

Effect of antioxidant protection by *p*-coumaric acid on low-density lipoprotein cholesterol oxidation

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Zang, Lun-Yi, Greg Cosma, Henry Gardner, Xianglin Shi, Vince Castranova, and Val Vallyathan. Effect of antioxidant protection by *p*-coumaric acid on low-density lipoprotein cholesterol oxidation. *Am J Physiol Cell Physiol* 279: C954–C960, 2000.—Mechanisms in which *p*-coumaric acid (CA) acts as an antioxidant are not well understood. This study investigated whether CA can act as a direct scavenger of reactive oxygen species (ROS) and whether it minimizes the oxidation of low-density lipoprotein (LDL). Rats were administered CA in drinking water at low or high doses for 10, 21, and 30 days (uptakes were 29 and 317 mg/day, respectively). Blood levels of 8-epiprostaglandin $F_{2\alpha}$ were monitored as a marker of LDL oxidation. Oral administration of CA (317 mg/day) for 30 days significantly inhibited LDL oxidation. CA also reduced LDL cholesterol levels in serum but had no effect on levels of high-density lipoprotein cholesterol. In vitro studies that used electron spin resonance in combination with spin trapping techniques were used to determine the ability of CA to scavenge ROS and alter LDL oxidation. CA effectively scavenged $\cdot OH$ in a dose-dependent manner. IC_{50} and maximum velocity for CA scavenging of $\cdot OH$ were 4.72 μM and 1.2 $\mu M/s$, respectively, with a rate constant of $1.8 \times 10^{11} M^{-1} \cdot s^{-1}$. Our studies suggest that the antioxidant properties of CA may involve the direct scavenging of ROS such as $\cdot OH$.

hydroxyl radical; lipid peroxidation; reactive oxygen species

MANY STUDIES HAVE ATTEMPTED to determine whether antioxidants prevent oxidation of low-density lipoprotein (LDL) and slow the progression of atherosclerosis (9, 22, 24). In this respect, an increasing awareness and importance are given to nutrition and specifically to plant products in the diet. *p*-Coumaric acid (CA) widely exists in fruits, such as apples and pears, and in vegetables and plant products, such as beans, potatoes, tomatoes, and tea. It is an intermediate product of the phenylpropanoid pathway in plants. CA has been suggested to exhibit antioxidant properties (3, 4, 12–14). It was reported that CA in vitro can provide antioxidant protection to LDL as a result of the chain-breaking

activity of CA (3). Diet supplementation with a crude extract of CA isolated from pulses resulted in the reduction of ester cholesterol, providing a protective mechanism against the development of atherosclerosis (23). The ability of CA to prevent excessive lipid peroxidation on the basis of its chain-breaking activity of α -tocopherol oxidation has also been demonstrated (12). More recently, Castelluccio et al. (3) reported that CA was effective in enhancing the resistance of LDL to oxidation. If CA is an efficient antioxidant for LDL, it may play a key role in the purported effect of oxidized lipoprotein on platelet activity to inhibit atherogenesis. In addition, the dehydrogenation polymer of CA was reported to have anti-human immunodeficiency virus activity (26). However, from these studies, the mechanism of action of CA is not fully understood, although it has been proposed that the antioxidant properties of CA are due to its ability to directly scavenge reactive oxygen species (ROS) (10, 11). To address whether CA can act as a direct scavenger of ROS and thereby modify the age-related changes in blood lipid profiles, we have investigated the effects of CA administered through drinking water in rats fed a diet containing 5% fat. At days 0, 10, 21, and 30, we monitored the oxidative by-product of lipid peroxidation, 8-epiprostaglandin $F_{2\alpha}$ (8-EPI), and cholesterol levels in serum of rats consuming a low or high dose of CA. To investigate further the scavenging efficiency of CA, we studied the effects of CA on ROS generation with the use of electron spin resonance (ESR) techniques. If CA scavenges ROS, the characteristic ESR signals of trapped ROS generated in a given reaction system should decrease with the addition of CA. We also report new experimental data showing that CA is capable of scavenging $\cdot OH$, inhibiting lipid peroxidation in vivo, and thereby reducing serum LDL cholesterol levels.

