

## Original Research Article

# Redox Cycles of Caffeic Acid, $\alpha$ -Tocopherol, and Ascorbate: Implications for Protection of Low-Density Lipoproteins Against Oxidation

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### Summary

This study addresses the dynamic interactions among  $\alpha$ -tocopherol, caffeic acid, and ascorbate in terms of a sequence of redox cycles aimed at accomplishing optimal synergistic antioxidant protection. Several experimental models were designed to examine these interactions: UV irradiation of  $\alpha$ -tocopherol-containing sodium dodecyl sulfate micelles, one-electron oxidations catalyzed by the hypervalent state of myoglobin, ferrylmyoglobin, and autoxidation at appropriate pHs. These models were assessed by ultraviolet (UV) and electron paramagnetic resonance (EPR), entailing direct- and continuous-flow experiments, spectroscopy and by separation and identification of products by HPLC. The  $\alpha$ -tocopheroxyl radical EPR signal generated by UV irradiation of  $\alpha$ -tocopherol-containing micelles was suppressed by caffeic acid and ascorbate; in the former case, no other EPR signal was observed at pH 7.4, whereas in the latter case, the  $\alpha$ -tocopheroxyl radical EPR signal was replaced by a doublet EPR spectrum corresponding to the ascorbyl radical ( $A^{\cdot -}$ ). The potential interactions between caffeic acid and ascorbate were further analyzed by assessing, on the one hand, the ability of ascorbate to reduce the caffeic acid *o*-semiquinone (generated by oxidation of caffeic acid by ferrylmyoglobin) and, on the other hand, the ability of caffeic acid to reduce ascorbyl radical (generated by autoxidation or oxidation of ascorbate by ferrylmyoglobin). The data presented indicate that the reductive decay of ascorbyl radical ( $A^{\cdot -}$ ) and caffeic acid *o*-semiquinone (Caf-O $\cdot$ ) can be accomplished by caffeic acid (Caf-OH) and ascorbate ( $AH^{\cdot -}$ ), respectively, thus pointing to the reversibility of the reaction  $Caf-O\cdot + AH^{\cdot -} \leftrightarrow Caf-OH + A^{\cdot -}$ .

Continuous-flow EPR measurements of mixtures containing ferrylmyoglobin,  $\alpha$ -tocopherol-containing micelles, caffeic acid, and ascorbate revealed that ascorbate is the ultimate electron donor in the sequence encompassing transfer of the radical character from the micellar phase to the phase. In independent experiments, the effects of caffeic acid and ascorbate on the oxidation of two low-density lipoprotein (LDL) populations, control and  $\alpha$ -tocopherol-enriched, were studied and results indicated that  $\alpha$ -tocopherol, caffeic acid, and ascorbate acted synergistically to afford optimal protection of LDL against oxidation.

These results are analyzed for each individual antioxidant in terms of three domains: its localization and that of the antioxidant-derived radical, its reduction potential, and the predominant decay pathways for the antioxidant-derived radical, that exert kinetic control on the process.

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**Keywords** Antioxidants;  $\alpha$ -tocopherol; ascorbic acid; atherosclerosis; caffeic acid; EPR; low-density lipoprotein; redox cycle; synergism.

### INTRODUCTION

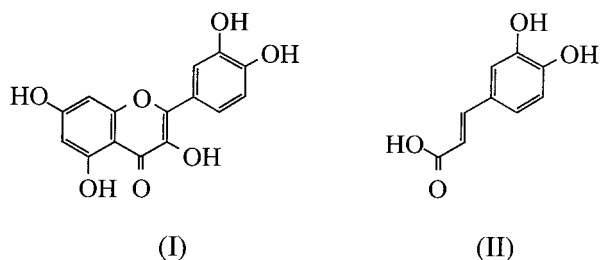
Phenolic compounds derived from plants are being intensively studied because of their potentially beneficial effects to human health (1). Although extensive research on the health benefits of these phytochemicals largely involves studies with flavonoids (2), phenolic acids have been shown to exhibit radical scavenging, antioxidant potential, and anticarcinogenic activity, properties that are not necessarily interdependent (3–6).

The structure of the phenolic compound caffeic acid (Caf-OH<sup>3</sup>; a dihydroxycinnamic acid derivative) retains the most relevant features that support the antioxidant activity assigned to flavonoids (Fig. 1) (7, 8), namely: (a) an *o*-dihydroxy (catechol) structure, the radical target site participating in the electron delocalization of phenoxy radical, and (b) a side-chain double-bond conjugated with the phenolic ring and with a

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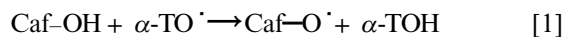
<sup>3</sup>Abbreviations:  $A^{\cdot -}$ , ascorbyl radical; AAPH, 2,2'-azo-bis(2-amidino-propane hydrochloride);  $AH^{\cdot -}$ , ascorbate; apoB, apoprotein B; Caf-OH, caffeic acid; Caf-O $\cdot$ , caffeic acid *o*-semiquinone; DTPA, diethylenetriamine pentacetic acid; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate;  $\alpha$ -TO $\cdot$ ,  $\alpha$ -tocopheroxyl radical;  $\alpha$ -TOH,  $\alpha$ -tocopherol.



**Figure 1.** Structures of quercetin (I) and caffeic acid (II).

carbonyl group, allowing further stabilization of the phenoxyl radical. This is not surprising, considering that the caffeic acid is a precursor of flavonoid biosynthesis in plants (9). Accordingly, the absence of either a lateral double bond (e.g., protocatechuic acid) or the second OH group in the phenolic ring (e.g., *p*-coumaric acid) results in lower antioxidant effects (10). Additionally, Caf-OH displays one of the highest reactivities toward peroxy radicals ( $k = 1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ ) among phenolic antioxidants (11) and, consequently, is an efficient inhibitor of low-density lipoprotein (LDL) oxidation initiated by 2,2'-azobis(2-amidino-propane hydrochloride) (AAPH)-derived peroxy radicals (5, 12). Of note, the second-order rate constant value for the reaction of Caf-OH with  $\text{O}_2^-$  ( $0.96 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ) is 2–3 orders of magnitude greater than that for the reaction of several flavonoids (13).

