Catechins Delay Lipid Oxidation and α-Tocopherol and β-Carotene Depletion Following Ascorbate Depletion in Human Plasma (44549)

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Abstract. **Blood plasma was incubated with 50 m*M* AAPH [2,2'-azobis-(2-amidinopropane) hydrochloride] in the absence or presence of catechins (5–100 μ*M*). Lipid oxidation was evaluated by measuring the formation of 2-thiobarbituric acid reactive substances (TBARS). The concentration of α-tocopherol (AT), β-carotene (BC), ascorbic acid (AA), and catechins was determined by reverse phase high performance liquid chromatography (HPLC) with electrochemical detection. All the assayed catechins inhibited plasma TBARS formation. Based on the calculated IC_{50}, the order of effectiveness was: epicatechin gallate (ECG) > epigallocatechin gallate (EGCG) > epigallocatechin (EGC) > epicatechin (EC) > catechin (C). Catechins protected plasma AT and BC from AAPH-mediated oxidation. The order of effectiveness for AT protection was ECG > EGCG > EC = C > EGC; and for BC protection, the order was EGCG > ECG > EGC > > EC > C. The addition of catechins modified the kinetics of TBARS formation and AT depletion, but the rate of AA depletion was not affected. Catechin oxidation did not start until the complete depletion of AA, and it preceded AT depletion. These results indicate that catechins are effective antioxidants in human blood plasma, delaying the lipid oxidation and depletion of endogenous lipid-soluble antioxidants (AT and BC).**

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Several epidemiological studies have shown correlations between a higher content of flavonoids in the diet and a risk of cancer and coronary heart disease mortality (1–4). These associations were mainly ascribed to the antioxidant capacity of these compounds (5, 6).

Catechins are a group of flavonoids that have attracted particular attention due to their relative high antioxidant capacity in biological systems (7–11) and their abundance in the human diet. Catechins are present in vegetables and plant-derived beverages and foods, like red wine, tea, and chocolate (6, 12–14). Chemically, catechins are polyhydroxylated flavonoids that exhibit water-soluble characteristics. The catechins that are most widely distributed in the diet are (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG), which differ in the number and position of the hydroxyl groups in the molecule (Fig. 1).

Increasing evidence suggests that lipoprotein oxidation is involved in the development of cardiovascular lesions (15–17). Thus, plasma antioxidants may play a role by protecting lipoproteins from oxidation, then delaying or preventing the development of cardiovascular pathologies (18, 19).

To assess the relevance of catechins as antioxidants in human plasma, the *in vitro* capacity of catechins to prevent plasma lipid oxidation was, and to delay the oxidation of other plasma antioxidants. Since the antioxidant capacity of the catechins has been largely related to the presence and

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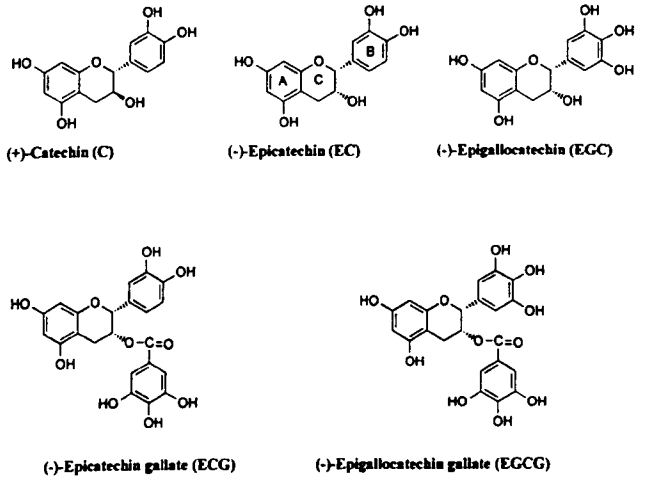

Figure 1. Chemical structure of catechins.

positions of the hydroxyl groups (20), the relationship between the chemical structure of several catechins and their antioxidant capacity in plasma was studied.

