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Inhibition of Lipid Peroxidation in Low-Density Lipoprotein by the Flavonoid Myrigalone B and Ascorbic Acid

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ABSTRACT. Lipid peroxidation in human LDL (0.05 mg protein/mL) incubated with Cu^{2+}-ions (5 μM) *in vitro* was dose-dependently inhibited by the flavonoid myrigalone B (MyB) and by ascorbic acid. MyB at 6 μM increased the oxidation lag time by 135 ± 24 min (approximately 5-fold compared to controls) and reduced the maximum oxidation rate by 46 ± 5%. Ascorbic acid, at 9 μM, increased the lag time by 179 ± 29 min (6-fold compared to controls) but did not affect the maximum oxidation rate. The increase in lag time induced by MyB was enhanced in the presence of ascorbic acid. Their effects were additive, except when both were present at the highest concentration tested, when a significant potentiation, giving an increase in lag time of approximately 2 hr more than the sum of separate effects, occurred. Concentration-time curves for MyB in the absence and presence of ascorbic acid showed that the vitamin protected MyB against deterioration during incubation, and indicated that the net consumption of MyB in the oxidation process was reduced. No differences were observed when ordinary ascorbic acid and Ester-C®, a commercial vitamin C product, were compared. In conclusion, MyB and ascorbic acid seem to interact in a way that further improves the antioxidant status of the LDL particle relative to each substance separately. BIOCHEM PHARMACOL 51;12:1719–1725, 1996.

KEY WORDS. myrigalone B; lipid peroxidation; ascorbic acid; oxidized LDL; flavonoids

The pathogenesis of atherosclerosis is complex, but available evidence suggests that oxidation of LDL[‖] may represent an important event in atherogenesis [1, 2]. Naturally occurring antioxidants, such as α-tocopherol, ascorbic acid, and ubiquinol, may protect LDL against oxidative modification [3] and, thus, inhibit the development of atherosclerotic disease [4]. Furthermore, it has been reported that probucol, a hypolipidemic drug with antioxidant properties, may prevent LDL oxidation [5]. In addition, the well-known antioxidant BHT has been shown to prevent the formation of aortic lesions in cholesterol-fed rabbits [6].

Flavonoids are naturally occurring phenols. They are ubiquitous in the plant kingdom and are consumed through intake of vegetables, fruits, tea, and wine. Many flavonoids are known to be antioxidants and some of these, such as quercetin, catechin, and morin, have been shown to inhibit LDL oxidation *in vitro* [7–10]. It has been suggested that members of this substance class may be responsible for the inhibitory activity of red wine against LDL oxidation [11, 12]. An increase in dietary flavonoids may be correlated to a reduced risk of death from coronary heart disease in elderly men [13].

The fruit exudate of the bog myrtle plant, *Myrica gale* L., is rich in C-methylated dihydrochalcones [14, 15]. The biological activity of this uncommon class of flavonoids is virtually unknown. One of the main components, MyB (Fig. 1), is a potent antioxidant in several systems [16, 17]. Its ability to inhibit oxidation of LDL from cholesterol-fed rabbits has recently been reported [18].

Ascorbic acid is a water-soluble antioxidant that appears to form the first line of defence in human plasma exposed to a variety of oxidant insults [19]. It also effectively protects LDL against Cu^{2+}-induced oxidation [20, 21]. Ascorbic acid can regenerate α-tocopherol from its oxidized form at the water-lipid interface *in vitro* [22, 23]. However, whether such a mechanism is operative *in vivo* remains controversial.

An additive interaction between ascorbic acid and the flavonoid rutin, as well as a supraadditive effect for ascorbic acid, rutin, and α-tocopherol in combination, has been

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‖ *Abbreviations:* AA, ordinary ascorbic acid; apoB-100, apolipoprotein B-100; BHT, butylated hydroxytoluene; DTPA, diethylenetriamine pentaacetic acid; EC, Ester-C®; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MyB, myrigalone B; TLC, thin-layer chromatography.

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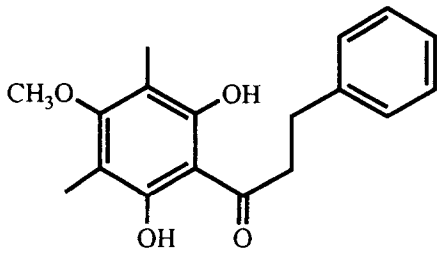

FIG. 1. Structure of myrigalone B (MyB; 2',6'-dihydroxy-4'-methoxy-3',5'-dimethyldihydrochalcone).

shown in different model systems [24, 25]. Similar interactions may occur with other flavonoids.

Ester-C® (EC), a commercial vitamin C product, is a complex mixture containing ascorbate (73%), calcium (10%), dehydroascorbic acid (8%), threonate (~1%), and trace levels of xylonate and lyxonate [26]. EC has been claimed to improve ascorbic acid availability in intact rats [26] and white blood cells [27], but no information is available on its antioxidative properties.