The nature of the interactions of Caf-OH with other antioxidants is essential for understanding the effects of this compound in oxidative stress situations in vivo. Along these lines, it has been recently suggested that Caf-OH is able to regenerate  $\alpha$ -tocopherol at the LDL surface, in LDL particles subjected to oxidation by the hypervalent state of myoglobin, ferrylmyoglobin (14). The proposed mechanism for the reaction between Caf-OH and  $\alpha$ -tocopherol ( $\alpha$ -TOH) may entail the reduction of  $\alpha$ -tocopheroxyl radical ( $\alpha$ -TO $\cdot$ ) by Caf-OH at the LDL surface with concomitant production of caffeic acid *o*-semiquinone (Caf-O $\cdot$ ; reaction 1). A structurally related compound, *p*-coumaric acid (monohydroxycinnamic acid), not only failed to regenerate  $\alpha$ -TOH but also increased its consumption in LDL particles (14).



This study is aimed at establishing the dynamic interactions among  $\alpha$ -TOH, Caf-OH, and ascorbate ( $\text{AH}^-$ ) in terms of the sequence entailing transfer of the radical character required to accomplish optimal synergistic action. The experimental models used involve generation of the respective radical species by UV radiation, ferrylmyoglobin catalysis, and autoxidation. By means of electron paramagnetic resonance (EPR) spectroscopy and absorption spectroscopy and HPLC analysis of products, a set of coupled electron-transfer reactions are described, which may support the strong synergistic antioxidant effect of these compounds on LDL oxidation.

## EXPERIMENTAL PROCEDURES

**Chemicals and Biochemicals.** Ascorbic and caffeic acids (Fluka Chemical Co., Milwaukee, WI),  $\text{H}_2\text{O}_2$  (Merck, Darmstadt, Germany),  $\alpha$ -TOH (Aldrich Chemical Co., Milwaukee, WI), and horse heart metmyoglobin (Sigma Chemical Co., St. Louis, MO) were obtained from their respective manufacturers. The metmyoglobin was dialyzed against the following phosphate buffer [20 mM phosphate, 110 mM NaCl, and 50  $\mu\text{M}$  diethylenetriamine pentaacetic acid (DTPA)] and Chelex-100. Stock solutions of metmyoglobin and  $\text{H}_2\text{O}_2$  were standardized by using  $\epsilon_{632\text{nm}} = 2.1 \text{ mM}^{-1}\text{cm}^{-1}$  and  $\epsilon_{240\text{nm}} = 43.6 \text{ M}^{-1}\text{cm}^{-1}$ , respectively. Ferrylmyoglobin concentration was calculated by measuring absorbance at 550 and 630 nm and using the formula:  $[\text{ferrylmyoglobin}], \mu\text{M} = 249 \times A_{550\text{nm}} - 367 \times A_{630\text{nm}}$  (15). Solutions of Caf-OH were prepared before the experiments in water saturated with  $\text{N}_2$ . All other reagents were of analytical grade.

**Isolation and Treatment of LDL.** LDL particles were isolated from fresh human plasma by density-gradient ultracentrifugation and dialyzed by ultrafiltration under  $\text{N}_2$  atmosphere as previously described in a rapid two-step method (16). Using this procedure, one can obtain within 3 h a dialyzed concentrated fraction of LDL particles free from water-soluble plasma antioxidants.  $\alpha$ -TOH content in LDL was increased according to a published method (17); briefly, before LDL isolation, plasma was incubated with 250  $\mu\text{M}$   $\alpha$ -TOH from a stock solution in dimethyl sulfoxide (DMSO) under gentle stirring at 37  $^\circ\text{C}$ . To quantify  $\alpha$ -TOH, we supplemented 1-ml aliquots (LDL protein 180  $\mu\text{g}/\text{ml}$ ) with 100  $\mu\text{g}$  of butylated hydroxytoluene and extracted into hexane by the sodium dodecyl sulfate (SDS) method (18). Hexane extracts were separated by HPLC (Beckman; System Gold) on a LiChrospher 100 RP-18 (5  $\mu\text{m}$ ) column (Merck) eluted at 1.5 ml/min with a solvent of 65% methanol and 35% ethanol/isopropanol (95/5); eluted compounds were measured by UV detection (292 nm).

**EPR.** Spectra were recorded with a Bruker ECS 106 spectrometer at room temperature (22  $^\circ\text{C}$ ). Samples were transferred to bottom-sealed Pasteur pipettes, immediately inserted in the EPR cavity, and analyzed at the instrument settings described in the figure legends. An open (not sealed) Pasteur pipette directly connected to a 1-ml mixing cell was used as the cell cavity in the continuous-flow experiments. In the flow system the solutions of Caf-OH,  $\text{AH}^-$ , and  $\alpha$ -TOH-containing SDS micelles were prepared in the above-described phosphate buffer at pH 7.4. Computer simulation of caffeic acid radical was performed by using the hyperfine coupling constants published in the literature (19).

**Absorption Spectroscopy.** Measurements were carried out with a Perkin-Elmer  $\lambda 6$  UV/VIS spectrophotometer. After subtracting the spectrum for myoglobin (in the phosphate buffer described above), we added Caf-OH,  $\text{AH}^-$ , or both to the reaction mixture and initiated the reaction by addition of  $\text{H}_2\text{O}_2$ . Metmyoglobin and ferrylmyoglobin exhibited similar spectra within the wavelength range used.

**HPLC.** A 1-ml mixture of 50  $\mu$ M DTPA, 20  $\mu$ M Caf-OH, and 10  $\mu$ M metmyoglobin in phosphate buffer, pH 7.4, at 37  $^{\circ}$ C, was supplemented with 15  $\mu$ M H<sub>2</sub>O<sub>2</sub> to initiate the reaction. At different times 40  $\mu$ M ascorbate was added to the reaction mixture, followed by immediate treatment with 20  $\mu$ l of HClO<sub>4</sub> (85%) and centrifugation at 10 000g. The supernatant (examined for vestigial contamination with hemoglobin by absorption spectroscopy) was analyzed by HPLC on a Beckman System Gold consisting of a model 126 binary pump and a model 166 variable UV detector. The column, a LiChrospher 100 RP-18 (5  $\mu$ m) (Merck, Darmstadt, Germany) was eluted with a mixture of 2% acetic acid/25% isopropanol at a flowrate of 1 ml/min. Detection was carried out at 320 nm.

## RESULTS

### Reaction of Caf-OH and AH<sup>-</sup> with $\alpha$ -TO<sup>•</sup>

UV irradiation of  $\alpha$ -TOH-containing SDS micelles is an effective means for producing the EPR signal for  $\alpha$ -TO<sup>•</sup> (20) (Fig. 2A). The typical spectrum of  $\alpha$ -TO<sup>•</sup> was also observed when the  $\alpha$ -TOH-containing micelles were incubated with ferrylmyoglobin (not shown). Of relevance to this experimental design is that the hydrophobic  $\alpha$ -TO<sup>•</sup> does not diffuse out of the micelle (21).