Materials and Methods

Chemicals. C, EC, and ECG were purchased from Sigma Chemical Co. (St. Louis, MO). Dr. Yukihiko Hara (Mitsuri Norin Co. Ltd., Shizuoka, Japan) kindly supplied EGC and EGCG. 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was from Polysciences Inc. (Warrington, PA). All the solvents used were HPLC-grade from Merck Quimica Argentina S. A. (Buenos Aires, Argentina) and J. T. Baker Inc. (Phillipsburg, NJ). All other chemicals used were of the highest purity available.

Blood Plasma Preparation. Blood was collected from healthy humans by venipuncture, using heparin sulfate to prevent clotting. After centrifugation at 1000*g* for 10 min at room temperature, plasma was separated from the blood cell package, and used immediately for analysis. When necessary, plasma samples from different subjects were combined in a single experimental pool. Plasma AA concentration defined the time for comparing values from different samples. The rationale used when it was necessary to compare different plasma samples (or pools) was to determine the different variables 180 min after the total depletion of AA. The initial concentrations of AA in the plasmas ranged from 50 to 80 μ*M*, which are within the range of a healthy population (21).

Human Plasma Oxidation. Human plasma was oxidized by incubation in the presence of AAPH, which generates alkyl peroxyl radicals at a constant rate (22). AAPH was dissolved in distilled water and prepared immediately before using. All catechins were dissolved in methanol at a concentration of 1 m*M* and then diluted in distilled water to the concentrations assayed. Plasma samples were incubated in the presence of 50 m*M* AAPH, with or without the previous addition of catechins (5–100 μ*M*). The incubations were performed at 37°C, with continuous shaking, under air. Aliquots were removed at different times and analyzed for lipid oxidation products, antioxidants, and catechins.

Determination of 2-Thiobarbituric Acid Reactive Substances (TBARS). The formation of lipid oxidation products was evaluated as TBARS (23). After incubation, plasma aliquots (100 μl) were added with 50 μl of butylated hydroxytoluene (4% w/v in ethanol), 500 μl of sodium dodecyl sulfate (0.3% w/v), 2 ml of 0.1 *N* HCl, and 0.3 ml of phosphotungstic acid (10% w/v). After vortexing, 1 ml of 0.7% (w/v) 2-thiobarbituric acid was added, and the samples were maintained in boiling water for 45 min. TBARS were extracted with 3 ml of butanol and quantitated fluorometrically (515 nm, excitation; 555 nm, emission). TBARS are expressed as malondialdehyde equivalents. Malondialdehyde standard was prepared from 1,1,3,3-tetraethoxy propane.

Determination of α-Tocopherol (AT) and β-Carotene (BC). The determination of lipid-soluble antioxidants was carried out by high performance liquid chromatography (HPLC) with electrochemical detection (24). After incubation, plasma samples (100 μl) were added to 30 μl of 4% (w/v) butylated hydroxytoluene, and proteins were precipitated with methanol (200 μl). Hexane (4 ml) was added, the samples were vortexed vigorously for 1 min, and the tubes were centrifuged at 600*g* for 10 min. A 3-ml aliquot of the hexane phase was dried under nitrogen, and the residue was resuspended in methanol:ethanol (1:1, v:v). The resulting suspensions were filtered through a 0.22-μm nylon membrane and subjected to isocratic reverse phase chromatography, using a C-8 column (3.3 cm × 4.6 mm), and 20 m*M* $LiClO_4$ in methanol:H_2O (97.5:2.5, v:v) as mobile phase. AT and BC were detected electrochemically at +0.6 V using a BAS LC4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN).

Determination of Ascorbic Acid. Plasma AA was determined according to Lykkesfeldt *et al.* (25) with modifications. Plasma aliquots (100 μl) were precipitated with 200 μl of 10% (w/v) metaphosphoric acid, vortexed, and centrifuged at 10 000*g* for 4 min. Samples were kept at 0°–4°C during the whole procedure. A volume from the supernatant (90 μl) was diluted with 300 μl of 0.8% (w/v) metaphosphoric acid, filtered through a 0.22-μm nylon membrane, and subjected to isocratic reverse phase chromatography, using a C-18 column (3.3 cm × 4.6 mm) and 0.8% (w/v) metaphosphoric acid as mobile phase. AA was electrochemically detected (+0.6 V) using an ESA Coulochem II coulometric electrochemical detector, equipped with a Model 5011 analytical cell.