MATERIALS AND METHODS

Hydrogen peroxide (H_2O_2) was obtained from Fisher Scientific (Pittsburgh, PA). CA, high-density lipoprotein (HDL)

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cholesterol reagent (PTA/MgCl₂), cholesterol diagnostic kits, cholesterol calibrator, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 3-cyano-proxyl free radical, and FeSO₄ were purchased from Sigma Chemical (St. Louis, MO). 8-EPI immunoassay kit EA-84 was obtained from Oxford Biomedical Research (Oxford, MI).

Animal treatment. Adult (60 days old) Sprague-Dawley male rats weighing 500–510 g were obtained from Hill Top Farms (Scottsdale, PA). Rats were fed a diet of standard Purina chow containing 5% fat and water ad libitum. Animals were housed in single cages and were given a low dose (1 mg/ml) or high dose (10 mg/ml) of CA in water. CA uptake was estimated from the daily consumption of water per 500 g body wt. The CA uptakes were 29 ± 3.6 and 317 ± 33.8 mg/day for the low and high doses, respectively. On a daily basis, there was an ~10–15% variability in the uptake of CA between animals. The animals were placed into four groups (6 in each group), based on CA treatment, and killed at predesignated times (0, 10, 21, and 30 days). All animals receiving 29 or 317 mg/day CA and all control (sham) rats were killed by an overdose of pentobarbital sodium (1.5 ml). Blood, ~15 ml from each rat, was collected directly from the heart. Blood was then allowed to clot at room temperature and then centrifuged at 600 *g* for 5 min, and the serum was collected. The serum samples were divided into several aliquots and stored at –70°C until used for the assays.

Measurement of lipid peroxidation. 8-EPI, a product of lipoprotein peroxidation, is a potent vasoconstrictor in rats and rabbits (16–19). This lipoprotein by-product can be used as a marker of oxidative stress in atherosclerosis and carcinogenesis (17–19). The method is based on a competitive enzyme-linked immunoassay for the determination of 8-EPI in biological samples (16). 8-EPI in the samples competes with 8-EPI conjugated to horseradish peroxidase for binding to the antibody coated on the plate. The peroxidase activity results in color development in the substrate, with color intensity being proportional to the amount of unconjugated 8-EPI in the samples. Unless otherwise stated, the reaction mixture consisted of 100 μ l of rat serum and 100 μ l of diluted 8-EPI horseradish enzyme conjugate in each well (antibody-coated plate). The plates were allowed to stand in the dark at room temperature for 2 h. They were then inverted, and the contents emptied by being patted dry upside down on lint-free towels. Each well was washed four times with 400 μ l of wash buffer, and 200 μ l of substrate were added to each well. After an incubation of 20 min, 50 μ l of 1 M sulfuric acid were added to each well, and the plates were read at 450 nm with the use of a Spectromax 250 (Molecular Devices, Sunnyvale, CA). The quantitative values for 8-EPI were calculated from a standard curve produced using the same reagents. All estimations were carried out in duplicate, using groups with and without spiked samples and negative controls. Butyl hydroxy toluene was used to inhibit lipid peroxidation in negative controls.

Assays of cholesterol. Measurements of total cholesterol and HDL cholesterol in the rat serum were made according to the standard procedures established by the Sigma diagnostics kits (nos. 352 and 352–4) using a Beckman DU 650 spectrophotometer (Beckman Instruments, Columbia, MD). LDL cholesterol values were calculated from the total and HDL cholesterol results.

Spin trapping and ESR measurements. The typical spin-trapping reaction mixture consisted of DMPO (250 μ M), H₂O₂ (1 mM), and FeSO₄ (100 μ M) in air-saturated phosphate buffer (10 mM, pH 7.4) in the absence or presence of CA. We used a low concentration of DMPO (250 μ M) because of the competition reaction between DMPO and CA for \cdot OH.

