Specific Antioxidant Activity of Caffeoyl Derivatives and Other Natural Phenolic Compounds: LDL Protection against Oxidation and Decrease in the Proinflammatory Lysophosphatidylcholine Production

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Specific antioxidant activity (SAA) (i.e., activity related to the molar or gallic acid equivalent amount of antioxidant) of natural polyphenolic mixtures or pure phenolic compounds was studied using their capacity to delay the conjugated diene production brought about by in vitro LDL copper-mediated or AAPH-mediated oxidation. The cinnamic acid series (caffeic, sinapic, ferulic acids) displayed a constant SAA over a large range of concentrations, whereas the benzoic acid series (gallic and protocatechuic acids) showed much higher SAA at low concentrations. The natural phenolic mixtures had a constant SAA. The highest SAA was obtained with caffeoyl esters (caffeoylquinic, rosmarinic, and caffeoyltartaric acids) and catechin for the copper-oxidation and the AAPH-oxidation system, respectively. Phenolic mixtures and acids delayed vitamin E depletion and decreased proinflammatory lysophosphatidylcholine production. As with polyphenols, probucol delayed lysophosphatidylcholine and conjugated dienes production, at higher concentrations, but was not effective at preventing vitamin E depletion. Polyphenols prevent the oxidation of LDL and its constituents (vitamin E, phosphatidylcholine), which is compatible with an antiinflammatory and antiatherosclerotic role in pathophysiological conditions.

Oxidized low-density lipoprotein (ox-LDL) plays a key role in atherogenesis. This type of lipoparticle is known to be cytotoxic to endothelial cells and chemotactic for monocytes, to decrease the mobility of macrophages, and to promote platelet aggregation and the adhesiveness of endothelial cells toward monocytes.[1,2] An antioxidant such as vitamin E can reduce in vitro ox-LDL formation.[3] It is also able to impair the macrophage or monocyte superoxide anion production,[4,5] generally considered to be partly responsible for LDL oxidation.[6]

It has clearly been shown that populations with a high saturated fat intake exhibit an elevated cholesterolemia and a higher risk of coronary heart disease (CHD) mortality.[7] However, these observations are not in accordance with those made in some other countries. Indeed, the French population presented a relatively high saturated fat intake and a rather high cholesterolemia, but a lower risk of CHD.[8] One possible, but currently debated, explanation is a higher consumption of polyphenols in France as compared to most Northern countries. Several epidemiological studies have shown a negative correlation between polyphenol consumption and the frequency of CHD.[9,10] More specifically, the red wine phenolic compounds have been shown to inhibit in vitro oxidation of human LDL.[11,12] We recently established their in vivo potency to increase both the plasma antioxidant capacity and the LDL vitamin E protection.[13]

LDL phospholipase A_2-mediated lysophosphatidylcholine (lysoPCho) production is plausibly another consequence of the LDL oxidation process.[14] Interestingly, lysoPCho is an important proinflammatory and atherogenic agent. In particular, it triggers the expression of the endothelial chemotactic proteins leading to monocyte recruitment,[15] inhibits the endothelium-dependent vascular relaxation,[16,17] promotes growth of the vascular smooth muscle cells,[18,19] and promotes or potentiates superoxide production by the vascular wall[20] and phagocytes,[21,22] respectively.

Taken together, these results indicate that antioxidant-rich diets may delay the development of atherosclerotic lesions by at least two different, but related ways, i.e., via their antioxidant properties and the decrease in the lysoPCho production.

The aim of this study was to compare several natural polyphenolic compounds on the basis of their capacity to protect LDL against oxidative modifications generated by two different oxidation systems in order (a) to define a molar antioxidant efficiency (a specific antioxidant activity, or SAA) for each compound and (b) to study the consequences of their antioxidant properties upon the lysoPCho production in LDL. Obviously, the findings are not expected to be directly representative of biological effects because in vivo interactions of these compounds with intestinal microflora and systemic (for example hepatic) metabolic reactions are not taken into account. However, in vitro screening of their action on a biological structure that plays an important role in atherogenesis is thought to be of great interest for the (re)orientation of future intervention studies in humans.

Results and Discussion

The SAAs of the natural polyphenol mixtures prepared from olive oil wastewaters (OOWW), red wine (RW), and green coffee beans (arabica and robusta GCB) are shown in Table 1. SAA assessed via Cu^{2+}-mediated LDL oxidation was unaffected by the concentration of the mixtures. The antioxidant efficiencies of OOWW and RW polyphenol compounds did not differ, whereas those of the GCB mixtures were higher. Under the same conditions, vitamin E—the main natural antioxidant of LDL —and probucol—a hypocholesterolemic and antioxidant drug—both residing inside the particle, were as efficient as RW or OOWW polyphenols, whose property generally recognized is located at the oil/water interface of and loosely associated with

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Table 1. Specific Antioxidant Activity (SAA) of Natural Polyphenolic Mixture, Vitamin E and Probucol

	SAA[a] (μGAE^{-1} L or μmol^{-1} L)	r^b	n	P^c
OOWW	7.2(1.5)*	0.96	9	<0.01
RWPC	7.7 (1.3)*	0.98	7	<0.01
arabica GCB	30.2 (4.0)**	0.98	9	<0.01
robusta GCB	25.0 (4.0)**	0.98	9	<0.01
vitamin E	7.2 (0.7)*	0.98	15	<0.01
probucol	5.8 (1.6)*	0.82	18	<0.01

[a] Slope (confidence intervel at 95%). Values with different numbers of asterisks are significantly different ($P \leq 0.05$). [b] Correlation coefficient of the linear equation $rT_{lag[A]} = SAA[A] + 100$. [c] Level of significance (Fisher test).