The purpose of this work was to study the effect of the flavonoid MyB and ascorbic acid, alone and combined, on Cu^{2+}-induced oxidation of human LDL as measured by the increase in absorbance at 234 nm caused by the formation of conjugated dienes. The concentrations of MyB and ascorbic acid during the peroxidation process were monitored by HPLC. Moreover, ordinary ascorbic acid, and EC were compared.

MATERIALS AND METHODS

Chemicals

MyB was isolated according to Uyar *et al.* [14]. L-Ascorbic acid (99.8%) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) and Ester-C® from Inter-Cal Corp. (Prescott, AZ, U.S.A.). Other chemicals were of analytical grade.

Isolation of LDL

Human LDL in the density range 1.019–1.063 g/mL was isolated from freshly prepared plasma obtained from healthy, female volunteers (n = 4) by sequential ultracentrifugation in a Centrikon T-2060 ultracentrifuge (Kontron Ltd, Zürich, Switzerland) with a TFT 70.38 rotor at 43,000 rpm; 10°C; 24 hr [28]. The purity of the lipoprotein fraction was ascertained by agarose gel electrophoresis [29]. LDL not immediately used was frozen at −70°C in a sucrose solution according to Rumsey *et al.* [30]. Prior to experiments, the LDL preparations were extensively dialysed against phosphate-buffered saline (PBS; 20 mM sodium phosphate with 0.15 M NaCl, pH 7.4), and filtered through a 0.45 μm filter (Millipore, Bedford, MA, U.S.A.). The protein concentration was determined with Coomassie Brilliant Blue G-250 reagent from Bio-Rad Laboratories GmbH (Munich, Germany), using a previously described method [31]. Each dialyzed batch of LDL was stored under N_2 in the dark at 4°C, and used for a maximum of 4 days. In separate experiments (results not shown), it was found that the freezing and thawing of the LDL did not alter its susceptibility to oxidation.

Oxidation of LDL

MyB was added to the incubation samples as an ethanolic solution. The resulting ethanol concentration (1%) in the samples did not influence the oxidation parameters. Ascorbic acid was added as AA or EC from stock solutions (either freshly prepared or stored at −196°C for a maximum of 1 week) in deionised water.

LDL (0.05 mg protein/mL) in PBS was incubated at 37°C in the presence of $CuSO_4$ (final concentration 5 μM). The peroxidation process was continuously monitored by the change in absorbance at 234 nm [32] in a UV-160A spectrophotometer equipped with a CPS-240A thermostatted cell positioner, both from Shimadzu (Kyoto, Japan). The samples were either unprotected or contained MyB alone, ascorbic acid alone (as AA or EC) or both antioxidants combined. MyB concentration was either 2 or 6 μM, and the ascorbic acid concentration either 3 or 9 μM. Each combination was made in triplicate. The end of the lag phase was defined as the intercept of the straight lines derived from the lag phase and the propagation phase. The maximum rate of formation of conjugated dienes (nmol/mg LDL protein/min), defined as the tangent of the steepest part of the oxidation curve, and the maximum amount formed (nmol/mg LDL protein) were calculated using a molar extinction coefficient of $2.95 \times 10^4\ M^{-1}\ cm^{-1}$ [32]. The intra-assay variation coefficients for the lag time, rate of formation, and amount of conjugated dienes formed were 4.1, 5.2, and 2.2%, respectively (n = 6).

For determination of MyB and ascorbic acid content during the peroxidation process, separate samples were incubated at 37°C in a covered, thermostatted waterbath, in parallel to the experiments described above. Aliquots were taken immediately before lipid peroxidation was started and at intervals during the process.

HPLC Analysis of MyB and Ascorbic Acid

MyB was analyzed by an unpublished method developed in our laboratory. The HPLC systems consisted of a high-performance liquid chromatograph model 2249 equipped with an auto sampler model 2157, both from LKB (Bromma, Sweden). Separation was achieved in an isocratic system on a C18 reversed-phase column (5 μm; 250 mm × 4.6 mm ID) from Supelco (Bellefonte, PA, U.S.A.) with Valco C18 guard columns (40 μm; 20 mm × 4.6 mm ID). The chromatograms were recorded and the concentrations computed (from peak heights) by an SP4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.).

Aliquots (50 μL) of the incubation sample were rapidly diluted with 450 μL mobile phase (whereby any MyB con-

tained in the LDL particles would be released), and 50 μL of this mixture was injected into the chromatograph. The mobile phase consisted of methanol:acetic acid (10% in water) 90:10 (v/v), and was continuously degassed with helium. The effluent was monitored at 280 nm by a variable wavelength monitor model 2151 from LKB (Bromma, Sweden). At a flow rate of 0.9 mL/min, MyB had a retention time of approximately 4.1 min. Appropriate standards (10–600 nM) were made from MyB stock solutions in the mobile phase. BHT, EDTA, or diethylenetriamine pentaacetic acid (DTPA), compounds normally added to inhibit further peroxidation [33, 34], caused serious interference with the analysis and could not be used here. Therefore, MyB had to be analyzed immediately after sampling.