The concentration of CA in stock phosphate buffer (pH 7.4) was measured by ultraviolet spectroscopy at 286 nm with an extinction coefficient of 1.9×10^4 M^{–1}cm^{–1} (1) and then adjusted to the desired final concentration. The reaction was initiated by mixing 20 μ l of 5 mM FeSO₄ stock solution (prepared in 0.01 N H₂SO₄ because Fe²⁺ can be oxidized at pH values higher than 3.0), 0.25 ml of 1 mM DMPO, 0.25 ml of 4 mM H₂O₂, and 0.48 ml (or 0.50 ml) of phosphate buffer (pH 7.4) in the presence or absence of various concentrations of CA stock solutions (20 μ l) in different concentrations. An ESR flat cell was calibrated and fixed in the ESR cavity. Immediately after samples were mixed, they were siphoned into the ESR flat cell assembly (60 \times 8.5 \times 0.25 mm ID), and the ESR spectrum was recorded at 1 min of reaction time at room temperature. The ESR signal of the DMPO- \cdot OH adduct produced by the standard reaction of H₂O₂ with Fe²⁺ was monitored for at least 15 min at room temperature, and its decay rate was found to be ~2%/min. Because the Fenton reaction is complete within seconds, commercially available 3-cyano-proxyl free radical was used as a nitroxide standard for determining relative concentrations of the spin adduct of DMPO with \cdot OH. The relative \cdot OH radical concentration was quantitated by measuring heights of the first peak of the DMPO- \cdot OH signals. The maximum concentration of DMPO- \cdot OH was calculated to be ~70 μ M. The recordings of ESR spectra were performed with a Varian 109 ESR spectrometer. Unless otherwise stated, the ESR parameters were set at 100 kHz, X-band, microwave frequency of 9.73 GHz, microwave power of 20 mW, modulation amplitude of 0.63 G, time constant of 0.25 s, scan time of 8 min, scan width of 200 G, and receiver gain of 2×10^4 .

Statistical analysis. The data presented are means \pm SD of three in vivo experiments for each time interval and a minimum of five or more in vitro experiments in duplicate. A Student's *t*-test was used to determine differences between control groups and animals treated with CA. A probability value of 0.05 or smaller was considered significant. Data analyses were made using Sigmapstat (Jandel Scientific, San Rafael, CA).

RESULTS

Effect of CA on rat serum lipid peroxidation. 8-EPI was used as a marker for the estimation of lipid peroxidation products resulting from oxidative injury (16–19). Figure 1 shows the in vivo effect of CA consumption on the formation of 8-EPI in rat serum. The accumulation of lipid peroxidation by-products in serum from control animals not receiving CA was found to increase with increasing age of the rats. At *day 0*, the average 8-EPI level in control group was 0.125 ng/ml. At *day 30*, the average 8-EPI level in the control group was 0.238 ng/ml. This age-related increase in 8-EPI after 30 days was significantly greater (1.9-fold) compared with the 8-EPI level (0.125 ng/ml) at *day 0*. In contrast, CA was found to inhibit significantly the formation of the lipid peroxidation product, 8-EPI. In animals administered 29 mg CA/day for 30 days, there was a modest inhibition of 8-EPI formation (~8.4%), whereas a significant inhibition of 8-EPI formation (~60%) was noted after administration of 317 mg CA/day for 30 days. A modest inhibition of 8-EPI production was also observed at *day 10* (~2%) and at *day 21* (~2.5%) in animals administered 317 mg CA/day. To examine the possibility of CA interaction with 8-EPI,

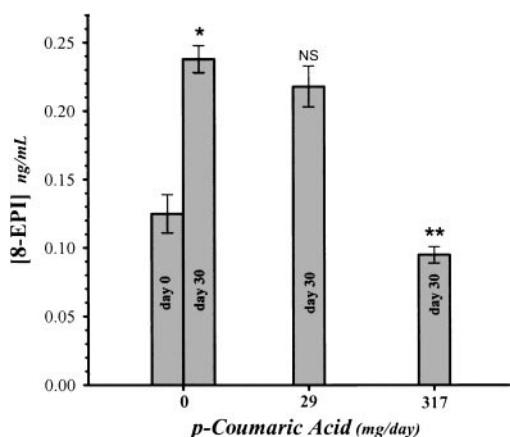


Fig. 1. Inhibition of lipid peroxide formation in the blood serum of rats administered *p*-coumaric acid (CA) in drinking water at low (29 mg/day) or high (317 mg/day) levels for 30 days. The amount of CA intake by rat was calculated from the daily consumption of water containing CA. Data are means \pm SD of 3 experiments in duplicates. Each assay sample contained 100 μ L of serum and the amount of reagents indicated in MATERIALS AND METHODS. *Value is significantly different from day 0 ($P = 0.006$). **Value is significantly different from day 30 control ($P = 0.002$). 8-EPI, 8-epiprostaglandin $F_{2\alpha}$; NS, not significant; brackets indicate concentration.

standards containing different concentrations of 8-EPI were mixed with CA and evaluated using the same protocol. There was no significant effect on absorbance at 450 nm, indicating that CA did not react with 8-EPI and that it was an effective inhibitor against lipid peroxidation.