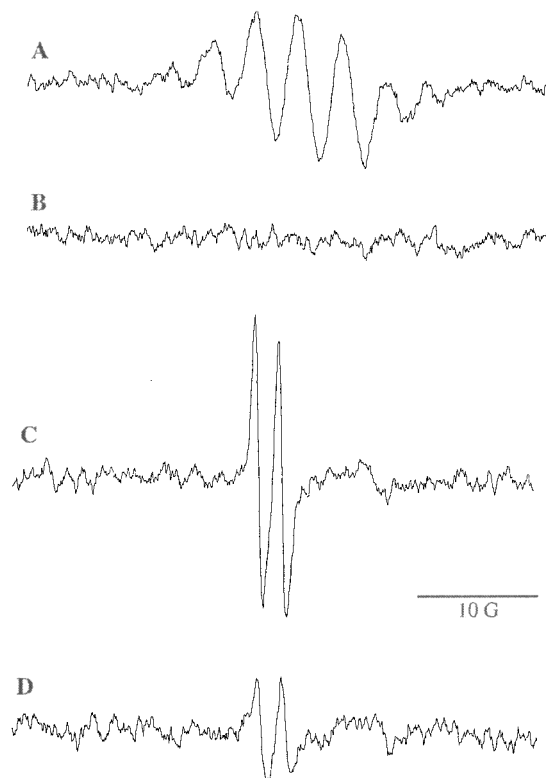
Addition of Caf-OH to the  $\alpha$ -TOH-containing micelles (after switching off UV radiation) resulted in suppression of the EPR signal (Fig. 2B). Under these conditions, no other EPR signal was observed, in agreement with the short-lived character of the Caf-O<sup>•</sup> formed (22). The EPR signal for the *o*-semiquinone can, however, be detected at pH 8.3 or by continuous flow (see below).

At variance with the Caf-OH results, in the presence of AH<sup>-</sup> the EPR spectrum of the  $\alpha$ -TO<sup>•</sup> was replaced by a doublet spectrum characteristic of the ascorbyl radical, A<sup>•-</sup> ( $a^H = 1.8$  G) (Fig. 2C), the lifetime of which is longer than that of other free radical species (23). The intensity of the A<sup>•-</sup> signal was diminished when Caf-OH was present in the assay mixture (Fig. 2D).

### Electron-Transfer Reactions between Caf-OH and AH<sup>-</sup>

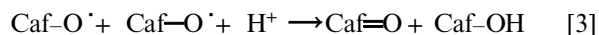
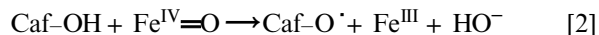
The decrease in the intensity of the signal for A<sup>•-</sup> by Caf-OH shown in Fig. 2D suggests an interaction between Caf-OH and AH<sup>-</sup>. This potential interaction was addressed with experimental models aimed at evaluating (a) by means of absorption spectroscopy and HPLC, the recovery of Caf-OH (oxidized by ferrylmyoglobin) by AH<sup>-</sup> (Fig. 3); and (b) by means of EPR spectroscopy, the effects of Caf-OH on the A<sup>•-</sup> (generated by incubation of AH<sup>-</sup> with ferrylmyoglobin or by autoxidation) (Figs. 4 and 5).

The time course of Caf-OH oxidation by ferrylmyoglobin is characterized by a decrease in Caf-OH by as much as 65% of the initial concentration, followed by a slight increase (Fig. 3A). The decrease in Caf-OH is consistent with the univalent oxidation of the phenolic acid by ferrylmyoglobin (reaction 2, where Fe<sup>IV</sup>=O is the oxoferryl moiety in ferrylmyoglobin). The slight increase

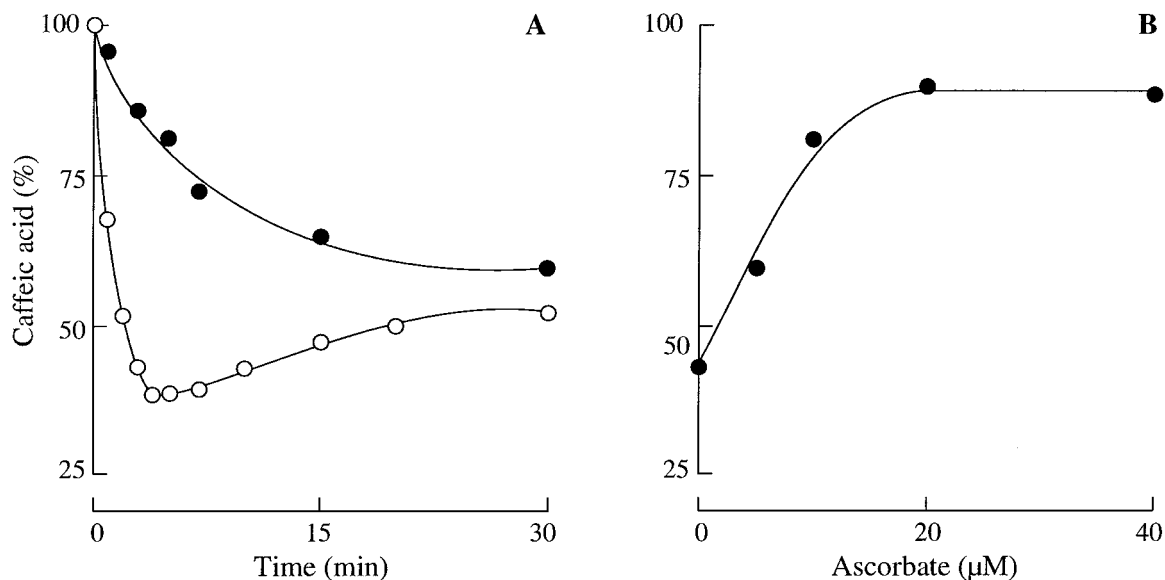


**Figure 2.** Reaction of caffeic acid and ascorbate with the  $\alpha$ -tocopheroxyl radical. (A) EPR spectra from UV irradiation of  $\alpha$ -TOH-containing SDS micelles: 1 mM  $\alpha$ -TOH was dispersed in SDS and irradiated for 5 min. (B) Effect of 0.2 mM Caf-OH on the spectrum in (A) after switching off the UV light. (C) As in (B) but with 0.2 mM AH<sup>-</sup> instead of Caf-OH, (D) As in (C) plus a subsequent addition of 0.2 mM Caf-OH. Instrument settings: microwave frequency, 9.8 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G; time constant, 0.65 s.

observed in the aforementioned time course may be attributed to the decay of Caf-O<sup>•</sup> by disproportionation (reaction 3), thereby regenerating the phenolic acid and the quinone form of Caf-OH; this is expected to be favored when high concentrations of the *o*-semiquinone build up.