Determination of Catechins. Plasma samples were analyzed for the content of EC and ECG. Plasma (100 μl) were treated with methanol (300 μl), vortexed, and centrifuged at 10,000*g* for 10 min. An aliquot of the supernatant was filtered through a 0.22-μm nylon membrane and subjected to isocratic reverse phase chromatography using a C-18 column (15.0 cm × 4.6 mm) (26). The mobile phases consisted of methanol:H_2O:formic acid, 19:70:1 (v:v:v) for

EC, and 49:50:1 (v:v:v) for ECG, containing 30 m*M* $LiClO_4$ in both cases. The catechins were determined electrochemically (+0.8 V) using the amperometric detector mentioned above.

Statistical Analysis. Student's *t* test was used to compare two groups of data. Analyses were performed using routines available in Staview for Windows version 5.0.1. (SAS Institute Inc., Cary, NC).

Results

Dose-Dependent Effects of Catechins on TBARS Formation, and α-Tocopherol and β-Carotene Depletion in Human Plasma: Inhibitory Concentration 50 (IC_{50}). Considering that the concentration of AA was 75 μ*M* in the pool of plasma used in these experiments, the IC_{50} for the different catechins was calculated from the values obtained after 300 min of incubation.

Oxidative damage to lipids was evaluated as TBARS in the plasma treated with 50 m*M* AAPH, during 300 min at 37°C. In the absence of catechins, TBARS formation reached a maximal value of 6.3 ± 0.3 μ*M* (79% increase above nonstimulated). Figure 2A shows the effect of the addition of catechins (5–100 μ*M*) on TBARS formation. All the catechins tested inhibited TBARS formation in a dose-dependent manner. The magnitude of the inhibition depended on catechin structure and concentration. The inhibition of TBARS formation attained with different catechin concentrations was used to calculate the IC_{50}(Table I). Based on these IC_{50} values, the order of antioxidant effectiveness was ECG > EGCG > EGC > EC > C.

The concentration of lipid-soluble antioxidants was determined in the same human plasma pool. Figure 2B shows the effect of the catechins on AT depletion. In the absence of catechins, the incubation with AAPH caused the complete depletion of plasma AT. The addition of catechins (5–100 μ*M*) prevented the depletion of AT, and this effect depended on the catechin structure and concentration. At a catechin concentration of 5 μ*M*, the remaining AT was less than 20% in plasma supplemented with any of the tested catechins. At a catechin concentration of 100 μ*M*, all the catechins were equally effective in the protection of AT. The inhibition of AT depletion attained with catechins was used to calculate the IC_{50} (Table I). Based on these IC_{50} values, the order for AT protection was ECG > EGCG > EC = C > EGC.

The concentration of BC in human plasma was also determined after 300 min of incubation with 50 m*M* AAPH. Figure 2C shows the effect of the catechins on BC depletion. In the absence of added catechins, the incubation with AAPH caused the complete depletion of plasma BC. The addition of catechins (5–100 μ*M*) prevented the depletion of BC, and this effect depended on the catechin structure and concentration. The inhibition of BC depletion attained with catechins was used to calculate the IC_{50} (Table I). Based on these IC_{50} values, the order for BC protection was EGCG > ECG > EGC > > EC > C.

Effect of Catechins on the Kinetics of Depletion of AT and TBARS Production. Plasma containing 50 μ*M* AA was used to test the effect of the catechins on the kinetics of α-tocopherol depletion and TBARS formation (Fig. 3). Human plasma was incubated with 50 m*M* AAPH, in the absence or presence of 100 μ*M* catechin, and the kinetics of AT depletion and TBARS formation were followed for up to 360 min.