Effect of CA on rat serum cholesterol. Atherogenesis is closely associated with circulating levels of LDL cholesterol and resulting reactions leading to injury (28). Elevated LDL has been associated with a higher

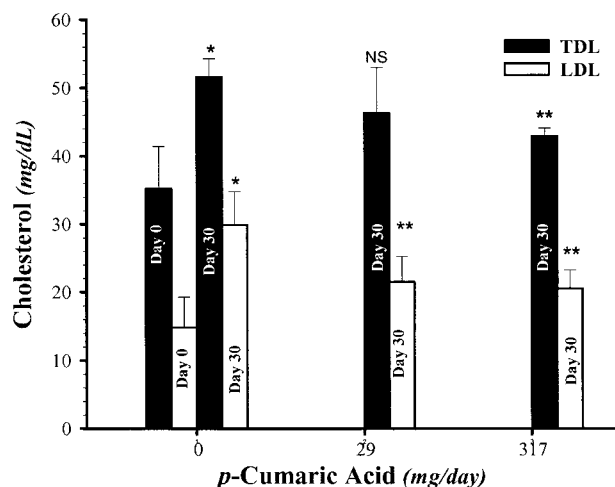


Fig. 2. Effect of CA supplementation on the levels of total serum cholesterol and low-density lipoprotein (LDL) cholesterol. Data are means \pm SD of values obtained from 3 rats administered CA in the drinking water (0, 29, or 317 mg/day). Each assay sample contains 50 μ L of serum and the amount of reagents indicated in MATERIALS AND METHODS. *Value is significantly different from day 0. **Value is significantly different from the day 30 controls. Total (TDL) cholesterol day 0 vs. day 30: $P = 0.02$; day 30 vs. day 30 CA: $P = 0.01$. LDL cholesterol day 0 vs. day 30: $P = 0.02$; day 30 vs. day 30, 29 mg CA/day: $P = 0.05$; day 30 vs. day 30, 317 mg CA/day: $P = 0.04$.

incidence of atherosclerosis (28, 27). LDL cholesterol levels in serum of control groups not receiving CA were found to increase with increasing age of the rats. As shown in Fig. 2, at day 30, the total serum cholesterol increased $\sim 40\%$ compared with day 0 and LDL cholesterol level increased $\sim 100\%$. In contrast, HDL cholesterol levels were not found to change significantly (data not shown). For rats treated with 29 mg CA/day, CA reduced total cholesterol by 12% and LDL cholesterol by 30% in 30 days. At 317 mg CA/day, CA inhibited total cholesterol by $\sim 17\%$ and LDL cholesterol by $\sim 33\%$ in 30 days. Animals administered 317 mg CA/day for 10 and 21 days also showed a remarkable decline in LDL cholesterol levels (~ 75 and 68% , respectively). There was no significant effect on HDL cholesterol in CA-treated animals at any of these time periods. These data indicate that CA is capable of decreasing LDL cholesterol levels in circulating blood of animals administered CA at all three time intervals monitored.

ESR spectra of the hydroxyl radical spin adduct. In vitro studies, the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$) was used as a source for $\cdot\text{OH}$ generation (6, 30). Figure 3 shows the ESR spectra of the DMPO- $\cdot\text{OH}$ adduct obtained in the reaction mixtures of H_2O_2 (1 mM) and FeSO_4 (100 μM) containing