LDL.[23] Interestingly, these results are not in complete accordance with others, in which the antioxidant activity of vitamin E, as measured by the similar LDL oxidation test, was reported to be considerably lower than that of phenolics present in olive oil[24] and in wine.[25] However, these results should be considered carefully. Vitamin E is inside and polyphenols are outside (see discussion below) the LDL particle.[13,23] Vitamin E prevents the lipoperoxidation chain reactions taking place inside the particle, whereas polyphenols regenerate the chromanol form from the chromanoxyl form of oxidized vitamin E at the oil/water interface, leading to the situation in which the antioxidant action of polyphenols is in fact mediated by vitamin E.[26] To some extent and from a strictly mechanistic viewpoint, such comparisons between vitamin E and polyphenols appear to be inconsistent.

The SAAs of purified (epi)catechin, some phenolic acids, and caffeoyl-esterified derivatives (rosmarinic, caffeoyltartaric, and caffeoylquinic acids) are presented in Table 2. The SAAs of cinnamic acids (*p*-coumaric, ferulic, sinapic, and caffeic acids) and caffeoyl derivatives were shown to be independent of their concentration range, as the mixtures were, which supports the notion of a linear relationship between lag phase and these polyphenols. This was true for the Cu^{2+}- and AAPH-mediated oxidation systems in the case of caffeic acid, caffeoylquinic acid, and catechin, for which both oxidation processes were carried out. Interestingly, when the Cu^{2+}-mediated oxidation was used, maximal values of SAA were obtained for the caffeoyl derivatives, whereas intermediary and low values of SAA were obtained for cinnamic acids and (epi)catechin, on one hand, and ferulic and *p*-coumaric acids, on the other, respectively. By contrast, when the AAPH-mediated oxidation was used, the highest value of SAA was obtained for catechin and lower—but not statistically different—values were found for caffeic and caffeoylquinic acids.

How can these discrepancies be explained? It is likely that differences in terms of polyphenol hydrophobic properties are not in question because we have already shown that phenolic acids (caffeic acid, sinapic acid, ferulic acid) and RW polyphenols are easily dissociated from LDL during dialysis, leading to the conclusion that they behave as hydrophilic substances (see ref 23 and Figure 1 for caffeic and gallic acids and RW polyphenols). This caffeic acid property could be, to some extent, reinforced by the quinic acid residue—a highly hydrophilic residue—in the caffeoylquinic acid mixture of GCB. Regarding the OOWW, it has been reported that some olive oil antioxidant polyphenolic compounds could be incorporated into the LDL.[24] However, it is difficult to assume that polyphenols from olive oil and OOWW are similar. It is well known that hydroxytyrosol is one of the major polyphenols in OOWW, whereas it is a trace element in olive oil.[27,28] Expectedly, this results from the olive oil process in which an initial

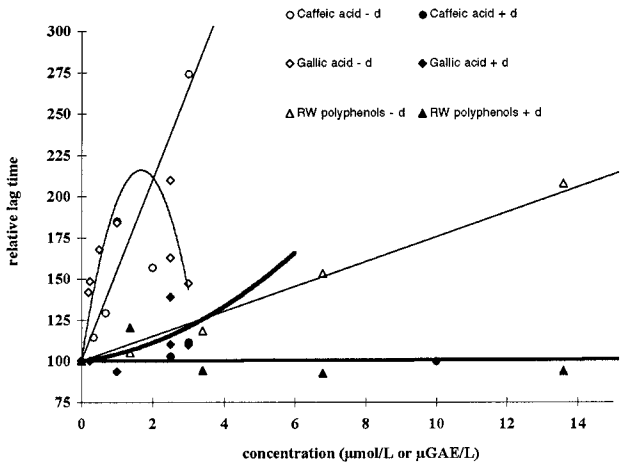


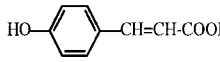
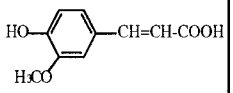
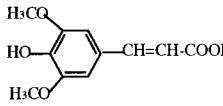
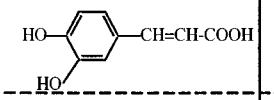
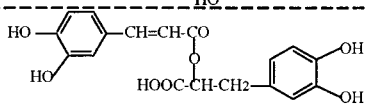
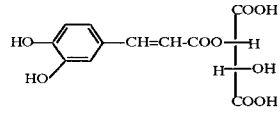
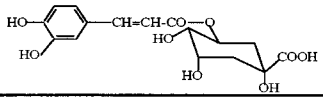
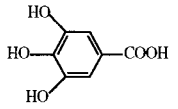
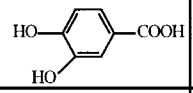
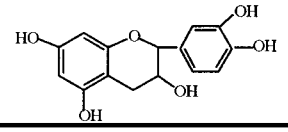
Figure 1. Effect of extensive dialysis on the LDL resistance to in vitro Cu^{2+}-oxidation. LDL (1 μmol apoB/L) was preincubated without or with phenolic compounds for 1 h at 37 °C under N_2, extensively dialyzed (solid symbols: caffeic and gallic acids and RW polyphenols, $R^2 = 0$, 0.43, and 0, respectively) or not (open symbols: caffeic and gallic acids and RW polyphenols, $R^2 = 0.41$, 0.85, and 0.97, respectively) and then diluted at 0.1 μmol apoB/L in PBS buffer for the in vitro Cu^{2+} (5 μmol/L)-oxidation. Results are presented as the relationship between the relative lag time and the phenolic concentrations expressed as μmol/L or μGAE/L.

biphasic (oil/vegetation waters) polyphenol partitioning takes place according to the hydrophilic/hydrophobic balance: the most hydrophilic molecule being driven to the aqueous phase.