For ascorbic acid determination, aliquots (20 μL) were rapidly mixed with 480 μL mobile phase containing DTPA (1 mM), a more effective copper chelator than EDTA [35], and immediately frozen at −196°C (liquid nitrogen). Prior to analysis, the samples were thawed one at a time and 50 μL injected into the chromatograph. Increasing the DTPA concentration 5-fold did not affect the results, indicating that no further Cu^{2+}-induced degradation of ascorbic acid occurred after sampling. DTPA did not interfere with the analysis, nor did the freezing and thawing affect the ascorbic acid content of the samples.

Ascorbic acid was determined by a modification of the method previously described [36]. The mobile phase consisted of 80 mM of a sodium acetate buffer pH 4.6, containing Na_2-EDTA 0.5 mM, NaCl 1 mM and *n*-octylamine 1 mM. The effluent was monitored by an electrochemical detector (HP 1049A, Hewlett-Packard, Waldbronn, Germany) operated at its most sensitive setting. At a flow rate of 1.3 mL/min, the retention time of ascorbic acid was approximately 5.9 min. Stock solutions of ascorbic acid in the mobile phase were freshly made or stored at −196°C for a maximum of 1 week. From these, working standards (15–360 μM) were made just before use by dilution in mobile phase that contained PBS and DTPA in the same concentrations as the samples.

The ascorbic acid and MyB content was calculated as percent of the concentration at zero time (i.e. prior to addition of copper). The curves were fitted to an exponential equation to obtain decline coefficients (k), and half-lives of the compounds were calculated from the formula $t_{1/2} = \ln 2/k$.

In control experiments, MyB (2 μM) was incubated in PBS without any further additives or in PBS containing either $CuSO_4$ or LDL in the usual amounts, and its rate of disappearance during incubation calculated as indicated above. Such controls were also made with ascorbic acid (3 μM) or EC, and with a combination of MyB (2 μM) and ascorbic acid (3 μM) or EC.

Statistical Calculations

For comparison between groups a two-sided Student's *t*-test with the Bonferroni correction was used. All results are expressed as mean values ± standard deviations.

RESULTS

The copper-catalyzed oxidation of LDL was significantly inhibited by MyB as well as by ascorbic acid. This was shown by the prolongation of the peroxidation lag time caused by both antioxidants (Fig. 2a–d). The combination of the two antioxidants at low doses, as well as the two intermediate combinations, gave effects equivalent to the sum of the individual effects. In contrast, the presence of high concentrations of both MyB and ascorbic acid caused a prolongation of the lag time that was significantly increased ($P < 0.01$) compared to the sum of their separate effects (Fig. 2d).

MyB also consistently reduced the maximum rate of conjugated diene formation (Table 1), both when given alone and when combined with ascorbic acid. In all our experiments, except one, ascorbic acid alone did not reduce the maximum rate (Table 1). None of the antioxidants influenced the maximum amount of conjugated dienes formed as measured by the increase in A_{234} during the propagation phase.

By pooling the different experiments, the dose-dependency of the antioxidant effects of MyB and ascorbic acid was evident. At 2 μM, MyB caused a mean increase in lag time of 46 ± 5 min and a reduction in the maximum oxidation rate of 37 ± 8% (n = 28); increasing its concentration to 6 μM gave values of 135 ± 24 min and 48 ± 5% (n = 29), respectively ($P < 0.001$ for both parameters compared to 2 μM). Ascorbic acid at 3 μM caused a lag time

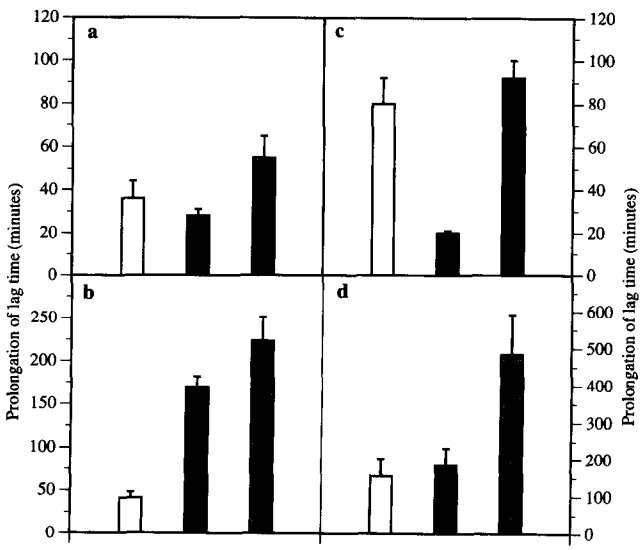

FIG. 2. The effects of myrigalone B (MyB) and ordinary ascorbic acid (AA) on the oxidation of LDL induced by Cu^{2+}-ions. LDL (0.05 mg protein/mL) was incubated at 37°C in the presence of 5 μM $CuSO_4$ in PBS, and the process was continuously monitored by the change in absorbance at 234 nm. The samples contained MyB (white), AA (black), or both antioxidants combined (grey) in the following concentrations: MyB 2 and AA 3 μM (a), MyB 2 and AA 9 μM (b), MyB 6 and AA 3 μM (c), and MyB 6 and AA 9 μM (d). Results are expressed as mean values ± SD (n = 6–9) and represent the prolongation of lag time compared to controls.