In the absence of added catechins, AT depletion started after 120 min of incubation in the presence of AAPH, at a rate of 85 n*M*/min, reaching a complete depletion at 360 min. When TBARS production was analyzed in the absence of catechins, a lag phase of 60 min was observed, after which TBARS production increased exponentially at an average rate of 12 n*M* TBARS/min. When catechins were added (100 μ*M* initial concentration), both AT depletion and TBARS production kinetics were affected. The lag phases for AT depletion and TBARS production were significantly increased by the presence of catechins, and the effect was different depending on the type of catechin assayed (Figs. 3A & 3B). For AT depletion, the longer lag phases were observed in the presence of ECG and EGCG, since no depletion of AT was detected after 360 min of oxidation. No effect on the lag phase of AT depletion was observed for EC and EGC (120 min), but both catechins lowered the AT depletion rate from 85 to 18 and 19 n*M*/min, respectively. In the presence of catechins, the lag phase of TBARS production was increased to 120 min for EC and EGC, and to 240 min for ECG. AAPH-dependent production of TBARS was completely prevented by 100 μ*M* EGCG during the 360 min of incubation.

Effect of Catechins on the Kinetics of AA Depletion. The effects of the addition of different catechins on AA depletion were studied in a human plasma pool that contained an initial concentration of AA of 57.0 ± 6.4 μ*M*. Figure 4 shows the remaining concentration of AA in human plasma as a function of the time of incubation in the presence of 50 m*M* AAPH. The addition of catechins (100 μ*M*, initial concentration) did not modify the kinetics of depletion of AA, which was completely depleted by 60 min.

Kinetics of Antioxidant Depletion and TBARS Formation. The kinetics of EC and ECG depletion were studied using the experimental conditions described above (Fig. 5). Plasma was incubated with 50 m*M* AAPH and supplemented with 100 μ*M* EC or ECG. A 60-min lag phase in the depletion of EC or ECG was observed, after which the depletion of the catechins started following a first-order kinetic decay. This lag phase was independent of the type of catechin tested, and of catechins' initial concentrations, finishing when AA depletion was almost complete (Lotto SB, Fraga CG, unpublished data). In the EC-supplemented plasma, a linear decrease in AT concentration was observed (15% depletion at 240 min). TBARS were detected only after 240 min of incubation, increasing exponentially until 360 min. In the ECG-supplemented plasma, AT concentra-