It could reasonably be assumed that the aforementioned discrepancies are partly due to transition metal-related properties of polyphenols. Indeed, in the studies reported herein both copper-dependent and copper-independent oxidation processes were examined. Whereas there were no different SAA values for caffeic acid—which is not in total agreement with others[29]—the SAA of caffeoylquinic acid was about three times higher in the copper-dependent as compared to the copper-independent system, which strongly suggests interactions of copper (or copper-linked processes) with caffeoylquinic acid. This is not due to the quinic acid moiety since no modification of LDL oxidation takes place with the copper oxidation system in the presence of quinic acid (not shown). The relative concentration Cu^{2+}/LDL used in the present study conforms to the case in which a low-affinity binding site is involved in LDL, leading to the reduced form Cu^{+}, which in turn leads to lipid peroxide formation.[30] Therefore, one possibility is that the peculiar conformation of caffeoylquinic acid is able to specifically interact with this hypothetical binding site, impairing the peroxidation reactions.

By contrast, catechin displayed a much higher SAA with the metal-independent oxidation system than with the metal-dependent one. AAPH, known to produce alkoxyl/peroxyl radicals in the presence of oxygen in the aqueous phase, initiates the lipid peroxidation, which can be impaired in the presence of substrates that efficiently scavenge these radicals in the lipid phase or earlier in the aqueous phase. The balance between the two means of antioxidant protection—interacting with the prooxidant transition metal and scavenging the peroxyl radicals—is poorly documented for (epi)catechin. However, data on flavonoids other than flavanols are more abundant[31−33] and generally in favor of efficient copper-chelating properties. We presently show that (epi)catechin is likely an efficient free-radical scavenger and a less efficient copper-chelator, supporting the essential role of the 3′,4′-*ortho*-dihydroxy structure in scavenging free radicals as compared with the

Table 2. SAA of Phenolic Acids and Flavonoids of Natural Poyphenolic Mixtures[a]

	Chemical formula	Cu^{2+}-oxidation SAA (μmol^{-1}L)*	n	r (P)*	AAPH-oxidation SAA (μmol^{-1}L)*	n	r (P)*
Cinnamic acid series							
p-coumaric acid		0.6 (0.3)[a]	20	0.68 (< 0.01)			
ferulic acid		8.5 (3.5)[a]	20	0.75 (< 0.01)			
sinapic acid		47.4 (13.3)[c]	14	0.9 (< 0.01)			
caffeic acid		54.0 (19.1)[c,e]	24	0.76 (< 0.01)	73.6 (5.2)[e]	8	0.99 (<0.01)
rosmarinic acid		149.7 (16.8)[d]	14	0.98 (< 0.01)			
caffeoyltartaric acid		125.3 (18.3)[d]	9	0.98 (< 0.01)			
caffeoylquinic acid		155.5 (12.1)[d]	8	0.99 (< 0.01)	55.3 (25.2)[e]	12	0.98 (< 0.01)
Benzoic acid series							
gallic acid		750 for 0.2 μmol/L 100 for 1 μmol/L			90 for 0.2 μmol/L 30 for 1 μmol/L		
protocatechuic acid		1000 for 0.2 μmol/L 100 for 1 μmol/L					
Flavanols							
catechin		34.6 (8.4)[c]	15	0.91 (< 0.01)	156.2 (19.2)[f]	10	0.99 (< 0.01)
epicatechin		37.1 (7.7)[c]	10	0.96 (< 0.01)			

[a] * = slope (confidence interval at 95%). Values with different letters are significantly different ($P \leq 0.05$).

2,3-double bond and the 4-oxo group, which flavanols lack. On the other hand, it is worthwhile mentioning that catechin and epicatechin SAA values were similar.

In the case of Cu^{2+}-mediated oxidation, rosmarinic and caffeoyltartaric acids were found to have similar SAA values, which in turn appeared to be the same as that of caffeoylquinic acid. This suggests that these caffeic acid derivatives interact with Cu^{2+} or the Cu^{2+}-binding site of LDL, as caffeoylquinic acid does.

In contrast with the cinnamic acid series, the gallic and protocatechuic acids belonging to the benzoic acid series displayed increasing SAA values when their concentration decreased (Figure 2 and Table 2), this being true with the Cu^{2+}- and AAPH-mediated oxidation processes (Table 2). Overall, gallic acid SAA was higher with Cu^{2+}- than with AAPH-mediated oxidation, suggesting that gallic acid acts in a way similar to that of caffeoyl derivatives. Likewise, within the high concentration range, gallic and protocatechuic acids exhibited SAA values comparable to those of the caffeoyl derivatives. On the other hand, that protocatechuic and gallic acids were found to have a very high SAA within the concentration range of 0.01−0.1 μmol/L has to be underscored. The SAA values were 5−10 times higher than those of the other most efficient polyphenols.