TABLE 1. The effect of myrigalone B (MyB) and ordinary ascorbic acid (AA) on the maximum rate of formation of conjugated dienes in LDL oxidized by Cu^{2+}

	Control	MyB	AA	MyB + AA
a		2 μM	3 μM	2 μM 3 μM
Rate ($nmolmg^{-1}min^{-1}$):	35.5 ± 6.3	19.4 ± 6.1	38.6 ± 1.6	24.4 ± 6.4
% reduction:		45 ± 20 ($P < 0.0012$)	−9 ± 20 (NS)	31 ± 22 ($P < 0.013$)
b		2 μM	9 μM	2 μM 9 μM
Rate ($nmolmg^{-1}min^{-1}$):	24.0 ± 3.1	15.7 ± 2.9	24.5 ± 1.2	19.3 ± 1.1
% reduction:		35 ± 15 ($P < 0.0007$)	−2 ± 14 (NS)	20 ± 11 ($P < 0.0055$)
c		6 μM	3 μM	6 μM 3 μM
Rate ($nmolmg^{-1}min^{-1}$):	32.6 ± 2.5	18.9 ± 1.8	32.5 ± 5.3	19.6 ± 1.6
% reduction:		42 ± 7 ($P < 0.0001$)	1 ± 18 (NS)	47 ± 5 ($P < 0.0001$)
d		6 μM	9 μM	6 μM 9 μM
Rate ($nmolmg^{-1}min^{-1}$):	23.7 ± 1.4	10.8 ± 2.2	19.3 ± 2.5	12.6 ± 1.0
% reduction:		54 ± 10 ($P < 0.0001$)	19 ± 12 ($P < 0.0005$)	47 ± 5 ($P < 0.0001$)

LDL (0.05 mg protein/mL) was incubated at 37°C in the presence of 5 μM $CuSO_4$ in PBS, and the process was continuously monitored by the change in absorbance at 234 nm. The samples were either unprotected or contained MyB and AA alone or combined in the concentrations indicated. The maximum rate was calculated using the molar extinction coefficient for conjugated dienes, 2.95×10^4 $M^{-1}cm^{-1}$. Results are presented as mean values ± SD (n = 6–9) and represent rates and reduction in rates compared to controls. NS: not significant.

prolongation of 24 ± 2 min (n = 12) which, at 9 μM, was increased to 179 ± 29 min (n = 15; $P < 0.001$). The effects of EC on the lipid peroxidation parameters did not differ significantly from those obtained with AA.

The estimated half-lives of MyB in the various lipid peroxidation experiments are presented in Table 2. At 6 μM, the value was twice that at 2 μM ($P < 0.001$). Addition of AA or EC increased the lifetime further, resulting in a 4–5-fold increase for 6 μM MyB with 9 μM AA or EC present, compared to MyB alone at 2 μM ($P < 0.001$). There were no significant differences between the two preparations of the vitamin. Figure 3 shows the concentration-time curves for MyB at 2 and 6 μM, alone or combined with 3 or 9 μM ascorbic acid.

In Fig. 4, corresponding curves for ascorbic acid are shown. Increasing the ascorbic acid concentration led to increased survival time, as evidenced by half-lives of 0.21 ± 0.07 and 0.55 ± 0.21 min at 3 and 9 μM, respectively. Again, no significant difference between AA and EC was observed. Coincubation with MyB did not influence the half-life values of either vitamin preparation.

TABLE 2. Estimated half-lives (min) for myrigalone B (MyB)

	MyB 2 μM	MyB 6 μM
Alone	5.3 ± 1.5	9.5 ± 1.2
+3 μM AA	8.6 ± 2.2	13.2 ± 1.9
+3 μM EC	7.5 ± 0.8	13.8 ± 2.6
+9 μM AA	14.8 ± 2.4	26.8 ± 9.8
+9 μM EC	14.7 ± 1.6	20.8 ± 4.3

MyB was present alone or combined with either ordinary ascorbic acid (AA) or Ester-C® (EC) in LDL samples (0.05 mg protein/mL in PBS) undergoing oxidation induced by Cu^{2+} (5 μM) at 37°C. Pooled data from all experiments. Results are expressed as mean values ± SD (n = 4–20) and are calculated according to first-order kinetics. Addition of AA or EC in all instances increased the half-life of MyB ($P < 0.005$).

