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Antioxidant and Prooxidant Effects of Ascorbic Acid, Dehydroascorbic Acid and Flavonoids on LDL Submitted to Different Degrees of Oxidation

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Although a high intake of antioxidants may decrease the risk of developing cardiovascular diseases, under certain circunstances they may promote free radical generation and lipid peroxidation. The objectives of the present study were to determine the antioxidant effects of ascorbic acid (AA), dehydroascorbic acid (DHA) and flavonoids on LDL submitted to different degrees of oxidation. LDL was submitted to oxidation with $CuCl_2$ (2.4 μM). Before or at different times after the propagation of the oxidation process, 28 μM (5 μg/ml) of either AA or DHA or 5 μg/mL flavonoids extract were added. Alpha-tocopherol, conjugated dienes, thiobarbituric acid reacting substances (TBARS) and LDL electrophoretic mobility were determined as indices of LDL oxidation. The presence of any of the three antioxidants from the onset of the incubation delayed the oxidation process. However, the addition of both DHA and flavonoids to the oxidation process when it was already initiated and alpha-tocopherol consumed, accelerated the oxidation. In contrast, AA delayed the oxidation process even when added after alpha-tocopherol was consumed. Nevertheless, it also accelerated LDL oxidation when added during the propagation phase of the oxidation process. In conclusion: although AA, DHA and flavonoids delay LDL oxidation when added before the initiation of the process, they accelerate the process if added to minimally oxidized LDL.

Keywords: ascorbic acid, dehydroascorbic acid, flavonoids, low density lipoprotein

INTRODUCTION

On the basis of in vitro and in cell culture experiments, it is widely accepted that LDL oxidation plays a central role in the development of cardiovascular diseases secondary to atherosclerosis.[1,2] Besides, a large body of epidemiological studies has shown that populations with a high intake of antioxidants, including ascorbic acid, vitamin E and flavonoids have a lower rate of cardiovascular diseases than populations with low intake.[3–5] In fact, it is assumed, that the antioxidant vitamins would prevent the changes

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in LDL that can lead to the development of atherosclerosis. Experimental studies have demonstrated that either liposoluble antioxidants as vitamin E[6,7] or water soluble antioxidants, including AA, DHA and flavonoids, decrease the rate of LDL oxidation.[8–11]

Despite the potential benefits of the administration of antioxidant vitamins on human health, some authors have raised questions on the potential side effects of the intake of large doses of these compounds. It is well established that most antioxidants, including vitamins, may promote free radical generation under certain circumstances, acting as prooxidant.[12,13] In fact, AA, when incubated with LDL stored for certain time, and probably minimally oxidized, may promote their oxidation.[14] It can not therefore be assumed that AA would always act as an antioxidant. In fact, when incubated with iron, AA is even used to propagate lipid peroxidation.[15,16]

The present study was addressed to determine whether such prooxidant effects of AA on minimally oxidized LDL could also be extended to other water soluble antioxidants, such as DHA and flavonoids.

MATERIALS AND METHODS

Reagents. Ascorbic and dehydroascorbic acid were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). The flavonoid rich extract used was the anthocyanoside extract from Vaccinium myrtillus, supplied as a gift by Sigma-Tau España (Alcala de Henares, Madrid, Spain).

Isolation of Lipoproteins. Samples were obtained from healthy non-smoking voluntary subjects (age range: 20–27 years) after 12 h fasting. LDL were isolated from EDTA-treated plasma by ultracentrifugation in a vertical rotor (NVT 65, from Beckman) at 50,000 rpm for 1 h as described by Chung, et al.[17] The isolated LDL were filtered through an 0.22 μm filter (Millipore, Molsheim, France), and sucrose was added to obtain a final concentration of 10% (w/v), in order to preserve the natural structure and the oxidation capacities.[18] Samples were stored at –80°C until the day of the experiment.