To verify whether other benzoic acids shared the properties found for protocatechuic and gallic acids, we tested salicylic and acetylsalicylic acids in the copper-dependent system. These acids displayed no antioxidant effect in these conditions. The results shown in Figure 1 could lead to a better understanding of the type of action underlying the peculiar properties of gallic acid. Indeed, by contrast to the aforementioned behavior of polyphenols during dialysis, gallic acid was not completely lost after dialysis, showing that this phenolic acid interacts more strongly with LDL. It could be proposed that the Cu^{2+}-binding site of LDL has a particular affinity for this type of polyhydroxylated molecule, implying weak but cooperative interactions of a different nature (namely, hydrophobic forces and hydrogen bonding). In line with this hypothesis, a polyhydroxylated molecule such as quinic acid, which has no interactions with this site on its own right, could, however, participate in these interactions by virtue of its vicinity when it is covalently linked to caffeic acid in the caffeoylquinic acid, thus explaining its much higher SAA than that of caffeic acid.

It is worthwhile to compare the concentrations used in the present study with those in the plasma of subjects receiving a normal diet in order to consider the physiological significance of the present results. Taking into account the apoB concentration (0.1 μmol/L) and the phenolic compound concentration range generally used (1−30 μmol/L) in in vitro tests, it might be assumed that the physiological counterpart of these conditions is a phenolic acid plasma concentration range of 10−300 μmol/L, which

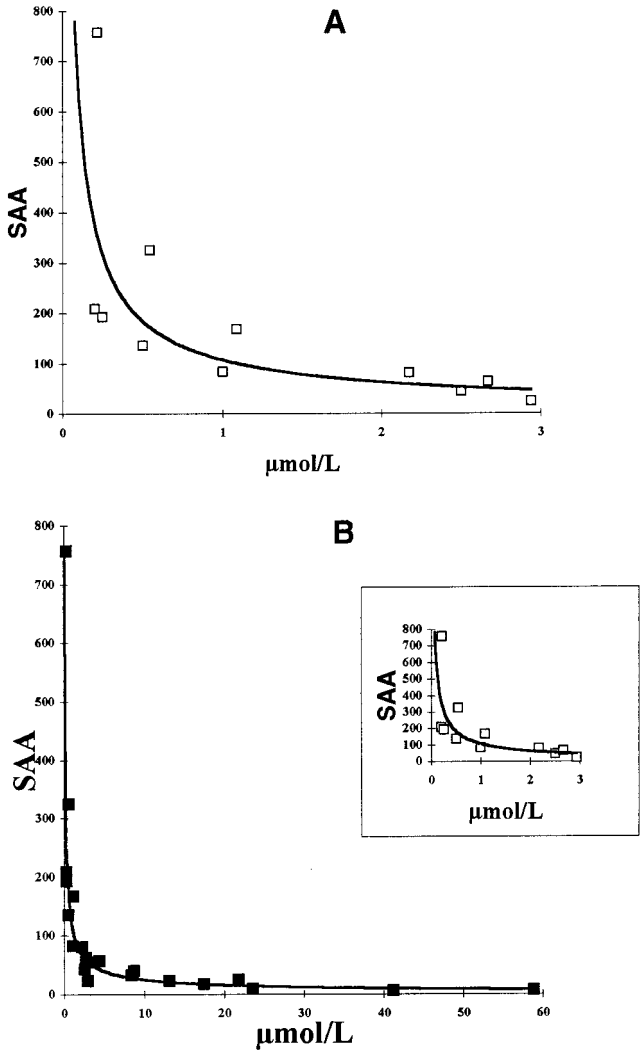


Figure 2. Change in the specific antioxidant activity (SAA) of benzoic acids: (A) protocatechuic acid and (B) gallic acid with regard to their concentrations in the medium (conditions of preincubation and oxidation are those of Figure 1). The inset represents an expanded scale for gallic acid concentrations.