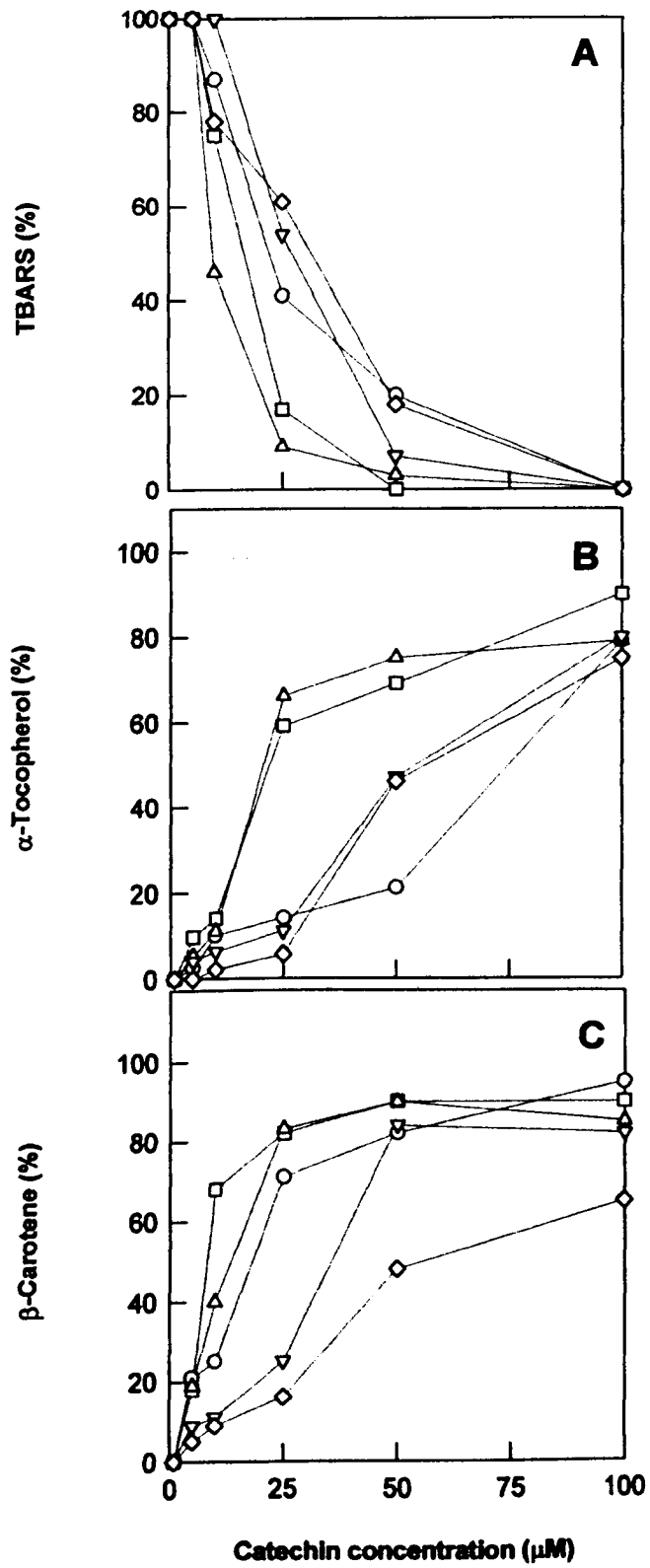

Figure 2. Effect of catechins on (Panel A) TBARS formation and (Panel B) AT and (Panel C) BC depletion. Human plasma was incubated at 37°C with 50 m*M* AAPH for 300 min in the absence or presence of different concentrations of catechins (5–100 μ*M*). TBARS formation is expressed as the percentage of the value obtained when plasma was incubated in the absence of added catechins. AT and BC are the percentages of the basal value. SEM was smaller than 5%. EGC (open circle); EGCG (square); ECG (triangle); EC (inverted triangle); C (diamond).

tion did not change during the 360-min incubation. Meanwhile, plasma TBARS were not modified until 240 min (Fig. 5).

Discussion

In this study, we investigated the effects of several catechins on both lipid oxidation and the depletion of antioxidants in human plasma treated with an oxidizing agent.

Using a pool of plasma incubated in the presence of 50 m*M* AAPH, it was observed that all the assayed catechins (i.e., C, EC, ECG, EGC, and EGCG) prevented TBARS formation. This antioxidant activity depended on the type of catechin assayed. Based on their capacity to inhibit TBARS formation, an order of effectiveness was established by calculating the IC_{50} for the different catechins. ECG and EGCG were the most effective in delaying TBARS formation, suggesting that the gallate group in position 3 is important for the antioxidant activity. By comparing EGC with EC and C, or EGCG with ECG, it can be concluded that the presence of a hydroxyl group in the position 5′ of the catechol structure did not increase the antioxidant capacity of these catechins significantly. The established order was in good agreement with a previous work that ranked the antioxidant activity of catechins by their capacity to scavenge water-soluble radicals generated in a pure chemical system (27). According to these findings, catechins could be acting as scavengers of radicals in plasma, preventing reactive species from reaching lipid domains and oxidizing plasma lipoproteins and other blood components.

The orders of effectiveness obtained from the IC_{50} for AT or BC were slightly different. However, the gallate group in position 3 again was relevant to the antioxidant capacity of catechins, as suggested by the fact that ECG and EGCG were the most effective. C and EC were very similar in antioxidant effectiveness, which indicates that the antioxidant activity is not determined by the stereoisomerism of these two molecules.