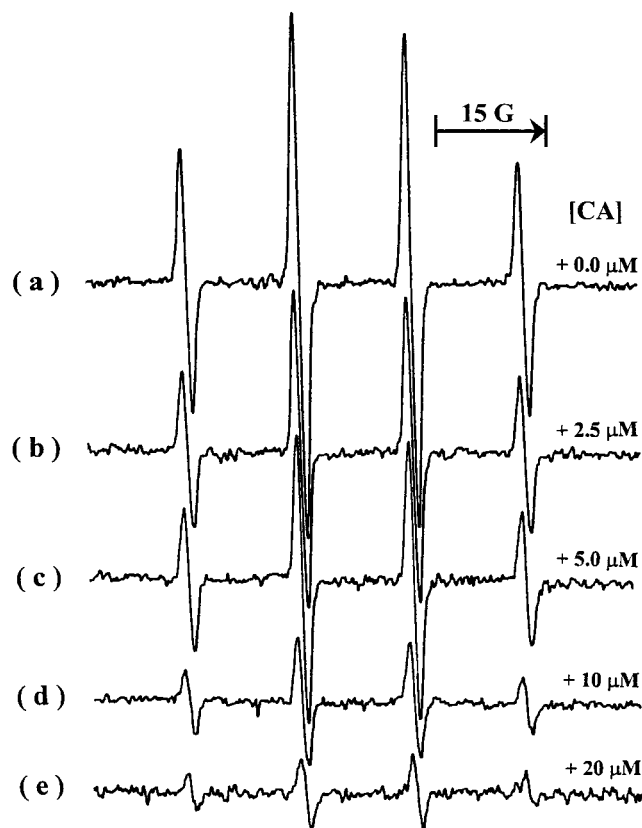


Fig. 3. Electron spin resonance (ESR) spectra of spin adducts of hydroxyl radical observed during the reaction of FeSO_4 (100 μM), H_2O_2 (1 mM), and 250 μM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in air-saturated phosphate buffer, pH 7.4, in the presence of 0 (a), 2.5 μM (b), 5.0 μM (c), 10 μM (d), and 20 μM (e) CA. ESR spectroscopy settings were as described in MATERIALS AND METHODS.

DMPO (250 μM) in the absence or presence of the indicated amount of CA. The ESR spectrum in Fig. 3a was obtained in a reaction mixture in the absence of CA. This spectrum exhibited four splitting lines with an intensity ratio of 1:2:2:1, which typically results from the interactions of an uncoupled electron with a primary nitrogen atom along with a secondary β -proton. The hyperfine splitting constants of the signal ($a_N = a_H = 14.9$ G) are consistent with previously reported values for DMPO- $\cdot\text{OH}$ (29–31), indicating that the signal results from trapped $\cdot\text{OH}$. CA was found to significantly inhibit the formation of the DMPO- $\cdot\text{OH}$ adduct. As shown in Fig. 3, *b–d*, at concentrations as low as 2.5 μM , CA inhibited $\sim 40\%$ of the EPR signal intensity and, at 20 μM , CA inhibited $\sim 86\%$ of the EPR signal intensity. To examine the possibility of CA interaction with DMPO- $\cdot\text{OH}$, 20 μM CA was added to the reaction mixture of DMPO (250 μM), H_2O_2 (1 mM), and FeSO_4 (100 μM) 1 min after initiation of the reaction, i.e., after DMPO- $\cdot\text{OH}$ adduct formation. There was no observed effect on ESR signal intensity (data not shown), indicating that CA is an effective scavenger for $\cdot\text{OH}$.

Effect of CA on ESR signal intensity. The dose dependence of CA inhibition of the DMPO- $\cdot\text{OH}$ adduct ESR signal intensity is depicted in Fig. 4. Its IC_{50} value, i.e., the concentration of CA required to cause 50% inhibition of ESR signal intensity, was estimated to be ~ 4.72 μM . To obtain a more precise value, the percentage of ESR signal intensity was plotted against the log of the CA concentration, which resulted in a linear curve with an equation $y = -52.783\log(x) + 85.576$ (Fig. 4, *inset*). Therefore, when $y = 50$, the IC_{50} value was calculated to be 4.72 μM , which is consistent with our roughly estimated value of CA concentration