Determination of LDL Susceptibility to Oxidation. Conjugated dienes formation was determined as described by Esterbauer et al.[19] In short, the stored LDL was passed through a Sephacryl-400 column (Pharmacia LKB, Biotechnology, Inc., Madrid, Spain) to remove the EDTA and the sucrose using PBS (10 mM sodium phophate buffer, pH 7.4, containing 0.15 M sodium chloride) as eluent buffer. The LDL protein concentration was determined immediately using the Lowry procedure.[20] Then, appropriate volumes of the LDL solution and PBS were pipetted into a set of quartz cuvettes, to have a final concentration of 0.1 mg of LDL protein/mL and the oxidation process was started with the addition of $CuCl_2$ (2.4 μM). The temperature was kept at 37°C during the whole process. Absorbance was read at 234 nm in a Beckman DU-640 spectrophotometer, every 10 min for a maximum of 8 h or until the rapid phase of LDL oxidation reached a plateau. The lag phase was estimated as the incubation time corresponding to the intersection of two lines drawn from the changes in optical density, one through the initial, slowly rising curve which corresponds to the utilization of endogenous antioxidants in the LDL and the other, a subsequent, rapidly rising curve which corresponds to the LDL oxidation following the exhaustion of endogenous antioxidants.[19] The lag phase is expressed as minutes from the addition of $CuCl_2$. Two other parameters, related to the degree of oxidation were also tested: 1, mobility on agarose gel electrophoresis and 2, the thiobarbituric acid reacting substances (TBARS) which were measured using malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane as a standard.[21]

Experimental Conditions. To determine whether, AA, DHA or flavonoids, could either prevent

LDL oxidation or to stop the ongoing oxidation once the process is started, LDL were incubated under the conditions described above and either 5 μg/ml of AA, DHA or the flavonoid rich extract were added at different times of incubation (final concentration 28 μM for AA and DHA). The formation of conjugated dienes was followed. The concentrations of AA, DHA and flavonoids used in these experiments did not induce changes in OD at 234 nm, therefore there was no interference with the determination of conjugated dienes. In parallel experiments, LDL was incubated under the same conditions and butylated hydroxytoluene (BHT) and EDTA (final concentration 0.1 mM) were added to aliquots obtained at different times to stop the ongoing process. These aliquots were used to test LDL alpha-tocopherol concentration, electrophoretic mobility and TBARS formation. Alpha-Tocopherol was determined by HPLC following the method previously described.[9,22]

Statistical Analysis. The mean ± S.E.M. are given. The significance of the difference between the means of two groups was obtained using the paired t test.

RESULTS

Effects of AA, DHA and Flavonoids on LDL Oxidation

To evaluate the antioxidant effects of these three compounds on the LDL oxidation process, oxidation was started with $CuCl_2$ and at different times of incubation either AA, DHA or the flavonoid extract were added into the media. When any of the three antioxidants was added at the beginning of the oxidation process (time 0 of incubation) all of them delayed the formation of conjugated dienes (Fig. 1, panel A to C), decreased the LDL electrophoretic mobility (Fig. 2, panel A to C) and reduced the TBARS production (Table I), indicating that, when initially present in the medium, any of these compounds delayed the LDL oxidation.

Different results were found when these antioxidants were added at different incubation times, after the oxidation process was initiated. The addition of either DHA or the flavonoid extract 10 min after the addition of $CuCl_2$ delayed the formation of conjugated dienes (Fig. 1, panel B and C), and decreased both the LDL electrophoretic mobility (Fig. 2, panel B and C) and the TBARS production (Table I). However, when DHA or the flavonoid extract were added after 30 min, they accelerated the oxidation process as shown by a faster rate in the formation of conjugated dienes (Fig. 1, panel B and C), an enhanced LDL electrophoretic mobility (Fig. 2, panel B and C) and an increased TBARS production (Table I). Unlike results with DHA or the flavonoid extract, AA added at 0, 10, 30 and at 60 min after the initiation of the oxidation process, decreased the rate of formation of conjugated dienes (Fig. 1, panel A), the electrophoretic mobility (Fig. 2, panel A) and the TBARS production (Table I). When AA was added during the LDL oxidation propagation phase (90 or 110 min after initiating the oxidation process) it slightly increased the formation of conjugated dienes (Fig. 1, panel A) and clearly increased both the electrophoretic mobility (Fig. 2, panel A) and the TBARS production(Table I), indicating that it accelerated the oxidation process.