Figure 5 illustrates the temporal relationship between the consumption of MyB and ascorbic acid* and the onset of lipid peroxidation for the experiments at high concentrations of the antioxidants, demonstrating the pronounced delay of the oxidation process beyond the life span of the antioxidants.

The control experiments (Table 3) showed that MyB deteriorated slowly, but at a measurable rate, in PBS. Ascorbic acid counteracted this deterioration completely within the observation time. With Cu^{2+} or LDL present, a higher disappearance rate for MyB was observed; here, too, ascorbic acid had a significant retarding effect ($P < 0.005$). Ascorbic acid itself was, as expected, short-lived in PBS alone and extremely short-lived in the presence of cupric ions. Addition to LDL, however, decreased its disappearance rate ($P < 0.025$). MyB did not influence the ascorbic acid disappearance rate either in the presence or in the absence of LDL.

DISCUSSION

Both MyB and ascorbic acid caused, in a dose-dependent manner, a prolongation of the peroxidation lag time in human LDL exposed to cupric ions. Presumably, MyB acts as a chain-breaking antioxidant [16, 17], scavenging peroxyl or alkoxyl radicals formed in the peroxidation cascade. MyB is a highly lipophilic compound* that probably acts within the LDL particles, whereas ascorbic acid exerts its action mainly in the water phase or at the water-lipid interface. Our results show that ascorbic acid not only protects MyB from oxidative deterioration in solution, but also indicate that its net consumption in LDL- and cupric ion-containing systems is reduced.

The effect in LDL oxidation experiments may be brought

* Unpublished results.

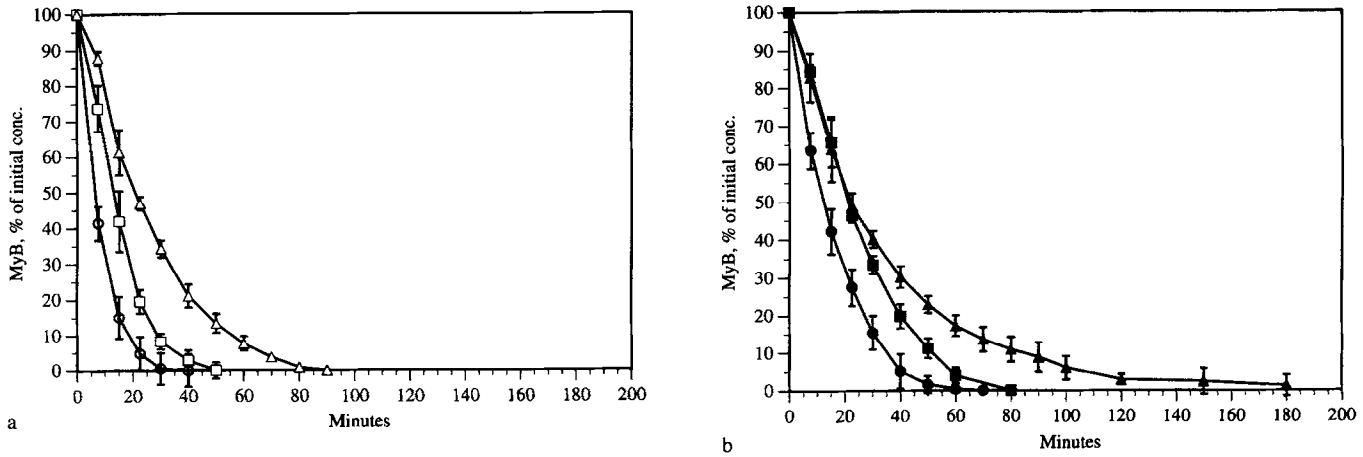

FIG. 3. Decay curves for myrigalone B (MyB) at 2 μM (a) and 6 μM (b), when present alone (○/●) or combined with ordinary ascorbic acid 3 μM (□/■) or 9 μM (△/▲), in LDL samples (0.05 mg protein/mL) incubated at 37°C in the presence of 5 μM $CuSO_4$ in PBS. Results are expressed as mean values ± SD, and represent pooled data from all experiments (n = 4–20).

about in two ways: directly, by regenerating MyB that is consumed by its antioxidative action, or indirectly, by a regeneration of endogenous antioxidants; thus, exerting a sparing effect on MyB. The direct mode of action will be analogous to the previously suggested [23] recycling mechanism for α-tocopherol by ascorbic acid. An involvement of MyB in the regeneration of endogenous antioxidants might also be possible.

When MyB and ascorbic acid were combined, most combinations led to additive effects, in accordance with a previous study using ascorbic acid and the flavonoid glycoside rutin in isolated membranes and membrane-like systems [24]. Rutin is, however, a much more polar compound than MyB, and its mechanism for interaction with ascorbic acid or lipophilic antioxidants such as α-tocopherol may well be different. High concentrations of MyB and ascorbic acid (6 μM and 9 μM, respectively) led to a potentiation of the antioxidative effect. Such effects were not observed at lower concentrations. Small potentiating effects might, however, not be detected, due to standard deviations induced by intraexperimental variation. Supra-additive effects have previously been reported for a triple combination of antioxidants, involving ascorbic acid, α-tocopherol, and rutin [25].