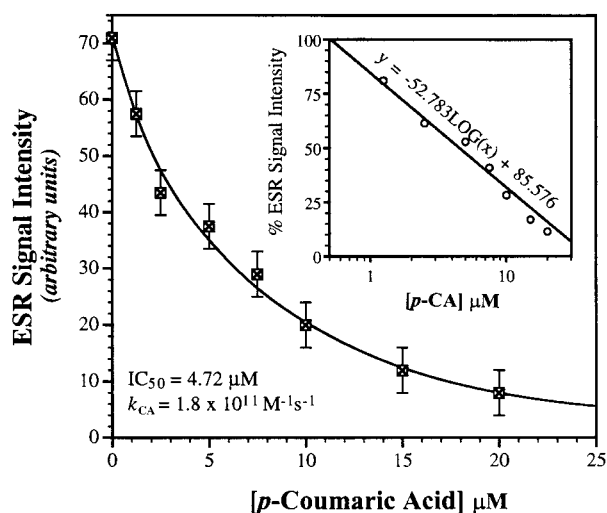


Fig. 4. Dose-dependent inhibition of DMPO- $\cdot\text{OH}$ signal intensity by CA. Reaction mixture was same as in Fig. 3 in the presence of the indicated concentration of CA. Values are means \pm 5 units SD of 5 determinations. *Inset*: data displayed as the percentage of ESR signal intensity against $\log[\text{CA}]$. ESR spectroscopy settings were the same as described in Fig. 3. τ , Time constant; β , ratio of slope to intercept; V_{max} , maximum velocity; k_{CA} , rate constant of CA scavenging $\cdot\text{OH}$.

that caused 50% inhibition of ESR signal intensity. No inhibition of ESR signal was observed in the reaction mixture incubated under identical conditions in the absence of CA, indicating that inhibition of the ESR signal of DMPO- $\cdot\text{OH}$ is entirely dependent on the presence of CA.

To quantitatively confirm the elimination of $\cdot\text{OH}$ by CA in this system, we used the methods reported by Buettner et al. (2). If we assume that the velocity of CA scavenging $\cdot\text{OH}$ is equal to the rate of formation of spin adducts, when the ESR signal intensity of DMPO- $\cdot\text{OH}$ is suppressed by 50%, then we can obtain the following equation

$$k_{\text{CA}}[\text{CA}][\cdot\text{OH}] = k_{\text{DMPO}}[\text{DMPO}][\cdot\text{OH}] \quad (1)$$

where brackets indicate concentration, k_{CA} is the rate constant of CA scavenging $\cdot\text{OH}$, and k_{DMPO} is the rate constant of DMPO trapping $\cdot\text{OH}$. Because the $\cdot\text{OH}$ concentration on both sides of Eq. 1 are equivalent, we can omit this item and obtain the following equation

$$k_{\text{CA}} = k_{\text{DMPO}}[\text{DMPO}]/[\text{CA}] \quad (2)$$

With the use of the IC_{50} value of CA described above, 4.72 μM , and k_{DMPO} of $3.4 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e., the value for DMPO trapping $\cdot\text{OH}$ (7), we obtained a rate constant of $1.8 \times 10^{11} \text{ M}^{-1} \cdot \text{s}^{-1}$ for CA scavenging $\cdot\text{OH}$ in this aqueous solution (pH 7.4).

Effect of CA on the rate of hydroxyl radical elimination. Because CA does not interact with DMPO- $\cdot\text{OH}$, there exists a competition between DMPO and CA for available $\cdot\text{OH}$. Therefore, the kinetic parameters of CA, as the competitor in the test system, can be obtained. In a typical reaction, Fe^{2+} and H_2O_2 generate $\cdot\text{OH}$, which in turn reacts with DMPO to form DMPO- $\cdot\text{OH}$ spin adducts. CA inhibits the spin adduct formation by scavenging $\cdot\text{OH}$. Because the maximum concentration of DMPO- $\cdot\text{OH}$ generated was determined to be ~ 70 μM (see MATERIALS AND METHODS) and the DMPO concentration in the reaction system is four times larger than the $\cdot\text{OH}$ concentration, we assume that all $\cdot\text{OH}$ can be trapped and that DMPO- $\cdot\text{OH}$ formation reaches a maximum velocity (V_{max}). Therefore, DMPO- $\cdot\text{OH}$ can represent the relative concentration of $\cdot\text{OH}$. Because one DMPO molecule can trap one $\cdot\text{OH}$ molecule with a high rate constant ($3.4 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$), the concentration of inhibited DMPO- $\cdot\text{OH}$ spin adducts should be identical to the concentration of $\cdot\text{OH}$ eliminated by CA. If the reduced ESR signal intensity is directly proportional to the concentration of CA, a first-order kinetic plot should yield a straight line. Under steady-state conditions, the decrease in concentration of $\cdot\text{OH}$ could be expressed as follows