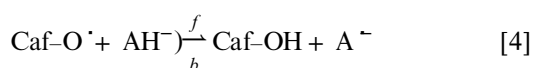
Recovery of Caf-OH by AH<sup>-</sup>, as analyzed by HPLC after protein precipitation with HClO<sub>4</sub>, was time- and concentration-dependent: Fig. 3A shows that the extent of recovery decreased as incubation time increased; the amount of Caf-OH recovered by AH<sup>-</sup> stayed constant for at least 30 min. Regardless of the time of addition, maximal recovery of Caf-OH is achieved with an AH<sup>-</sup> concentration of  $\sim$ 20  $\mu$ M (Fig. 3B); higher concentrations did not improve recovery. Similar results were obtained when Caf-OH recovery by AH<sup>-</sup> was assessed by UV spectroscopy (not shown). The recovery of Caf-OH by AH<sup>-</sup> can be



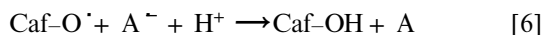
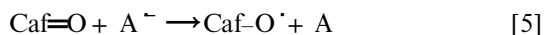
**Figure 3.** Effect of ascorbate on caffeic acid oxidation by ferrylmyoglobin. (A) (○) Time course of Caf-OH oxidation by ferrylmyoglobin. Assay conditions: 10  $\mu\text{M}$  metmyoglobin and 20  $\mu\text{M}$  Caf-OH in phosphate buffer, pH 7.4, containing 50  $\mu\text{M}$  DTPA, were supplemented with 15  $\mu\text{M}$   $\text{H}_2\text{O}_2$  to initiate the reaction. (●) 40  $\mu\text{M}$  ascorbate was added to the above reaction mixture at the times indicated, followed by protein precipitation with  $\text{HClO}_4$  and analysis of the supernatant by HPLC (as described in text). The levels of Caf-OH thus recovered by  $\text{AH}^-$  were maintained for 30 min. Results are the mean of 3 separate experiments. (B) Dependence of Caf-OH recovery on  $\text{AH}^-$  concentration. Assay conditions as in (A); the effects of the different ascorbate concentrations were examined after a 3-min incubation.

partly understood in terms of the transfer of the radical character shown in reaction 4.

Reaction 4<sub>r</sub> is thermodynamically feasible in view of the reduction potentials of the redox couples involved [ $E(\text{Caf-O}^\bullet, \text{H}^+/\text{Caf-OH}) = 0.54 \text{ V}$ ;  $E(\text{A}^{\bullet-}, \text{H}^+/\text{AH}^-) = 0.28 \text{ V}$ ] (23, 24). Because of the short life time of  $\text{Caf-O}^\bullet$ ,



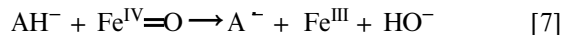
the effect of  $\text{AH}^-$  cannot be explained only as in reaction 4. Similarly to the interaction of flavonoid (aroxyl) radicals with  $\text{AH}^-$  (25), other reactions may contribute to the recovery of Caf-OH, namely, the reduction of caffeic acid quinone ( $\text{Caf}=\text{O}$ ) by  $\text{A}^{\bullet-}$  (reaction 5) and a radical-radical recombination involving  $\text{A}^{\bullet-}$  and  $\text{Caf-O}^\bullet$  (reaction 6). The reducing power inherent in the  $\text{A}^{\bullet-} \rightarrow \text{A}$  (dehydroascorbate) transition ( $E^{\circ'} = -0.17 \text{ V}$ ) (22) makes these reactions feasible.



None of the reactions listed above accounts for the decreased recovery of Caf-OH by  $\text{AH}^-$  as the incubation time increased (Fig. 3A); it is likely that oxidation products of Caf-OH, other than the quinone are formed and that these products cannot be reduced back to the phenolic compound by  $\text{AH}^-$ .

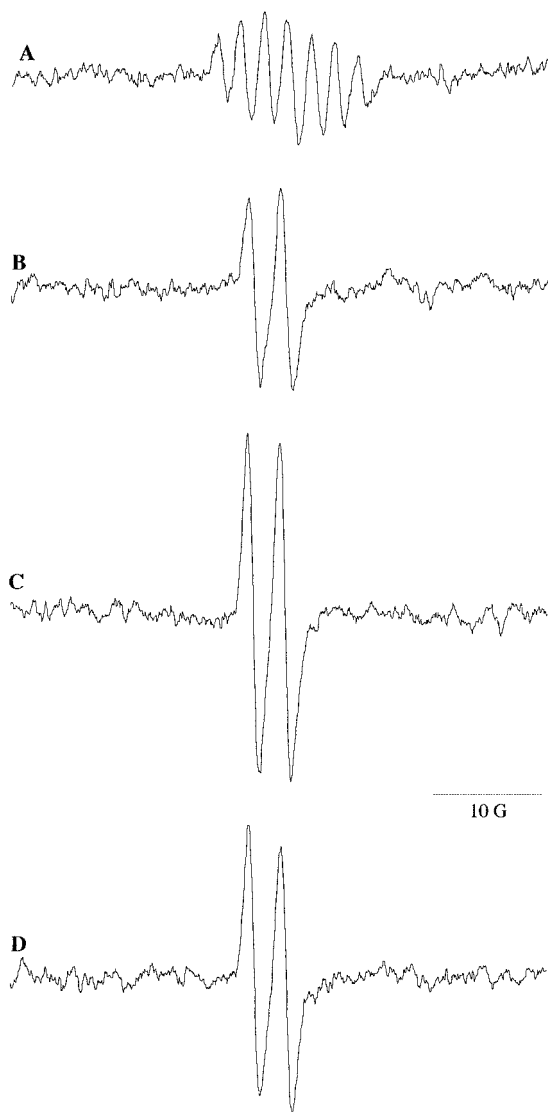
Incubation of Caf-OH and ferrylmyoglobin at pH 8.3 yielded a 7-line EPR spectrum characteristic of the  $\text{Caf-O}^\bullet$  (Fig. 4A) (19), in agreement with the electron transfer depicted in reaction 2 above. In the presence of  $\text{AH}^-$ , the EPR spectrum of the *o*-semiquinone was replaced by a doublet spectrum characteristic of  $\text{A}^{\bullet-}$  (Fig. 4B). As previously reported (26), the  $\text{A}^{\bullet-}$  signal may also be observed when  $\text{AH}^-$  is incubated with ferrylmyoglobin (reaction 7) in the absence of Caf-OH (Fig. 4C).