A dose increase for each antioxidant alone led to an increase in their life span (Table 2). When the two antioxidants were combined, ascorbic acid dose-dependently caused a further prolongation of the half-life of MyB. In other words, the results of increased doses of both compounds will be elevated, as well as better maintained, antioxidant concentrations (particularly for MyB). Consequently, one may assume that the uptake of MyB into the LDL particles will proceed faster and probably be more extensive than under other conditions, and the recycling of lipophilic antioxidants will be more effective. This may, at least in part, explain the potentiated effect observed.

MyB not only increased the lag time of the oxidation reaction, but also reduced the maximum rate of diene formation in the propagation phase, as recently shown for LDL from cholesterol-fed rabbits [18]. This effect was not shared by ascorbic acid, in accordance with a previous report [20]. Recently, paracetamol [37] and α-tocopherol [38] have, likewise, been shown to decrease the maximum rate of diene formation during LDL oxidation. The maximum amount of dienes formed did not appear to be influenced by MyB.

No differences in antioxidative effects were observed between AA and EC in the present study. Thus, the claimed improved bioavailability of EC [26] is probably not caused by increased lipid solubility, because this, most likely, would have affected the results in our study.

The slow deterioration of MyB in PBS shown in the

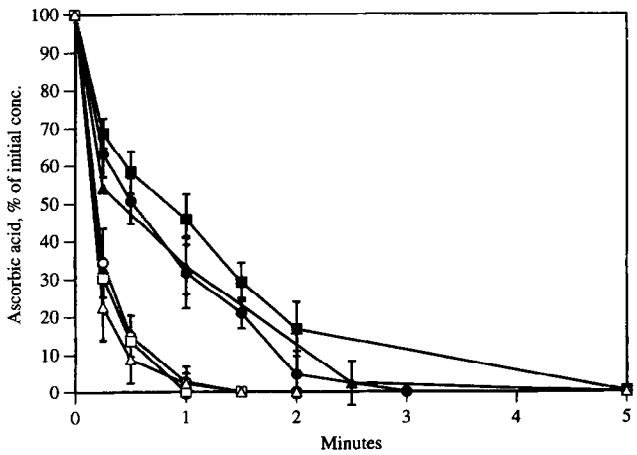

FIG. 4. Decay curves for ordinary ascorbic acid (3 μM: open symbols/9 μM: filled symbols) when present alone (○/●) or combined with myrigalone B (MyB) 2 μM (□/■) or 6 μM (△/▲), in LDL samples (0.05 mg protein/mL) incubated at 37°C in the presence of 5 μM $CuSO_4$ in PBS. Results are expressed as mean values ± SD (n = 4–16), and represent pooled data from all experiments. For one experiment (□) n = 2 and only mean values are shown. However, the absolute variation from the mean value was maximum 4%.

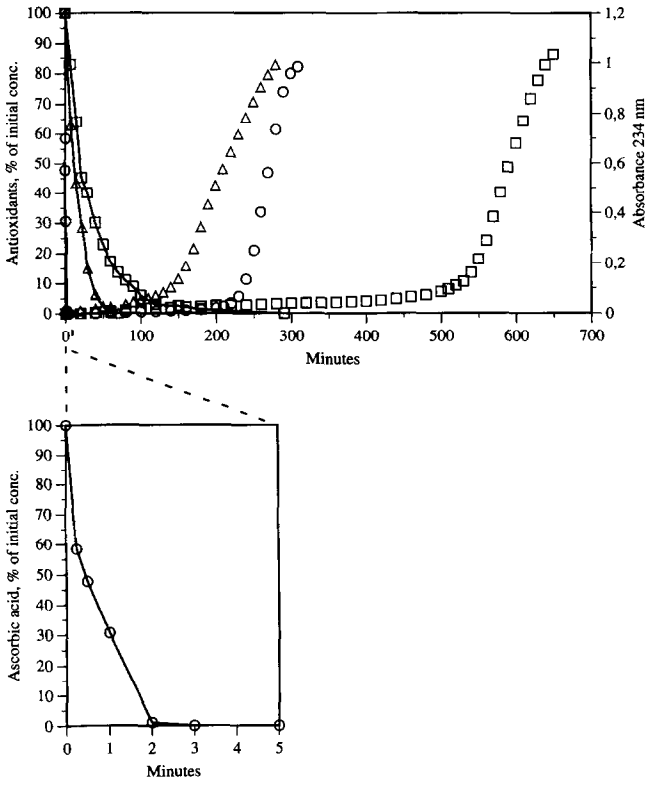

FIG. 5. Temporal relationship between consumption of ordinary ascorbic acid (AA) and myrigalone B (MyB) and onset of lipid peroxidation. LDL (0.05 mg protein/mL) in PBS was incubated at 37°C in the presence of 5 μM $CuSO_4$, and AA (○), or MyB (△), or both (□). At the times indicated, AA and MyB (△/□) were determined by HPLC (solid lines). The degree of oxidation was continuously monitored by the change in absorbance at 234 nm (symbols only). Initial concentrations were 9 μM AA and 6 μM MyB. The 0–5-min interval is expanded to show the decline in ascorbic acid concentration.

control experiments (Table 3) may be caused by phenol oxidation catalyzed by trace metals present. Addition of cupric ions increased the rate of deterioration. The loss of MyB was completely abolished by the more readily oxidizable ascorbic acid in the absence of added cupric ions, and partially counteracted with cupric ions present.