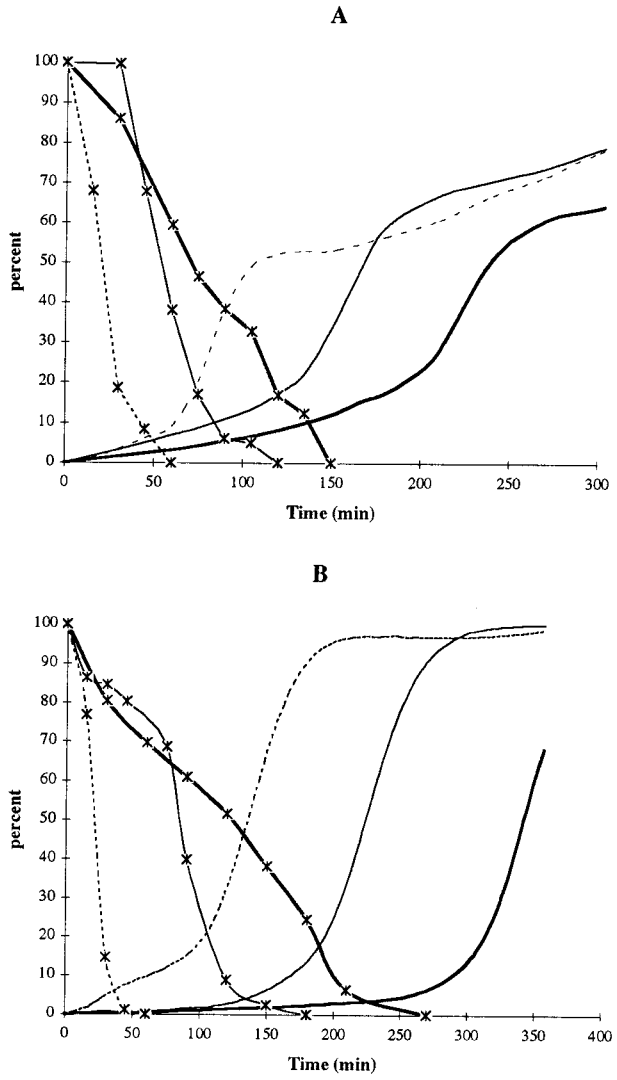


Figure 3. Time course of CD production and vitamin E consumption during in vitro Cu^{2+}-oxidation of LDL preincabated without or with different phenolic compounds (conditions of preincubation and oxidation are those of Figure 1). (A**)** RW (dashed line 0 μGAE/L, thin line 3.7 μGAE/L, and bold line 7.3 μGAE/L); (B) caffeic acid (dashed line 0 μmol/L, thin line 1.6 μmol/L, and bold line 3.1 μmol/L). Results are presented as percentage of the maximal CD production. The vitamin E time course (−×−) was followed up during Cu^{2+}-oxidation by collecting 200 μL of LDL (0.1 μmol/L) every 15 min. Results are presented as percentage of the initial vitamin E/apoB molar ratio.

appears to be 100−10 000 times as high as the concentrations found in the plasma.[34] In this context, benzoic acids are of particular interest because they appear to be active at submicromolar concentrations, which prevail in postprandial plasmas (unpublished data). Therefore, it is likely that some phenolic acids play a role in protecting LDL oxidation in vivo. Since protocatechuic acid has been reported to be released during the oxidative attack of anthocyanins by a peroxyl radical,[35] present findings reinforce the hypothesis of an antioxidant role of anthocyanins, which are abundant in red wine, as precursors of a specifically efficient antioxidant.

The present data confirm other results[25] on the antioxidant efficiency of structures with two adjacent OH substituents on the benzene ring (the *ortho*-diphenol array). This is clear, for example, when the SAA of *p*-coumaric acid is compared with that of caffeic acid. Nevertheless, additional information has been collected on more complex molecules. Although the rosmarinic acid molecule contains two *o*-dihydroxybenzene rings—equivalent to two molecules of caffeic acid—its SAA for Cu^{2+}-mediated oxidation was more than double that of caffeic acid. This suggests that the entire molecule plays a role in the antioxidant action and that a specific positioning of each *o*-diphenol functionality could occur after its interaction at the oil/water interface of the lipoprotein particle. Cooperative interfacial interactions could also be involved as previously discussed for polyhydroxylated molecules. On the other hand, the methoxy groups efficiently increase, in a manner dependent on the number of groups, the antioxidant properties. This is evident from a comparison of ferulic and sinapic acids; the mono- and dimethoxylated forms of *p*-coumaric acid, respectively, are compared to *p*-coumaric acid (Table 2). In contrast, the *o*-methylation of one or two hydroxy group(s) in caffeic and gallic acids (leading to ferulic and sinapic acids, respectively) considerably decreased the antioxidant properties, as was also previously demonstrated[31] in the case of flavonoids. The length of the side chain of phenolic acids appears also to play an important role, as highlighted by comparing cinnamic to benzoic acids series. It is clear that the structure−activity relationship of phenolic acids and their derivatives needs to be more thoroughly studied.

Figures 3 and 4 show the influence of caffeic acid or RW polyphenols on the time course of LDL vitamin E consumption and lysoPCho production, in parallel with the CD