However, the intensity of the  $\text{A}^{\bullet-}$  signal thus obtained is attenuated by Caf-OH (Fig. 4D). The extent of this



attenuation increases as pH increases (up to pH 9.3), an effect that may be attributed to the monoanion form of caffeic acid ( $\text{Caf-O}^-$ ), considering the reported pK value for this compound (7.6) (24). Superoxide dismutase in catalytic concentrations ( $\sim 30 \text{ nM}$ ) had no effect on this experimental model, thus suggesting that  $\text{O}_2^{\bullet-}$  was not involved in the free radical chain.

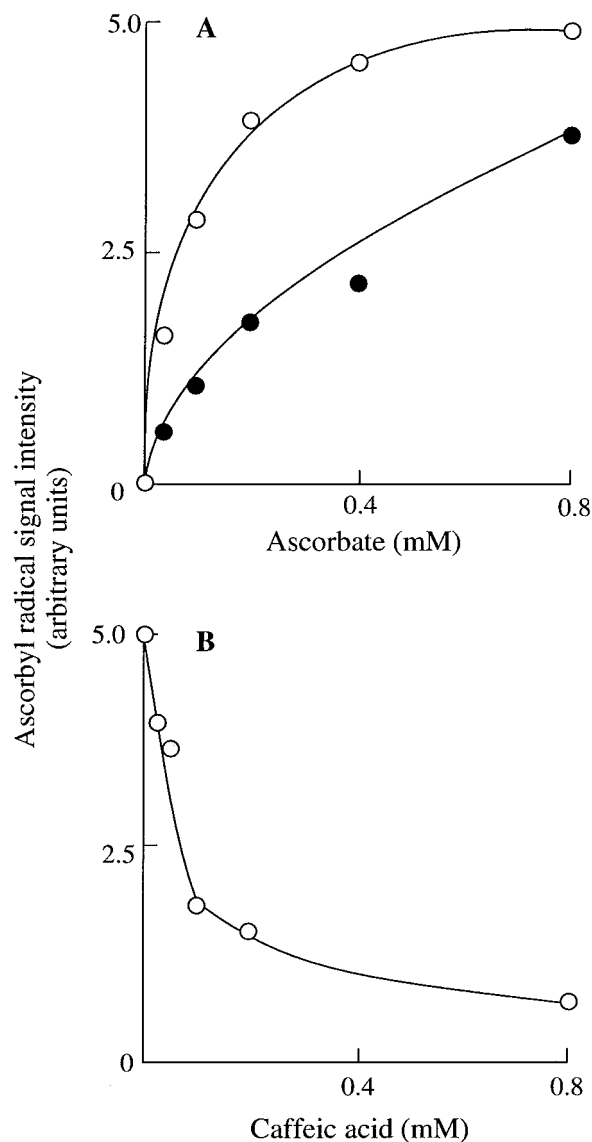
Further insight in the attenuation of the  $\text{A}^{\bullet-}$  signal by Caf-OH is provided by the experiments shown in Fig. 5. The  $\text{A}^{\bullet-}$  signal was obtained by  $\text{AH}^-$  autoxidation at pH 9.3, believed to arise from the ascorbate dianion ( $k \approx 10^2 \text{ M}^{-1}\text{s}^{-1}$ ) (27, 28). Under these conditions, the EPR signal intensity increases with increasing  $\text{AH}^-$  concentrations up to  $\sim 0.4 \text{ mM}$  (Fig. 5A) and is attenuated by Caf-OH in a concentration-dependent manner (Fig. 5B).



**Figure 4.** Ferrylmyoglobin-mediated oxidation of caffeic acid and ascorbate. (A) EPR spectrum of Caf-O $\cdot$ . Assay conditions: 0.2 mM Caf-OH and 10  $\mu$ M metmyoglobin in phosphate buffer, pH 8.3, were supplemented with 15  $\mu$ M H $_2$ O $_2$  to initiate the reaction. (B) 0.2 mM Caf-OH, 0.2 mM AH $^-$ , and 10  $\mu$ M metmyoglobin in phosphate buffer, pH 8.3, were supplemented with 15  $\mu$ M H $_2$ O $_2$  to initiate the reaction. (C) 0.2 mM AH $^-$  and 10  $\mu$ M myoglobin in phosphate buffer, pH 8.3, were supplemented with 15  $\mu$ M H $_2$ O $_2$  to initiate the reaction. (D) As in (C) plus 0.02 mM Caf-OH. Instrument settings as in Fig. 2.

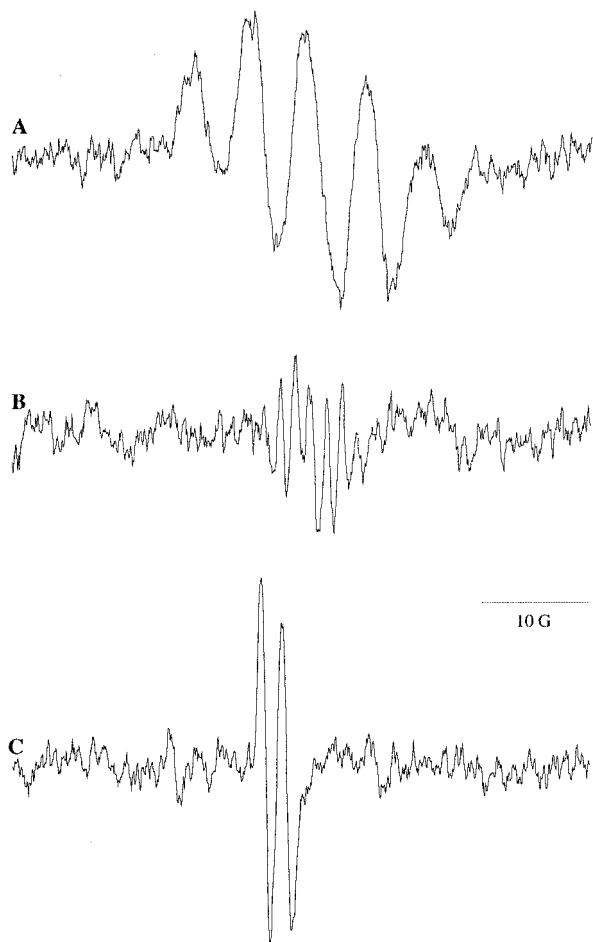
The attenuation of the A $\cdot$  signal [obtained by either incubation of AH $^-$  with ferrylmyoglobin (Fig. 4) or AH $^-$  autoxidation (Fig. 5)] by Caf-OH may be interpreted, in part, as reaction 4 $_b$  being operative or as a competition between reactions 2 and 7.