From the data for TBARS formation and AT and BC depletion, it can be concluded that the presence of the gallate group affords a significant increase in the antioxidant capacity of catechins. That property goes beyond the increased number of hydroxyl groups per molecule, since no significant differences were found between ECG and EGCG antioxidant capacities. A gallate group in position 3 of the C ring could favor the reaction with oxygen-centered radicals, and/or could determine a higher chemical stabilization of the catechin radical.

To further assess the mechanisms involved in the antioxidant capacity of catechins, we studied the kinetics of catechins, AA, AT, and BC depletion in oxidizing human plasma. As we found previously for C (28), none of the assayed catechins protected AA from oxidation at a catechin concentration range of 10–100 μ*M*. On the contrary, AA acted, preventing catechin depletion as indicated by the fact that no depletion of catechins was observed within the first 60 min of incubation, which corresponded to the time of

Table I. IC_{50} for Catechin Inhibiting Plasma TBARS Formation, and AT and BC Depletion

	IC_{50} (μM)				
	EGC	EGCG	ECG	EC	C
AT oxidation	66.3^{a}	22.8^{b}	20.7^{b}	56.5^{a}	58.7^{a}
BC oxidation	$17.7^{a,d}$	8.3^{b}	$13.5^{b,d}$	35.4^{c}	57.3^{c}
TBARS production	20.8^{a}	15.6^{b}	9.4^{b}	28.1^{a}	32.3^{a}

Note. Human plasma was incubated at 37°C with 50 m*M* AAPH for 5 hr in the absence or presence of different concentrations of catechins (5–100 μ*M*).

* IC_{50} is the concentration of catechin that inhibited the 50% of TBARS formation, or AT or BC depletion. Data in the same row having different superscripts are significantly different ($P < 0.05$).

complete AA depletion. The reduction potentials of catechins depend strongly on the electron-properties of the substituents in the B-ring (29). Bors *et al.* (30) found that flavonoids bearing a catechol structure in the B-ring, generally have higher reduction potentials than AA. The reduction potentials of C, EGC, and EGCG were reported to be +0.54, +0.43, and +0.43 V (pH 7.0 and 20°C), respectively (29), values that are in agreement with that reported for the catechol group (+0.53 V), and far above the reduction potential of AA (+0.28 V) (31). Recently, Jürgensen and Skibsted (32) reported lower reduction potential values for catechins and AA (pH 7.4), but the value for AA was still below the catechin value. Considering these reports and the present results, a redox couple AA-catechin is thermodynamically feasible, and AA could regenerate the flavonoid from the respective aroxyl radical, similarly to the postulated redox coupling AA-AT (30, 31). The hypothetical occurrence of such recycling *in vivo* is supported by the present results obtained in whole human plasma, as well as by previous results obtained in a pure chemical system, in which the rate of C oxidation was dependent on the concentrations of AA (28). Furthermore, when oxidation occurred in plasma depleted of AA, we did not observe a lag time for C depletion after incubation in the presence of AAPH (26).

In the absence of added catechins, lipid oxidation (TBARS formation) did not start until complete AA depletion. This observation agrees with a previous report using a similar system (33). But, in the presence of EC or ECG, TBARS formation did not start until AA and catechins were almost depleted. These results support the idea that catechins could protect lipids from oxidation in human plasma following ascorbate depletion.

Moreover, catechins exert a significant protection to AT and BC, suggesting that these compounds could act as antioxidants of intermediate reactivity, between the water-soluble AA and the lipid-soluble AT and BC, as was suggested by the comparison of catechin reduction potential with those for AT and BC. This AT protection has been observed previously in plasma depleted of AA (26) and in *ex vivo* LDL oxidation (27, 34). The maintenance of the concentration of AT, which is claimed to be important for different cell functions beyond its antioxidant capacity (35, 36), could explain the health effects linked to a high consumption of foods rich in polyphenols.