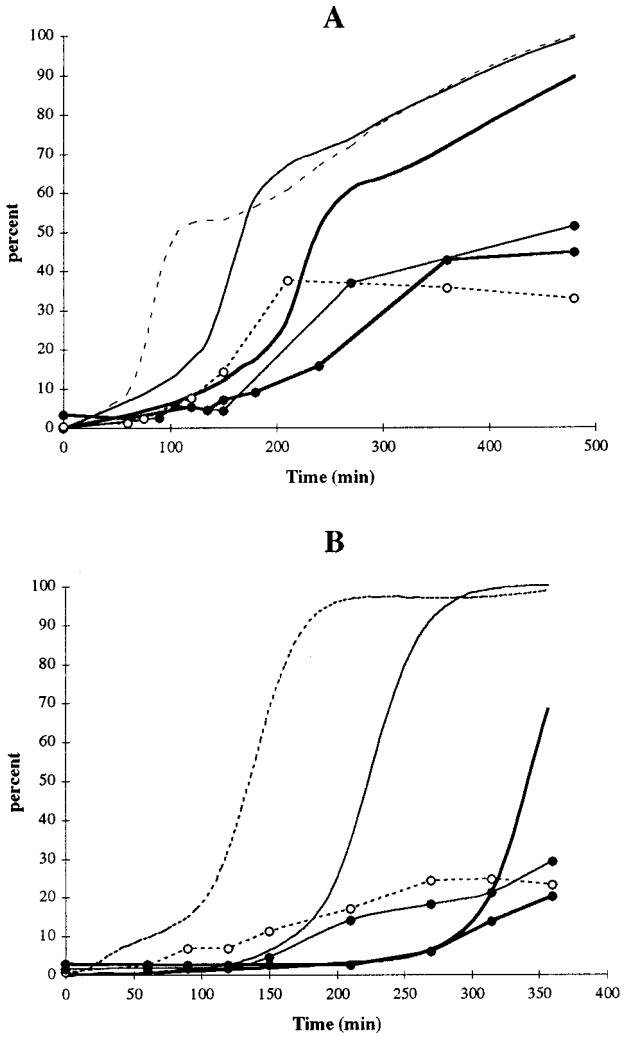


Figure 4. Influence of polyphenols on lysoPCho production during in vitro LDL Cu^{2+}-oxidation with different polyphenols and comparison with CD production (conditions of preincubation and oxidation are those of Figure 1). (A**)** In the presence of RW (dashed line 0 μGAE/L, thin line 3.7 μGAE/L, and bold line 7.3 μGAE/L; (B) in the presence of caffeic acid (dashed line 0 μmol/L, thin line 1.6 μmol/L, and bold line 3.1 μmol/L. The lysoPCho production without (−○−) or with an antioxidant (−●−) was followed up by collecting 2 mL of LDL (0.1 μmol/L) every hour. Results are presented as percentage of the lysoPCho/(lysoPCho + Pcho) molar ratios.

production. These two types of phenolic compounds were chosen only because they were representative of the types of polyphenols under study. We found that vitamin E consumption and lysoPCho production, as well as CD production, were delayed in a dose-dependent manner, regardless of the nature of the phenolic compound. Interestingly, the very beginning of the propagation phase (i.e., the end of the inhibition period) of the CD production coincided with the time of the total consumption of vitamin E and with the starting time of the production of detectable lysoPCho. In fact, this means that both CD and lysoPCho productions started when LDL vitamin E was completely depleted. Similar results have been previously reported[36] in the case of oxidizing LDL by reactive oxygen species produced in macrophages. In addition, polyphenols were found to be consumed before vitamin E, vitamin C, and β-carotene, showing a direct protection of these compounds by polyphenols. Likewise, caffeoylquinic acid and caffeic acid were rapidly consumed during the initiation period of *cis*-parinaric acid peroxidation.[37] Accordingly, it is likely that in the situation shown in Figures 3 and 4 phenolic acids were consumed during the initiation phase of LDL oxidation, before the vitamin E decay, thus protecting vitamin E during the oxidation process, as has recently been suggested to occur in vivo after ingestion of RW polyphenols by human volunteers.[23]

The probucol effect during LDL Cu^{2+}-mediated oxidation upon vitamin E consumption and CD and lysoPCho production is shown in Table 3. Probucol delayed the CD and lysoPCho production in a dose-dependent manner. The time course of vitamin E consumption was not significantly modified by probucol. The consumption of probucol was much slower than that of vitamin E and largely independent of the probucol level at the oxidation starting time. Taken together, these results show for the first time that probucol does not protect vitamin E against oxidation in vitro, although (i) it is a potent free-radical scavenger in the same oxidation system,[38] (ii) it is as efficient as vitamin E in protecting LDL against oxidation,[39] and (iii) it resides in the LDL lipid phase as vitamin E does. This finding is consistent with the absence of protection of LDL vitamin E in patients consuming daily 0.9 g of probucol for more than 2 years.[40] This could result from an independent antioxidant effect of these lipophilic antioxidants attributable to different physical localizations inside the lipid phase.

LysoPCho formation has to be attributed to an LDL-specific PLA_2 (LDL-PLA_2) activity. The enzyme responsible for this activity has been thought to be an intrinsic LDL phospholipase,[41] then a platelet-activating factor acetyl hydrolase (PAF-AH).[42,43] Finally, PAF-AH and LDL-PLA_2 have been thought to be one and the same enzyme.[43] It has a similar apparent K_m for both its natural substrates, PAF and oxidized PCho.[44] Since polyphenols were found not to directly interfere with LDL-PLA_2 activity (the LDL-PLA_2 activity was not affected by polyphenols, results not shown) and since oxidative processes are known to be crucial for the cleavage of the PCho *sn*-2 ester bound by LDL-PLA_2/PAF-AH, the lysoPCho production-delaying effect is likely due to an antioxidant protection by polyphenols of the polyunsaturated fatty acids, which specifically occupy the *sn*-2 position of PCho. LysoPCho production is prevented by antioxidants,[41] including vitamin E. This is the first time this action is shown for polyphenols. Interestingly, probucol has a similar action.

It is worthwhile mentioning that the quantitative lysoPCho production we observed in our conditions appears to be similar to the level of lysoPCho effective in decreasing superoxide production by intact aorta and to the level found in human atherosclerotic lesions.[20] The polyphenol effect we have observed sheds new light on the potential pathophysiological role of this type of natural product. This action results first in a decreased formation of several types of oxidized PCho and a decreased formation of lysoPCho, all considered as inflammation/atherogenesis initiators.[20,45] As illustrated in Figure 5, this decreased formation may then lead to an increase in the platelet-activating-factor degrading activity[46] by an enzyme substrate-transfer (because of the similar K_m for PAF and oxidized PCho), and thus to the inactivation of PAF, one of the most potent inflammatory mediators. Both the antioxidant and the anti-lysoPCho actions of polyphenols may be beneficial for preventing inflammation and atherosclerosis. In addition, the prevention of LDL vitamin E depletion is considered to be a beneficial action, since it also prevents increased production of superoxide anion.[4] Further research on these multiple effects of polyphenols is needed, as this area of bioactivity remains largely unexplored.