Because of the different timing between the antioxidant and prooxidant effects of DHA, flavonoids and AA, it was of interest to determine whether these differences were due to the ability of these compounds to regenerate alpha-tocopherol. Since it was previously found that the flavonoid extract is unable to regenerate alpha-tocopherol,[9] in the present manuscript only AA and DHA were tested. In Figure 3 (panel A and B), it is shown that neither AA nor DHA were able to regenerate alpha-tocopherol when added to LDL solutions at different times of the oxidation process, although both of them delayed the rate of alpha-tocopherol consumption. Similar

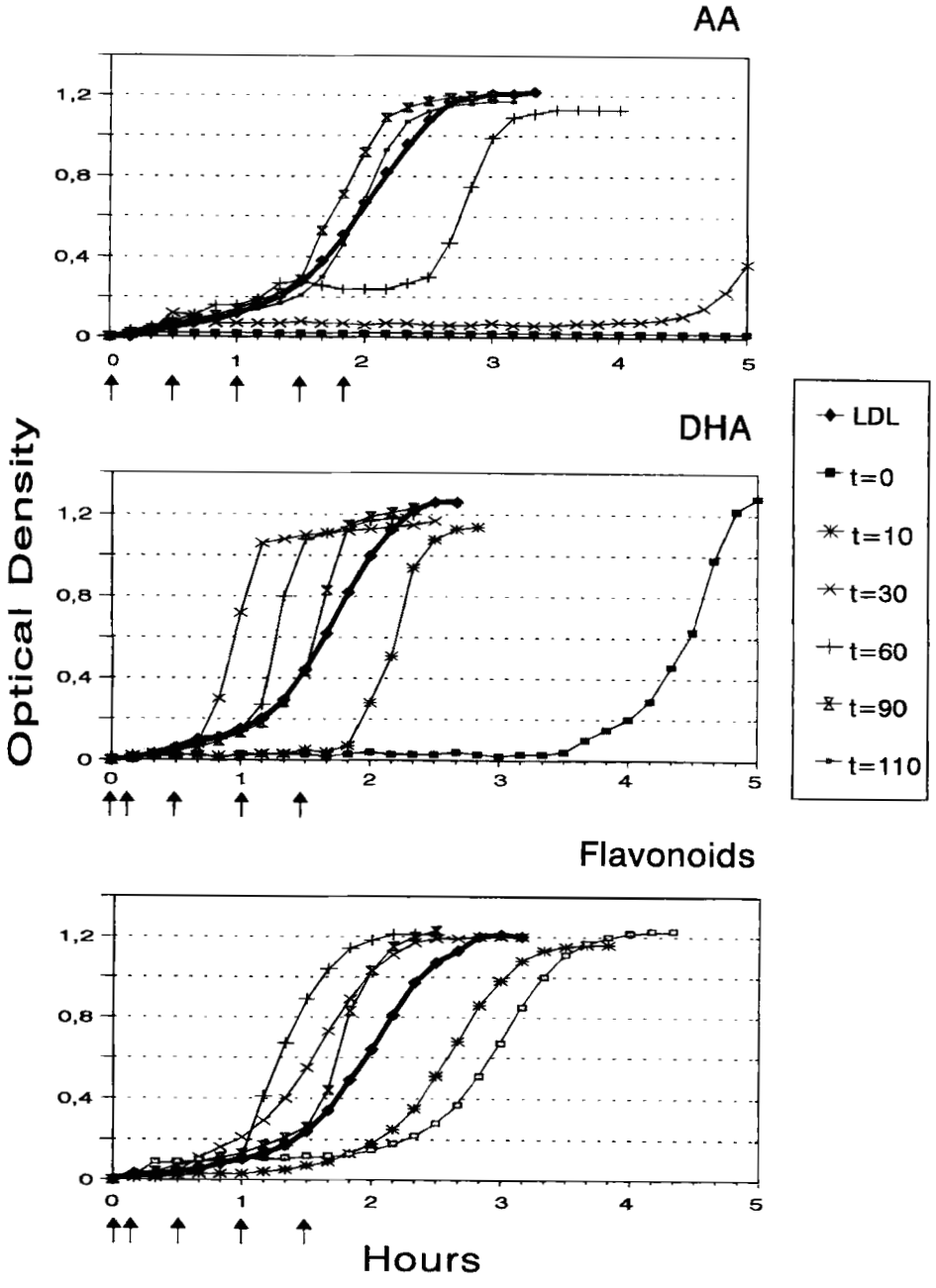

FIGURE 1 Effects of AA, DHA and flavonoids on LDL submitted to different degrees of oxidation. Oxidation was induced by the addition of $CuCl_2$ (2 μM) as described in the methods section and the formation of conjugated dienes was determined by changes in the light absorbance at 234 nm every 10 min. In one sample the formation of conjugated dienes was followed without any antioxidant addition, in the remaining samples, before (t = 0) or at different times after the initiation of the oxidation process 5 μg/mL of either AA, DHA and flavonoids (panel C) were added into the media. Notice that DHA and flavonoids were added at t = 10, 30, 60 and 90 min and because of its longer effect as antioxidant, AA was added at t = 30, 60, 90 and 110 min. These representative experiments were replicated with LDL from 6 different donors, with similar results.

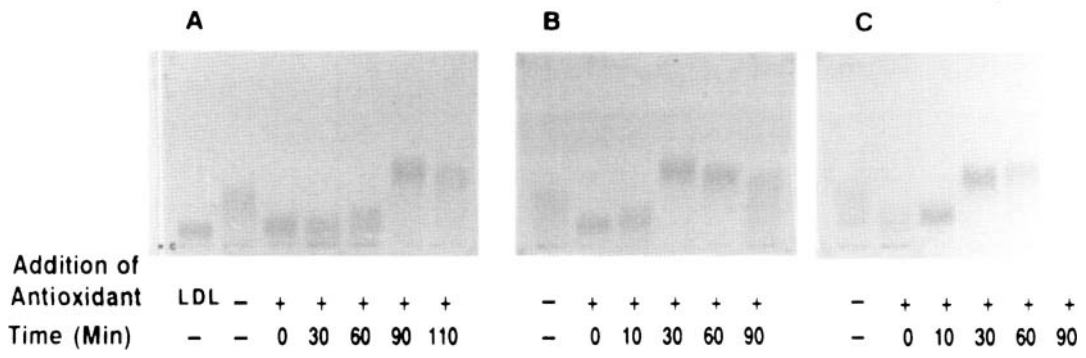