$$\begin{aligned} dV &= -d[\cdot\text{OH}]/dt \\ &= -d[\text{DMPO} \cdot \text{OH}]/dt \\ &= k_{\text{CA}}[\text{CA}][\cdot\text{OH}] \end{aligned} \quad (3)$$

where dV is the velocity of scavenging of $\cdot\text{OH}$ by CA. Under a given condition, dV values can be obtained at different concentrations of CA. Figure 5 depicts the

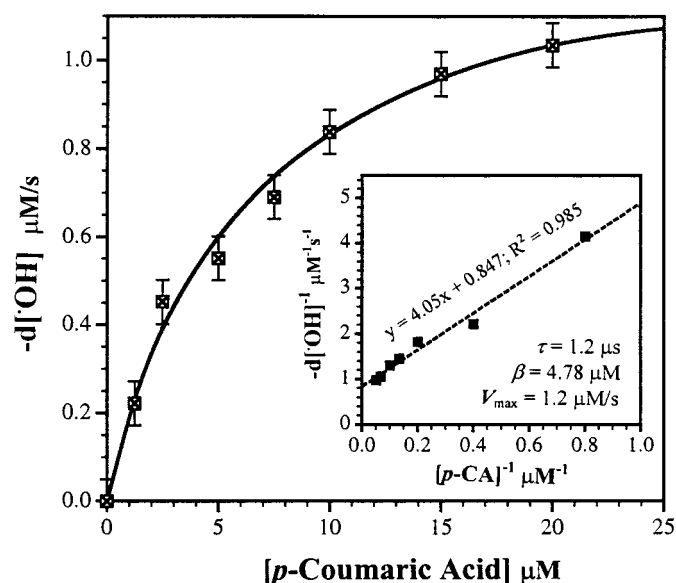


Fig. 5. Rate of $\cdot\text{OH}$ scavenging as a function of CA concentration during the reaction of FeSO_4 (100 μM) and H_2O_2 (1 mM) in air-saturated phosphate buffer, pH 7.4, in the presence of 250 μM DMPO. Values are means \pm SD of 5 determinations. *Inset*: double reciprocal plot of the rate of DMPO- $\cdot\text{OH}$ reduction vs. CA concentration. ESR spectroscopy settings were the same as described in Fig. 3.

velocity of CA scavenging $\cdot\text{OH}$ as a function of CA concentration. The hyperbolic nature of the curve in Fig. 5 indicates that CA scavenged $\cdot\text{OH}$ to completion. The Lineweaver-Burk plot (Fig. 5, *inset*) demonstrated linearity with a correlation coefficient of 0.985. The reciprocal of the y-intercept value of the plot, V_{max} , i.e., the maximum rate of CA scavenging $\cdot\text{OH}$ in this system, was found to be 1.2 $\mu\text{M/s}$. The ratio of the slope to intercept from Fig. 5 provides k_d/k_{CA} (where k_d is the dissociation constant), the β -value (8, 32), which is the concentration of CA that eliminates one-half of the $\cdot\text{OH}$. This β -value was found to be 4.78×10^{-6} M, which is similar to the IC_{50} value (4.72 μM) obtained in Fig. 4. Thus, when $k_{\text{CA}} = 1.8 \times 10^{11} \text{ M}^{-1}\cdot\text{s}^{-1}$, i.e., the value obtained above for CA scavenging $\cdot\text{OH}$, the k_d value for the decay of $\cdot\text{OH}$ was calculated to be $8.6 \times 10^5 \text{ s}^{-1}$. These data strongly support the hypothesis that CA is an efficient scavenger for $\cdot\text{OH}$.

The scavenging rate constants for the reactions of several compounds with $\cdot\text{OH}$ have been reported (5, 15, 25) and are presented in Table 1. As shown in Table 1, the rate constant value obtained using CA as an antioxidant is one order of magnitude larger than all of the listed compounds. Because all the listed compounds were reported to have been tested at pH 7.0 at room temperature, we believe the phenomenal scavenging property of CA is probably also due to its phenolic structure. Phenolic compounds are also good reductants that may contribute to this antioxidant property. Thus it appears that CA is a powerful antioxidant with a specific scavenging efficiency for $\cdot\text{OH}$.