The experiments shown in Figs. 3–5 support the electron transfer depicted in reaction 4. The reduction potential of the caffeic radical is higher than that of AH $^-$  and, on thermody-



**Figure 5.** Effect of caffeic acid on the intensity of the ascorbyl radical signal. (A) EPR spectra of A $\cdot$  were recorded with different AH $^-$  concentrations (as indicated in the figure) in 0.1 M Tris buffer, pH 9.3, in the absence ( $\circ$ ) and presence ( $\bullet$ ) of 0.2 mM Caf-OH, (B) Effect of Caf-OH on A $\cdot$  EPR signal intensity obtained from a 0.2 mM AH $^-$  solution in .01 M Tris buffer, pH 9.3. Instrument settings: microwave frequency, 9.8 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.6 G; time constant, 0.65 s.

amic grounds, it is expected that  $k_{4f} > k_{4b}$ . If the concentration of Caf-OH is increased over that of AH $^-$ , the equilibrium for reaction 4 can be shifted to the left and a less intense A $\cdot$  signal is observed. The direction of electron flow depends on the relative concentrations of redox reactants; these results do not necessarily support an A $\cdot$  scavenging activity of Caf-OH *in vivo* (29).



**Figure 6.** Continuous-flow EPR measurements of mixtures of  $\alpha$ -tocopherol, caffeic acid, and ascorbate. Ferrylmyoglobin,  $\alpha$ -TOH-containing SDS micelles, caffeic acid, and ascorbate were sequentially introduced in the mixing cell. (A)  $\alpha$ -TO $\cdot$  detected after flowing  $\alpha$ -TOH-containing SDS micelles and the mixture metmyoglobin/ $\text{H}_2\text{O}_2$ . (B) Caf-O $\cdot$  detected after the flow of Caf-OH into the mixture. (C)  $\text{A}^-$  detected after the flow of  $\text{AH}^-$  into the system. Solutions containing  $100\ \mu\text{M}$  DTPA were partially saturated with He. Flow rate was  $15\ \text{ml}\ \text{min}^{-1}$ , and concentrations of  $\alpha$ -TOH, Caf-OH, and  $\text{AH}^-$  in the mixing cell were maintained at  $1\ \text{mM}$ ; those of metmyoglobin and  $\text{H}_2\text{O}_2$  were  $50\ \mu\text{M}$  and  $60\ \mu\text{M}$ , respectively. Instrument settings as in Fig. 2.

#### **Interaction of $\text{AH}^-$ , Caf-OH, and $\alpha$ -TOH Assessed by Continuous-Flow EPR**

Although the above data indicate that caffeic acid can independently interact with  $\alpha$ -TOH and  $\text{AH}^-$ , resulting in synergistic antioxidant effects, the potential dynamic interactions involving transfer of the radical character are not clear. The feasibility of coupled redox reactions among these compounds was further investigated by continuous-flow EPR.

Continuous-flow EPR measurements of a mixture of ferrylmyoglobin and  $\alpha$ -TOH-containing SDS micelles (in the amounts described in Fig. 6) resulted in an  $\alpha$ -TO $\cdot$  signal (Fig. 6A).

Flowing Caf-OH into the system resulted in the immediate replacement of the  $\alpha$ -TO $\cdot$  signal by the Caf-O $\cdot$  signal (Fig. 6B). Subsequent introduction of  $\text{AH}^-$  in the flowing mixture yielded the doublet spectrum characteristic of  $\text{A}^-$  (Fig. 6C).

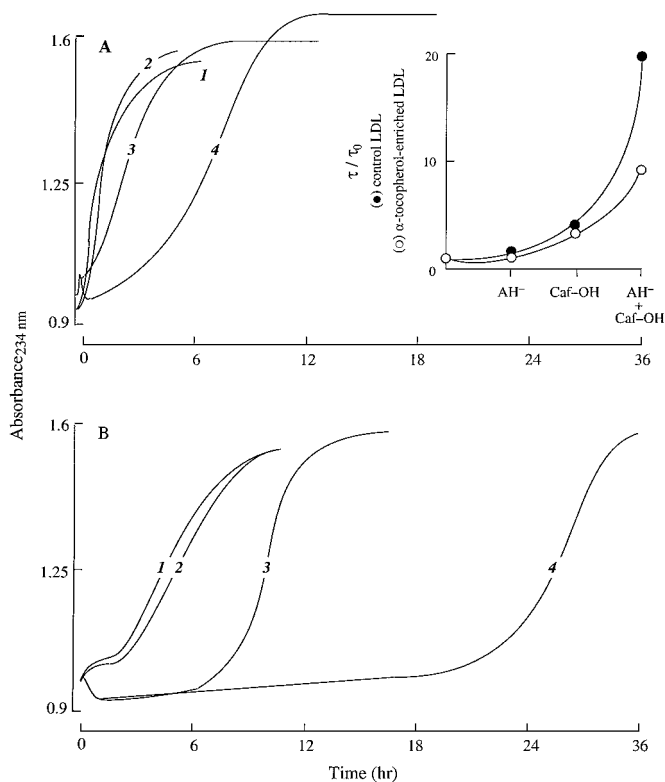
Continuous-flow EPR measurements of a mixture of ferrylmyoglobin and  $\text{AH}^-$  resulted in the  $\text{A}^-$  signal (not shown). Albeit of a lower intensity, this signal persisted when  $\alpha$ -TOH-containing micelles and Caf-OH were introduced sequentially into the system. When biological fluids are examined by EPR spectroscopy, the  $\text{A}^-$  signal would be most likely observed, thus suggesting a role for  $\text{AH}^-$  as the terminal small-molecule antioxidant (28).

These data suggest that  $\text{AH}^-$  is the ultimate electron donor and that reduction of the  $\alpha$ -TO $\cdot$  by Caf-OH and  $\text{AH}^-$  efficiently competes with the oxidation of Caf-OH and  $\text{AH}^-$  by ferrylmyoglobin.