FIGURE 2 Effects of AA, DHA and flavonoids on LDL submitted to different degrees of oxidation. LDL oxidation was induced as described in figure 1 and 5 μg/mL of either AA (panel A), DHA (panel B) or flavonoids (panel C) were added at different times. In this experiment LDL represents the electrophoretic mobility of non-oxidized LDL. At 140 min the reaction was stopped by the addition of BHT and EDTA (final concentration 0.1 mM). The results from this representative experiment were reproduced with LDL from six different donors.

result have been previously described for the flavonoid extract.[9]

DISCUSSION

The present results show that, in vitro, AA, DHA and flavonoids can act as both antioxidants and prooxidants in the LDL oxidation process, depending on the degree of oxidation at the time when they are added to the system. In the early stages of the LDL oxidation process, when alpha-tocopherol was still present, AA, DHA and flavonoids delayed the oxidation process, clearly acting as antioxidants. However, when these compounds were added to LDL submitted to more prolonged period of oxidation, major differences were observed between them. When added after 30 min since the addition of $CuCl_2$ and alpha-tocopherol was completely consumed, both DHA and flavonoids accelerated the oxidation process. Whereas other authors have found that DHA only acts as an LDL prooxidant when added at the same time as $CuCl_2$,[23] present results show that DHA only acts as prooxidant, when LDL is oxidized to a minimal degree, at a time when there are no changes in conjugated dienes formation, but vitamin E is already consumed. The same effect is shown by the flavonoid extract, and present findings agree