The shorter half-life of MyB in the presence of LDL than in PBS alone is likely to reflect a consumption of internalized MyB during spontaneous oxidation in the LDL particles. Ascorbic acid counteracted the loss of MyB, probably in the manner discussed above. It is not unexpected that ascorbic acid, unlike MyB, was more stable in the presence of LDL than in its absence, because trace metals in the medium may bind to LDL, thereby reducing their effective concentration in the water phase. Whereas ascorbic acid generally was shown to cause protection of MyB, the flavonoid did not show any such effect on ascorbic acid, neither under control conditions nor during induced lipid peroxidation. This is probably related to a difference in redox potentials [39].

Despite their increased life span, the two antioxidants were, nevertheless, completely consumed long before significant amounts of conjugated dienes were formed (Fig. 5). This effect was most pronounced at high concentrations of both substances, but a prolonged latency was also evident at other concentrations. Thus, ascorbic acid was undetectable after a few min, but still caused inhibition of lipid peroxidation for a significant time. This may indicate that endogenous antioxidants in the LDL particles may be responsible for much of the effect in the later phase of the lag period, having been efficiently regenerated or spared by the action of ascorbic acid or MyB. Dehydroascorbic acid, an oxidation product of ascorbic acid, has, however, recently been reported to possess antioxidant activity [21] and may contribute to the delay.

It has been suggested [7, 9] that dietary flavonoids may have an antiatherogenic effect if sufficiently high plasma levels can be obtained. It follows, then, that an important task would be to subject flavonoids showing antioxidant effects *in vitro* to a thorough pharmacokinetic examination.

TABLE 3. Half-lives (min) for myrigalone B (MyB, 2 μM) and ordinary ascorbic acid (AA, 3 μM) during incubation at 37°C under control conditions

	MyB	AA
MyB/PBS	171	
AA/PBS		0.70 ± 0.03
MyB/Cu*/PBS	38.3 ± 0.6	
AA/Cu/PBS		0.10 ± 0.02
MyB/LDL†/PBS	36.7	
AA/LDL/PBS		4.6 ± 1.8
MyB/AA/PBS	stable	0.96 ± 0.13
MyB/AA/Cu/PBS	59.5 ± 3.0	0.09 ± 0.03
MyB/AA/LDL/PBS	140.3 ± 33.4	6.0 ± 3.1

Results are calculated as described in Table 2. * Cu, 5 μM $CuSO_4$; † LDL, 0.05 mg protein/mL.

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References

1. Ross R, The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362:** 801–803, 1993.
2. Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL, Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* **320:** 915–924, 1989.
3. Esterbauer H, Gebicki J, Puhl H and Jürgens G, The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad Biol Med* **13:** 341–390, 1992.
4. Steinberg D, Antioxidant vitamins and coronary heart disease. *N Engl J Med* **328:** 1487–1489, 1993.
5. Parthasarathy S, Probucol inhibits oxidative modification of low density lipoprotein. *J Clin Invest* **77:** 641–643, 1986.
6. Björkhem I, Henriksson-Freyschuss A, Breuer O, Diczfalusy U, Berglund L and Henriksson P, The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler Thromb* **11:** 15–22, 1991.