#### **LDL Lipid Peroxidation in the Presence of $\alpha$ -TOH, $\text{AH}^-$ , and Caf-OH**

The effects of  $\alpha$ -TOH,  $\text{AH}^-$ , and Caf-OH (acting individually or synergistically) on lipid peroxidation were assessed in two LDL populations obtained from the same plasma sample: normal LDL and  $\alpha$ -TOH-enriched LDL ( $\alpha$ -TOH concentrations were 10.5 and 36.1 nmol/mg apoB protein, respectively). Each LDL population was incubated with ferrylmyoglobin, and Caf-OH or  $\text{AH}^-$  was added, alone or together. Ferrylmyoglobin-mediated oxidation of LDL has been shown to be a useful model for studying the reactions of  $\alpha$ -TOH with co-antioxidants in LDL. In fact, the rate of  $\alpha$ -TOH oxidation in LDL increases with increasing ferrylmyoglobin concentrations (14), and  $\alpha$ -TOH depletion is followed by the propagation phase of lipid peroxidation. Also, ferrylmyoglobin induces a typical pattern of LDL lipid peroxidation, the oxidation rate of cholesteryl esters being a function of unsaturation (30).

Fig. 7 shows a typical experiment of conjugated diene formation for the two LDL samples, the normal (Fig. 7A) and the  $\alpha$ -TOH-enriched (Fig. 7B), during incubation with ferrylmyoglobin. Expectedly, the lag phase observed with  $\alpha$ -TOH-enriched LDL particles was longer ( $\sim 7.5$ -fold) than that observed with the normal particles. The same pattern was observed when either LDL population was incubated with Caf-OH,  $\text{AH}^-$ , or both.  $\text{AH}^-$  had little effect on the duration of the lag phase (regardless of the  $\alpha$ -TOH content): The increase of lag phase duration was only 1.2- to 1.5-fold. Caf-OH was more effective in prolonging the lag phase, giving a 4.0- and 3.3-fold increase in control and  $\alpha$ -TOH-enriched LDL, respectively. The greatest effect was obtained when both  $\text{AH}^-$  and Caf-OH were present in the mixture: The combination gave 19.5- and 9.3-fold increases for native- and  $\alpha$ -tocopherol-enriched LDL, respectively. These values, which clearly differ from the sum of the lag phase durations elicited by the individual compounds, suggest a strong synergism among  $\alpha$ -TOH, Caf-OH, and  $\text{AH}^-$ . These relationships are summarized in the insert in Fig. 7, in which the ratio of the duration of the lag phase in the presence and the absence of the antioxidant ( $\tau/\tau_0$ ) is plotted for both LDL populations.

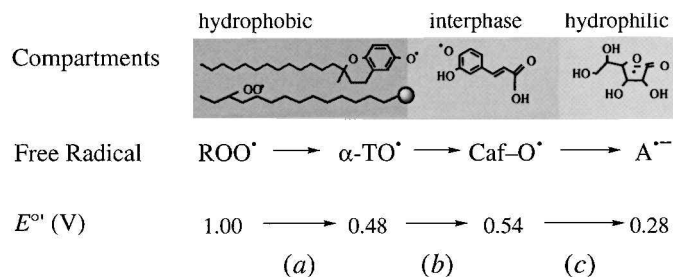


**Figure 7.** Effects of  $\alpha$ -tocopherol, caffeic acid, and ascorbate on LDL oxidation by ferrylmyoglobin. Assay conditions: (A) Control LDL particles (containing 10.5 nmol  $\alpha$ -TOH per milligram of apoB) and 9  $\mu$ M metmyoglobin in phosphate buffer, pH 7.4, were supplemented with 9  $\mu$ M H<sub>2</sub>O<sub>2</sub> to initiate the reaction. Line 1: no further addition; line 2: 2  $\mu$ M AH<sup>-</sup> added; line 3: 2  $\mu$ M Caf-OH added; line 4: mixture containing 2  $\mu$ M each of AH<sup>-</sup> and Caf-OH added. (B) As in (A) but experiments were carried out with  $\alpha$ -TOH-enriched LDL particles (36.1 nmol  $\alpha$ -TOH per milligram of apoB protein). Insert in (A) shows the ratio of the lag phase in the presence of antioxidants ( $\tau$ ) to that in the absence ( $\tau_0$ ) for control (●) and  $\alpha$ -TOH-enriched (○) LDL particles. Measurements as described in text.

## DISCUSSION

Analysis of the data presented in this study requires consideration of at least 3 domains for each antioxidant: (a) localization of the antioxidant and of the antioxidant-derived radical, (b) reduction potential (important to assess the thermodynamic feasibility of the reactions), and (c) the predominant decay pathways for the antioxidant-derived radical, which establish a kinetic control of these reactions. These relationships are addressed for the individual antioxidants below and summarized in the scheme in Fig. 8.

$\alpha$ -TOH is a chain-breaking antioxidant, acting by means of a typical reaction encompassing H transfer to secondary lipid peroxy radicals; a property of the  $\alpha$ -TO<sup>•</sup> produced in these reactions (in  $\alpha$ -TOH-containing micelles—as in this study—or in LDL) is that it does not diffuse out of the micelle. The polar nature of



**Figure 8.** Proposed redox transitions of  $\alpha$ -tocopherol, caffeic acid, and ascorbate and synergistic protection against free radicals.

the phenol group of  $\alpha$ -TOH results in this part of the molecule being at or near the water-membrane interface. This physical arrangement allows the reaction of the antioxidant-derived radical ( $\alpha$ -TO<sup>•</sup>) with water-soluble antioxidants. The reduction potential of the  $\alpha$ -TO<sup>•</sup>; H<sup>+</sup>/ $\alpha$ -TOH couple is 0.48 V. Within the scope of this study, the free radical decay pathways for the  $\alpha$ -TO<sup>•</sup> are largely encompassed by its reduction by AH<sup>-</sup> and Caf-OH. The reduction of 5,7-diisopropyl-TO<sup>•</sup> in Triton X-100 micelles by these compounds proceeds with similar second-order rate constants ( $\sim 10^3 \text{ M}^{-1}\text{s}^{-1}$ ), as determined by stopped-flow spectrophotometry (K. Mukai, personal communication).