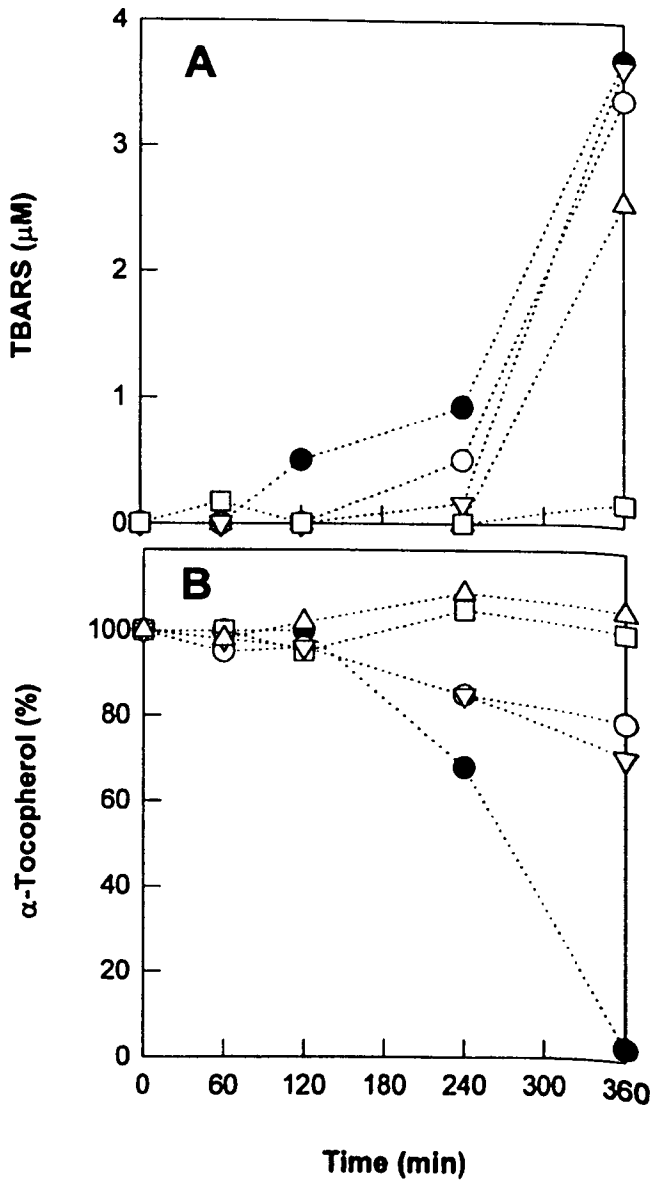

Figure 3. Effect of catechins on the kinetics of (Panel A) TBARS formation and (Panel B) AT depletion. Human plasma was incubated at 37°C with 50 m*M* AAPH in the absence (filled circle) or presence of 100 μ*M* catechins: EGC (open circle); EGCG (square); ECG (triangle); EC (inverted triangle). TBARS formation is expressed as the amount of fluorophere formed during the incubation. AT is the percentage of the zerotime value. SEM was smaller than 5%.

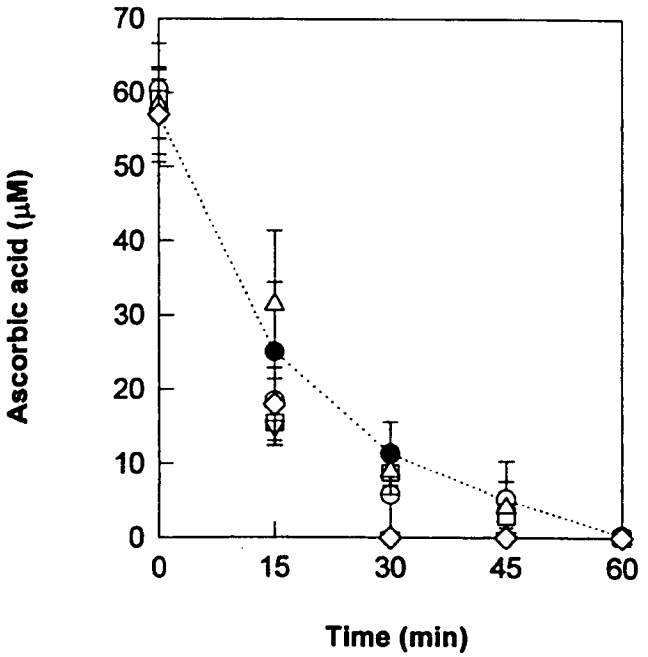

Figure 4. Effect of catechins on AA depletion. Human plasma was incubated at 37°C with 50 m*M* AAPH in the absence (filled circle) and presence of 100 μ*M* catechins: EGC (open circle); EGCG (square); ECG (triangle); EC (inverted triangle). SEM was smaller than 10%.