TABLE I Effects of AA, DHA and flavonoids on TBARS formation (nmol of MDA/0.1 mg of LDL protein) in LDL submitted to different degrees of oxidation

TIME OF ANTIOXIDANT ADDITION (MIN)		0	30	60	90
	LDL without Antioxidant				
AA	4.41 ± 0.59	0.86 ± 0.24 ***	2.27 ± 0.72 *	4.65 ± 0.90	7.03 ± 0.63 ***
DHA	5.10 ± 1.42	1.62 ± 0.70	9.06 ± 1.37 **	9.57 ± 1.75 *	—
FLAVONOIDS	3.48 ± 0.45	2.15 ± 0.26	5.67 ± 0.41 **	5.59 ± 0.69 *	—

The LDL were submitted to oxidation as described in the methods section, the antioxidants were added at different times (0 to 90 min) and after 130 min the TBARS concentration was determined. Mean ± S.E.M. of four different experiments are shown. *:LDL incubated without v.s. with antioxidant. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

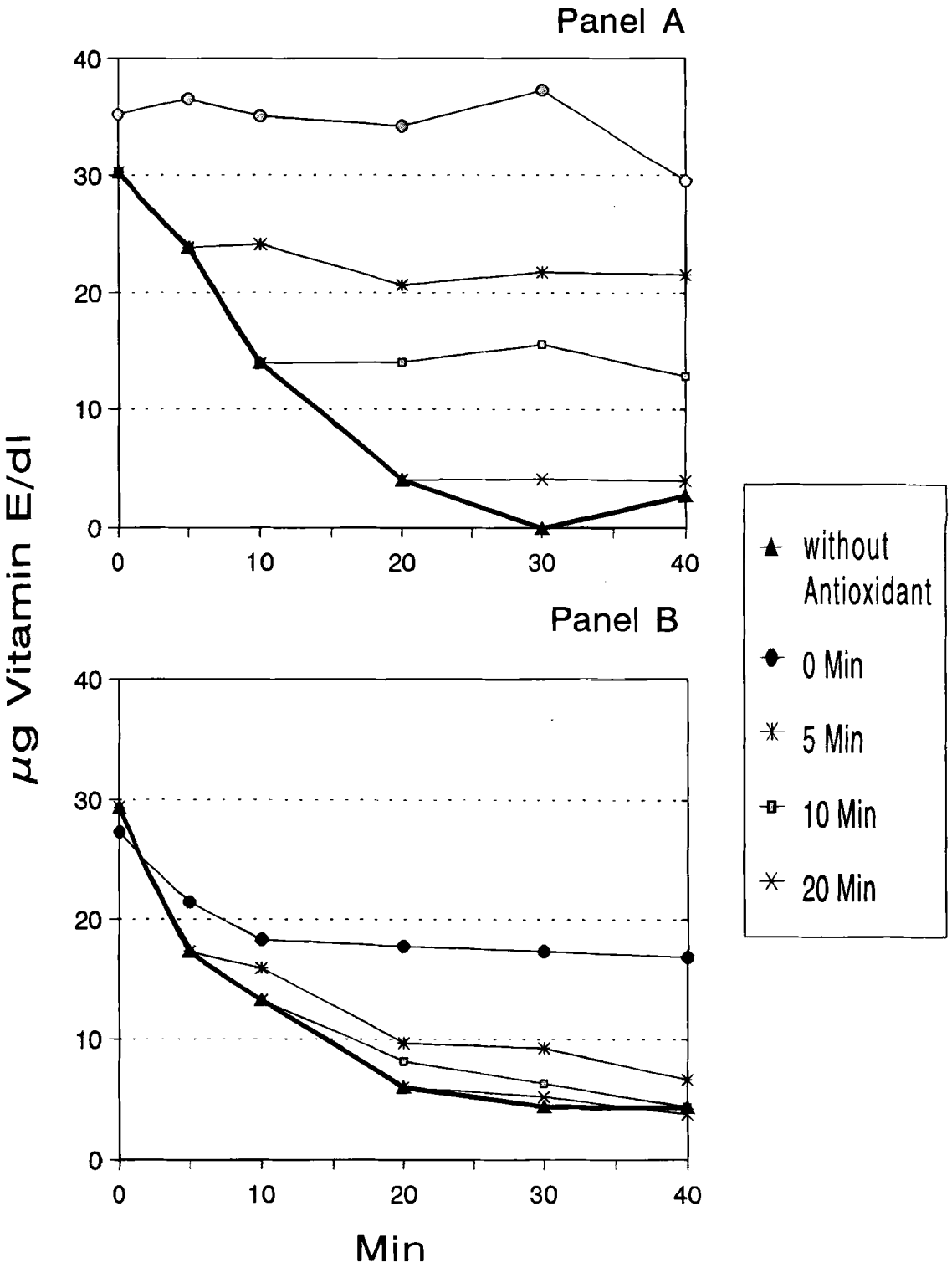

FIGURE 3 To determine if AA and DHA could arrest vitamin E consumption once the LDL oxidation was initiated, LDL was submitted to oxidation with $CuCl_2$ as indicated in Fig. 1. At 0, 5, 10 and 20 min either AA (panel A) or DHA (panel B) were added. Aliquots were obtained at different times, as described in the methods section and alpha-tocopherol measured. This representative experiment was reproduced with LDL from two different donors with similar results.

with other authors showing that flavonoids act as prooxidant on LDL minimally oxidized.[24] In contrast, AA delayed the oxidation process when added up to 60 min after the addition of $CuCl_2$, when alpha-tocopherol was completely consumed, and the oxidation phase was in the early stages of the propagation period. These results could explain contradictory results reported by other authors showing that AA act as an antioxidant on LDL minimally oxidized,[10] whereas others found that AA was prooxidant.[14] Our findings, demonstrate that depending on the degree of LDL oxidation, AA may act as anti or prooxidant.

Several mechanisms have been suggested to explain the antioxidant effect of AA on LDL submitted to oxidation by $CuCl_2$. Although, it has been shown that AA could regenerate the oxidized alpha-tocopherol[25] decreasing the rate of LDL oxidation, in our experimental model, AA was unable to raise the alpha-tocopherol concentration, although it decreased its consumption when added to oxidizing