The hydrophilic properties of polyphenols and flavonoids facilitate their localization at the interface of the lipid bilayers in membranes (31), thereby suggesting two advantages: both effective inhibition of attack by free radicals in the aqueous phase and effective repair of lipophilic free radicals (such as the  $\alpha$ -TO<sup>•</sup>). The reported reduction potential at pH 7 for the Caf-O<sup>•</sup>; H<sup>+</sup>/Caf-OH couple is 0.54 V (24), a value that may be lower at pH 7.4, given the Caf-OH pK of 7.6 (24). Despite the fact that Caf-OH displays one of the highest reactivities toward peroxy radicals ( $1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ ) among phenolic antioxidants (7, 11), only moderate chain-breaking antioxidative efficiencies were obtained with it, as measured by the ratio of chain-terminating to chain-propagating rate constants during the oxidation of methyl linoleate in SDS micelles (32). Most likely, this is related to the limited access of Caf-OH to lipid peroxy radicals inside SDS micelles. This supports the view that the antioxidant activity of Caf-OH is largely related to its localization at the bilayer surface where it can encompass the reduction of  $\alpha$ -TO<sup>•</sup> (reaction 1) rather than intercept directly any secondary lipid peroxy radicals. At least two free radical decay pathways for the Caf-O<sup>•</sup> relevant in this context may be considered: its recovery by aqueous-phase electron donors, such as AH<sup>-</sup> (reaction 4), and its disproportionation (reaction 3).

A similar analysis may be applied to the redox transitions of the water-soluble AH<sup>-</sup>, which is present in a gradient from plasma (0.01–0.1 mM) to the cell (up to 1 mM). AH<sup>-</sup> is a powerful electron donor in biosystems, a property consistent with its role as the terminal small-molecule antioxidant (28). The one-electron reduction potential of most antioxidants is greater than that of ascorbate (0.28 V), and the antioxidant-derived radical

(A<sup>•</sup>) decays largely by disproportionation ( $2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ) (28) or may be recycled by enzyme systems.

Steps (b) and (c) in Fig. 8, implying transfer of the radical character from the  $\alpha\text{-TO}^\bullet$  to Caf-OH and from the Caf-O<sup>•</sup> to AH<sup>-</sup>, respectively, require special consideration. In step (b), the reduction potentials of the  $\alpha\text{-TO}^\bullet$  and the Caf-O<sup>•</sup> indicate that the latter is a slightly stronger oxidant than the former. However, these reduction potentials are of little prognostic value when the actual concentrations of free radicals and electron donors are taken into account. The concentration of vitamin E in membranes and LDL, calculated in an integrative kinetic model from the molar ratio between phospholipids and vitamin E, is  $\sim 10^{-4} \text{ M}$ , and the ratio  $[\alpha\text{-TO}^\bullet]/[\alpha\text{-TOH}]$  in membranes has been estimated as  $1.7 \times 10^{-6}$  (33). On the other hand, Caf-OH can reach micromolar levels in plasma in a diet-dependent manner (34). Hence, one may surmise that the plasma Caf-OH concentration is far more than that of  $\alpha\text{-TO}^\bullet$ , thereby overcoming the thermodynamic constraints referred to above. Interestingly, it was recently reported that dietary supplementation of Caf-OH in rats (35) and of wine phenolics (including Caf-OH) in humans (36) resulted in an increase of  $\alpha\text{-TOH}$  in LDL.

Step (c) in Fig. 8 assumes that AH<sup>-</sup> is the ultimate reductant; however, the present study does not provide unequivocal support for this. That reaction 4 is reversible is suggested by the recovery of Caf-OH by AH<sup>-</sup> (reaction 4<sub>r</sub>) (Fig. 3) and by the decrease of the A<sup>•</sup> signal by Caf-OH (reaction 4<sub>b</sub>) (Figs. 4 and 5). In a physiological situation, however, the reduction of Caf-O<sup>•</sup> by AH<sup>-</sup> is expected to prevail, given that the plasma concentration of AH<sup>-</sup> (albeit within large variations) may be 10- to 100-fold more than that of Caf-OH. We do not provide the rate constant for the reaction of Caf-O<sup>•</sup> with AH<sup>-</sup>; however, the second-order rate constant for the reaction of AH<sup>-</sup> with flavonoid radicals (e.g., quercetin) is quite high ( $4.75 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ) (25). From the structural similarities between quercetin and Caf-OH (Fig. 1), one may presume that the reduction of Caf-O<sup>•</sup> by AH<sup>-</sup> (reaction 4<sub>r</sub>) proceeds rapidly. In this context, AH<sup>-</sup> inhibits the oxidation of Caf-OH by soluble and cell-wall peroxidases (enzymes involved in the lignification process) in plants, through a mechanism probably involving the reduction of phenoxy radicals by AH<sup>-</sup> (37).

As mentioned above, ferrylmyoglobin-mediated oxidation of LDL is a useful model for studying the reactions of  $\alpha\text{-TOH}$  with co-antioxidants in LDL. However, this experimental model adds a level of complexity to the above analysis. Ferrylmyoglobin is a strong oxidant with a reduction potential of 0.99 V (38) and a high chemical reactivity attributable to its oxoferryl moiety or a protein radical delocalized onto an aromatic amino acid, or both (39, 40). Because ferrylmyoglobin catalyzes the one-electron oxidation of the 3 antioxidants studied here ( $\alpha\text{-TOH}$ , Caf-OH, and AH<sup>-</sup>), complex mutual interactions can occur in the system. Thus, it is possible that in experiments addressing the effect of Caf-OH on LDL oxidation (Fig. 8), the interaction of the phenolic compound with ferrylmyoglobin could temporarily prevent the oxidation of  $\alpha\text{-TOH}$ . However, the data in Fig. 8 clearly

show that the combined effects of Caf-OH and AH<sup>-</sup> result in a synergistic antioxidant protection against LDL oxidation and that the sequence of redox cycles depicted in Fig. 8 amplifies the antioxidant capacities of the individual compounds.

A similar synergistic interplay involving Caf-OH and AH<sup>-</sup> accounts for the increase in the lag phase preceding the accumulation of cholesteryl linoleate hydroperoxide (quantitatively the major peroxide in LDL) and 7-ketocholesterol in LDL (30). At variance with Caf-OH, AH<sup>-</sup> alone failed to delay the onset of formation of cholesteryl linoleate hydroperoxide and 7-ketocholesterol. These findings strengthen the view implying regeneration of Caf-OH by AH<sup>-</sup>. At a cellular level, Caf-OH was shown to protect endothelial cell lines against apoptosis induced by oxidized LDL; this acquires further relevance when considering that endothelial defects are associated with platelet adhesion and thrombi formation (6).

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