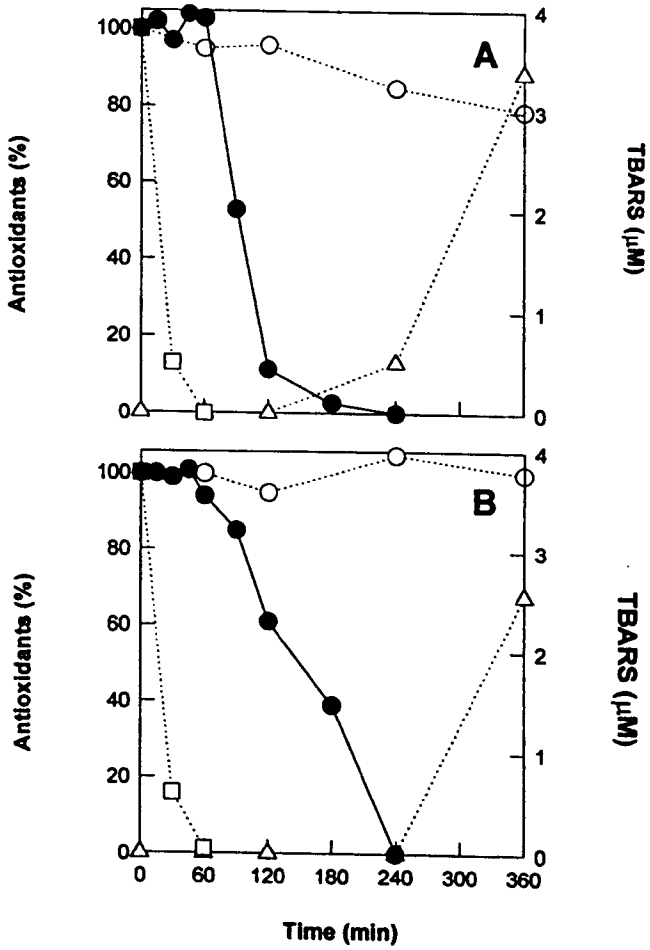

Figure 5. Kinetics of antioxidant depletion and TBARS formation. Human plasma was incubated at 37°C with 50 m*M* AAPH in the presence of 100 μ*M* (Panel A) EC or (Panel B) ECG. EC and ECG (filled circle), AA (open square), and AT (open circle) are expressed as a percentage of the zerotime value. TBARS formation (open triangle) is expressed as the amount of fluorophere formed during the incubation. SEM was smaller than 10%.

The physiological significance of the present study can also be discussed considering the very low amount of nonconjugated catechins present in plasma and/or other target tissues. It was shown that most of the catechins and plant polyphenols consumed in the diet are conjugated in the intestine and liver, and are mainly present in the tissues as methylated, glucuronides, and/or sulfated metabolites (37, 38). The antioxidant activity of these conjugates seems to be similar to that of nonconjugated catechins, as suggested by a study of Manach *et al.* (39) showing that conjugated derivatives of the flavanol quercetin retain the antioxidant capacity of the nonconjugated quercetin.

This work describes the relevance of different catechins as antioxidants in plasma, and their relationships with relevant nonenzymatic antioxidants, supporting the inclusion of catechins in the antioxidant network of human plasma. These findings are of significance considering both that catechins are plant-derived nutrients widely present in human diet, and the correlation found between a high consumption of vegetables and a lower risk of several chronic diseases. The protection of plasma lipid oxidation and lipid-soluble antioxidant depletion by catechins could be a mechanism for the prevention or delay of the development of pathologies related to free radical damage.

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