LDL. Since AA can scavenge water soluble free radicals[13] it is proposed that it can also scavenger the free radicals generated in the surface of the LDL particle, therefore preventing the oxidation of the LDL lipid core. In contrast, in more advanced phases of the LDL oxidation, the lipid core may be already oxidizing and the free radicals generated may not be neutralized by the AA. Since it has been previously shown that AA can bind Cu[8,10] this mechanism could be also interfering with the propagation of the oxidation process.

What is more relevant from the present results is the fact that in contrast with DHA and flavonoids, AA is able to decrease the oxidation rate of LDL when alpha-tocopherol is consumed, indicating that other mechanism not related to alpha-tocopherol must be involved in the antioxidant effects of AA on oxidized LDL. This antioxidant effect of AA clearly differs from the effects of either DHA or flavonoids, which are only able to decrease the LDL oxidation in the early phases of the process, when alpha-tocopherol is still present, and their respective effects could be related to their capacity to arrest the alpha-tocopherol disappearance.

However, we do not know why the three compounds studied act as prooxidants on LDL submitted to different degrees of oxidation. Since it is known that under certain circumstances[12,13,26,27] antioxidants may promote both reduction and oxidation, it is possible, that during the process of LDL oxidation, in the presence of any of the compounds studied, products that accelerate the LDL oxidation may be generated. These compounds do not seem to be the same for AA, DHA or flavonoids since their activity as prooxidants starts in different stages of the LDL oxidation process, being AA the last one to start acting as prooxidant.

Although the biological relevance of the present results remains to be established, they suggest that when minimally oxidized LDL are present in plasma or in the atherosclerotic plaque, AA, DHA or flavonoids could accelerate the LDL oxidation process, therefore promoting damage rather than preventing it, especially if small amounts of transition metal ions are present in the arterial wall, as has been shown.[28]

Acknowledgments

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