider range of free radical scavenging activity than an individual antioxidant, clinical and nutritional studies in humans should be directed towards the use of combinations of several types of dietary antioxidants, including combinations of flavonoids together with the other nutritional antioxidants, such as vitamin E and carotenoids. It is most important to use reliable biological markers of oxidative stress, and to identify populations suitable for antioxidant treatment, as antioxidant treatment may be beneficial only in subjects who are under oxidative stress.

Compounds called antioxidants may possess activity beside their antioxidant properties. Antioxidants differ in their ability to react with different reactive oxygen/nitrogen species (ROS/RNS). Some antioxidants exhibit additional anti-atherogenic activities beyond their antioxidant effects.

The exact roles of humoral (PON1) and cellular (PON2) PONs in macrophage foam cell formation under oxidative stress during the development of atherosclerosis is still not understood (Aviram 2003c, Aviram and Rosenblat 2004). Strategies to reduce LDL oxidation and to attenuate atherosclerosis may include appropriate antioxidant combinations that can, on the one hand, reduce oxidative stress as a first line of defense, and on the other hand increase PON activity as a second line of defense against CVDs.

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