7. Nègre-Salvayre A and Salvayre R, Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cell lines. *Free Rad Biol Med* **12:** 101–106, 1992.
8. Mangiapane H, Thomson J, Salter A, Brown S, Bell GD and White DA, The inhibition of the oxidation of low density lipoprotein by (+)-catechin, a naturally occurring flavonoid. *Biochem Pharmacol* **43:** 445–450, 1992.
9. Jessup W, Rankin SM, De Whalley CV, Hoult JRS, Scott J and Leake DS, α-Tocopherol consumption during low-density-lipoprotein oxidation. *Biochem J* **265:** 399–405, 1990.
10. De Whalley CV, Hoult JRS, Jessup W and Leake DS, Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem Pharmacol* **39:** 1743–1750, 1990.
11. Frankel EN, Kanner J, German JB, Parks E and Kinsella JE, Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **341:** 454–457, 1993.
12. Fuhrman B, Lavy A and Aviram M, Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* **61:** 549–554, 1995.
13. Hertog MGL, Feskens EJM, Hollman PCH, Katan MB and Kromhout D, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342:** 1007–1011, 1993.
14. Uyar T, Malterud KE and Anthonsen T, Two new dihydrochalcones from *Myrica gale*. *Phytochemistry* **17:** 2011–2013, 1978.
15. Malterud KE, C-methylated dihydrochalcones from *Myrica gale* fruit exudate. *Acta Pharm Nord* **4:** 65–68, 1992.
16. Malterud KE, Diep HO and Sund RB, C-Methylated dihydrochalcones from *Myrica gale* L.: Effects as antioxidants and as scavengers of 1,1-diphenyl-2-picrylhydrazyl. *Pharmacol Toxicol* **78:** 111–116, 1996.
17. Mathiesen L, Malterud KE and Sund RB: Antioxidant activity of fruit exudate and C-methylated dihydrochalcones from *Myrica gale* L. *Planta Med* **61:** 515–518, 1995.
18. Mathiesen L, Malterud KE, Nenseter MS and Sund RB: Inhibition of low density lipoprotein oxidation by myrigalone B, a naturally occurring flavonoid. *Pharmacol Toxicol* **78:** 143–146, 1996.
19. Frei B, Stocker R and Ames BN, Antioxidant defences and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci USA* **85:** 9748–9752, 1988.
20. Esterbauer H, Striegl G, Puhl H, Oberreither S, Rotheneder M, El-Saadani M and Jürgens G, The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. *Ann NY Acad Sci* **570:** 254–267, 1989.
21. Retsky KL, Freeman MW and Frei B, Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. Anti- rather than prooxidant activity of vitamin C in the presence of transition metal ions. *J Biol Chem* **268:** 1304–1309, 1993.
22. Niki E, Interaction of ascorbate and α-tocopherol. *Ann NY Acad Sci* **498:** 186–198, 1987.
23. Kagan VE, Serbinova EA, Forte T, Scita G and Packer L, Recycling of vitamin E in human low density lipoprotein. *J Lipid Res* **33:** 385–397, 1992.
24. Nègre-Salvayre A, Affany A, Hariton C and Salvayre R, Additional antilipoperoxidant activities of alpha-tocopherol and ascorbic acid on membrane-like systems are potentiated by rutin. *Pharmacology* **42:** 262–272, 1991.
25. Nègre-Salvayre A, Mabile L, Delchambre J and Salvayre R, α-Tocopherol, ascorbic acid, and rutin inhibit synergistically the copper-promoted LDL oxidation and the cytotoxicity of oxidized LDL to cultured endothelial cells. *Biol Trace Element Res* **47:** 81–91, 1995.
26. Bush MJ and Verlangieri AJ, An acute study on the relative gastro-intestinal absorption of a novel form of calcium ascorbate. *Res Comm Chem Pathol Pharmacol* **57:** 137–140, 1987.
27. Wright JV and Suen RM, *A clinical study of Ester-C® vs. L-ascorbic acid*. Report. Meridan Valley Clinical Laboratory, 1987.
28. Havel RJ, Eder HA and Bragdon JH, The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* **34:** 1345–1353, 1955.
29. Noble RP, Electrophoretic separation of plasma lipoproteins in agarose gel. *J Lipid Res* **9:** 693–700, 1968.
30. Rumsey SC, Galeano NF, Arad Y and Deckelbaum RJ, Cryopreservaton with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. *J Lipid Res* **33:** 1551–1561, 1992.
31. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt Biochem* **72:** 248–254, 1976.
32. Esterbauer H, Striegl G, Puhl H and Rotheneder M, Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Rad Res Comms* **6:** 67–75, 1989.
33. Jialal I, Vega GL and Grundy SM, Physiological levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis* **82:** 185–191, 1990.
34. Kuzuya M, Yamada K, Hayashi T, Funaki C, Naito M, Asai K and Kuzuya F, Role of lipoprotein-copper complex in copper catalyzed-peroxidation of low-density lipoprotein. *Biochim Biophys Acta* **1123:** 334–341, 1992.
35. Gerhartz W, ed. *Ullmann's encyclopedia of industrial chemistry*. 5. ed. Verlag Chemie, Weinheim, 1987.
36. Wang S, Schram IM and Sund RB, Determination of ascorbic acid in plasma: method and stability studies. *Eur J Pharm Sci* **3:** 231–239, 1995.
37. Nenseter MS, Halvorsen B, Rosvold Ø, Rustan AC and Drevon CA, Paracetamol inhibits copper-catalyzed, azo compound- and mononuclear cell-mediated oxidative modification of low density lipoprotein. *Arterioscler Thromb Vasc Biol* **15:** 1338–1344, 1995.
38. Suzukawa M, Ishikawa T, Yoshida H and Nakamura H, Effect of in-vivo supplementation with low-dose vitamin E on susceptibility of low-density lipoprotein and high-density lipoprotein to oxidative modification. *J Am Coll Nutr* **14:** 46–52, 1995.
39. Bors W, Michel C and Schikora S, Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: A pulse radiolysis study. *Free Rad Biol Med* **19:** 45–52, 1995.