Table 3. Production of Conjugated Dienes (CD) and Lysophosphatidylcholine (LysoPCho) and Consumption of Vitamin E (Vit E) and Probucol (Prob) during LDL Cu^{2+}-Oxidation with or without Probucol Added[a]

	probucol added (μmol/L)															
	0				5				10				20			
time (min)	C D[1]	Lyso PCho[2]	Vit E[3]	Prob[4]	C D[1]	Lyso PCho[2]	Vit E[3]	Prob[4]	C D[1]	Lyso PCho[2]	Vit E[3]	Prob[4]	C D[1]	Lyso PCho[2]	Vit E[3]	Prob[4]
0	0	2.7	100		0	2.3	100	100	0	0.3	100	100	0	0.8	100	100
15			41.9				45.0	96.0			51.8	86.6			38.2	97.0
30			18.2				15.5	94.8			17.8	82.0			19.9	91.8
45			4.9				7.5	113.9			11.0	73.0			7.5	87.7
60	11.5	4.1	0		4.1	2.5	0	117.9	0.6	0.7	0	34.1	1.3	1.2	5.5	51.7
90	69.7	7.8	0		37.9	4.1	0	55.3	4.0	0.0	0	27.2	0.7	1.1	0	44.1
120	77.9	13.3	0		78.6	10.6	0	56.5	39.4	1.3	0	37.2	0.2	1.0	0	43.2
150	76.8	27.2	0		78.3	17.5	0	28.6	77.2	3.0	0	19.8	1.1	1.3	0	41.2
180	78.6	37.2	0		79.5	19.4	0	45.3	77.3	6.2	0	31.2	2.6	1.5	0	34.4
210	81.1	37.4	0		80.8	23.1	0	36.8	76.3	9.1	0	21.6	2.6	1.4	0	38.3
270	88.4	48.5	0		87.1	26.1	0	22.5	79.9	12.9	0	15.8	2.6	1.5	0	34.6
360	100	53.8			100	29.1	-		100	19.2			2.3	1.9		

[a] Results are expressed in: [1]percent of the maximal CD production (CD/apoB = 396 mol/mol at 360 min); [2]percent of LysoPCho/LysoPCho + PCho (LysoPCho/apoB = 94.5 mol/mol at 360 min and without probucol); [3]percent of maximal LDL-Vit E (Vit E/apoB = 5.75 mol/mol at 0 min); [4]percent of the maximal probucol concentration at 0 min).

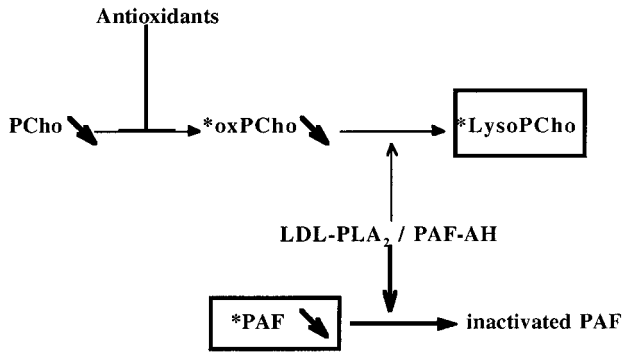


Figure 5. Possible implication of polyphenols as antioxidant agents leading to maintaining low levels of proinflammatory and thromboatherogenic substances (designated by an asterisk). The decrease in oxPCho due to the antioxidant effect results in a subtrate transfer of the LDL-PLA_2/PAF-AH from oxPCho to PAF.

Experimental Section

Chemicals. The red wine (RW) phenolic compounds were prepared and analyzed by INRA (Institut National de la Recherche Agronomique, Narbonne, France) from a Cabernet-Sauvignon grape variety. This preparation involved three steps: phenolic adsorption on an ADS-4 preparative column (a stationary phase from Applexion, Epone, France), alcoholic desorption (ethanol/water, 46:4, v/v), and eluent concentration by gentle evaporation. The concentrated residue was then sprayed to obtain a dry powder. The red wine produced 1.3 g/L containing 100 mg/g of total catechins *plus* proanthocyanidins (expressed as catechin, with only 1.0% of catechin and epicatechin), 64 mg/g of total flavonols with 85% of quercetin and quercetin-3-glucoside, and 8.7 mg/g of total phenolic acids with 19.5% of caffeoyltartaric acid. Olive oil wastewater (OOWW) was the aqueous phase obtained after olive crushing and separation of the lipid phase by centrifugation (Clermont l'Hérault, France). One kilogram of olive produced 1 L of OOWW, containing 7 g/L of total phenols with 0.4 g/L of hydroxytyrosol. This resulted in concentrations 10−20 times higher than olive oil itself. The phenol content of these natural mixtures was expressed as μmol of gallic acid equivalent/L (μGAE/L): 1 mg/L of RW and OOWW phenolics corresponded to 13.7, 14.2 μGAE/L, respectively. Pure phenolic acids were synthesized by S. Labidalle (Œnologie, UFR de Pharmacie, Toulouse, France). The chlorogenic acid mixtures, extracted from green coffee beans of the arabica and robusta varieties by esterification, purified by recrystallization from ethyl acetate, and identified by mass spectrometry, were a gift of B. Guyot (Centre de Coopération Internationale de Recherche Agronomique pour le Développement, Montpellier, France). They mainly consisted of quinyl esters of caffeic acid and ferulic acid (70%−80% of caffeoylquinic acid, 8%−12% of feruloylquinic acid, and 1%−2% of caffeoylferuloylquinic acid). A 1 mg/L solution of arabica and robusta varieties corresponded to 10.2 and 10.5 μGAE/L, respectively. Probucol, caffeoyl-5-quinic, salicylic, and acetylsalicylic acids were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The fluorescent substrates, NBD-C_6-HPC, 2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-1-hexadecanoyl-*sn*-glycerol-3-phosphocholine, and NBD-X, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid, were from Interchim (Montluçon, France). AAPH, 2−2′-azobis(2-amidinopropane) hydrochloride was from Biovalley (Conches, France)

Preparation and Oxidizability of LDL. Plasma was collected from non-hypercholesterolemic and non-hypertriglyceridemic patients undergoing plasmapheresis sessions in the Hematology Department of Hospital Lapeyronie (Montpellier, France).[47] LDL preparation was carried out according to the usual procedure in the laboratory.[4] LDL dialysis and subsequent oxidizability measurements (monitored at 234 and 245 nm for Cu^{2+}- and AAPH-oxidation, respectively) were performed as already reported.[13] To investigate the antioxidant efficiency of the phenolic compounds, isolated LDL from plasmapheresis was arbitrary diluted to 1 μmol/L, preincubated for 1 h at 37 °C in the presence of various antioxidant compounds, and oxidized either by 5 μmol/L Cu^{2+} after a 10-fold dilution in oxygenated phosphate-saline buffer (PBS: 10-mmol/L phosphate buffer, 150 mmol/L NaCl) or by 2.5 mmol/L AAPH after a 20-fold dilution in oxygenated PBS.

Biochemical Parameters of LDL. The apoB and vitamin E contents of LDL were determined as already described.[13,48] The range of LDL vitamin E was between 6 and 14 mol vitamin E/mol apoB. Probucol was determined simultaneously with the vitamin E. The assessment of the lysoPCho production was adapted from ref 49. It was determined by HPLC using a light diffusion detector (DDL 21, Eurosep Instruments, Cergy-Pontoise, France) equipped with a DOS Chemstation. Briefly, 2 mL of LDL from the oxidation medium was extracted by 4 mL of chloroform/methanol (2:1, v/v). The lipid extract was then dissolved in 100 μL of chloroform/methanol (2:1, v/v), and 50 μL was injected on a Lichrospher column (125 × 4 mm, 5 μm particle size). The gradient used consisted of a mixture of two solvents: A (chloroform/methanol/NH_4OH (800:193:7, v/v/v)) and B (chloroform/methanol/NH_4OH/water (600:342.5:2.5:55, v/v/v/v) with 8 mg/L of *dl*-serine). The flow rate was 1.3 mL/min. The initial conditions were 75% of A and 25% of B, and the mobile phase changed linearly. At the end of the analysis (20 min) the mobile phase contained 25% of A and 75% of B. Phospholipids were detected by their retention time (t_R of lysoPCho and PCho = 15.90 and 8.35 min, respectively),

and the quantitative measurement was performed by integration of the peak area and comparison with external standards.

The LDL-PLA_2 activity was measured by a fluorescence method using NBD-C_6-HPC and NBD-X, respectively, as substrate and standard of released fatty acids,[50] by means of a LS-3B fluorescence spectrometer (Perkin-Elmer, Paris, France). Isolated LDL from plasmapheresis was diluted 100 times in 10 mmol/L Tris-HCl, pH 7.4 buffer containing 100 mmol/L KCl, and the substrate was added just before measuring the PLA_2 activity. Fluorescence wavelengths were at 470 nm for excitation and 540 nm for emission. The effect of phenolic compounds on LDL-PLA_2 activity was measured after LDL incubation under the same conditions as that used for oxidation.

Expression of the Specific Antioxidant Activity. The production of CD was continuously monitored (see above) in order to assess its lag time (T_{lag}) as already described.[51] The T_{lag} was plotted versus increasing concentrations of the tested antioxidant.[52] This generally produces the linear relationship $y = \alpha x + 100$, where y represents the "relative" T_{lag} (designated by $rT_{lag[A]} = [T_{lag[A]}/T_{lag[0]}] \times 100$, $T_{lag[0]}$ being the T_{lag} in the absence of antioxidant) for a given concentration of antioxidant $x = [A]$, whereas α represents the coefficient of regression of the linear relationship. The coefficient α is equivalent to the expression $[rT_{lag[A]} - 100]/[A]$ considered here as the specific antioxidant activity (SAA) for one antioxidant product and expressed in a specific antioxidant unit (μmol^{-1} L for purified compounds or μGAE^{-1} L for antioxidant mixtures). The $rT_{lag[A]}$ was designed to render the measure of the lag time independent of the LDL used. The final concentrations of LDL in the oxidation medium were of 0.1 and 0.05 μmol apoB/L for Cu^{2+}- and AAPH-oxidation, respectively. The Cu^{2+}/apoB and AAPH/apoB molar ratios of 50:1 and 50 000:1 were chosen because they allow us to make the T_{lag} independent of apoB concentration[53] and, in such conditions, to take into account the actual LDL concentration in the oxidation medium by reporting values of rT_{lag} to 0.1 μmol/L in apoB for Cu^{2+}-oxidation or to 0.05 μmol/L in apoB for AAPH-oxidation.

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