DISCUSSION

Increasing evidence indicates that oxidative modification of LDL is a major causative factor in the patho-

genesis of atherosclerosis, and endogenous antioxidant supplementation may protect against oxidation of LDL and ameliorate the progression of atherosclerosis. The results reported here clearly demonstrate that CA is able to directly scavenge $\cdot\text{OH}$, protect LDL from oxidation, and reduce serum cholesterol levels. In addition, we also demonstrated that CA significantly reduced the levels of a lipid peroxidation by-product, 8-EPI, in serum of rats administered CA. The spin trapping-ESR techniques that we applied in these studies also substantiated the scavenging efficiency of CA, which was 10-fold greater than other antioxidants. It is known that other antioxidants such as vitamin C, vitamin E, β -carotene, ubiquinol-10, and others contribute to the protection of LDL from oxidation. Therefore, during a propagation phase of enhanced lipid peroxidation, supplementation with CA may prove beneficial.

Although CA has been reported to act as an antioxidant (3, 4, 12–14, 23), little direct evidence was available to support this claim. Our ESR studies demonstrate that CA is indeed an effective antioxidant with a scavenging rate for $\cdot\text{OH}$ one order of magnitude greater than other common antioxidants (Table 1). The biological role of this potent $\cdot\text{OH}$ scavenger was confirmed by its abilities to inhibit the formation of a lipid peroxidation by-product, 8-EPI, in serum and to reduce serum cholesterol levels. Such *in vivo* actions may be due to the ability of CA to scavenge $\cdot\text{OH}$, as demonstrated *in vitro* using ESR techniques in which CA decreased DMPO- $\cdot\text{OH}$ formation (Fig. 3). Additional support for this conclusion came from the ESR kinetic study, in which the Lineweaver-Burk plots resulted in a linear relationship with a correlation coefficient of 0.985 (Fig. 5, *inset*), indicating that there is a stoichiometric relationship between CA concentration and scavenging of $\cdot\text{OH}$. The value for k_{CA} was found to be $1.8 \times 10^{11} \text{ M}^{-1}\cdot\text{s}^{-1}$, which is close to the diffusion controlled rate. V_{max} of $\cdot\text{OH}$ scavenged by CA was found to be 1.2 $\mu\text{M/s}$. The IC_{50} value of CA required to cause 50% inhibition of ESR signal intensity was found to be 4.72 μM , which is equal to the β -value, the concentration of CA required to scavenge one-half of the $\cdot\text{OH}$ generated in the test system. The lifetime of $\cdot\text{OH}$ in aqueous solution was found to be 1.2 μs . These data strongly demonstrate that CA is a powerful $\cdot\text{OH}$ scavenger, capable of virtually eliminating the $\cdot\text{OH}$ generated. Thus CA may block LDL peroxidation and reduce serum cholesterol levels by scavenging $\cdot\text{OH}$.

Table 1. Comparison of scavenging rate constants of *p*-coumaric acid with some antioxidants on hydroxyl radical

Compound	k , $\text{M}^{-1}\cdot\text{s}^{-1}$	Reference
<i>p</i> -Coumaric Acid	1.8×10^{11}	Our data
Ascorbate	1.3×10^{10}	25
Glutathione	1.5×10^{10}	25
Cysteine	1.5×10^{10}	25
Azide	1.1×10^{10}	25
Lipoic Acid	1.9×10^{10}	26
Hypotaourine	1.6×10^{10}	27

It is known that cholesterol can be obtained from diet or it can be synthesized in the liver. Cholesterol is transported to peripheral tissues by lipoproteins and regulates de novo cholesterol synthesis. LDL is the major carrier of cholesterol in blood. LDL contains a core of about 1,500 esterified cholesterol molecules; the most common fatty acyl chain in these esters is linoleate, a polyunsaturated fatty acid. A shell of phospholipids and unesterified cholesterol surrounds this highly hydrophobic core. If the LDL shell ruptures, serum cholesterol levels will increase. $\cdot\text{OH}$ is well known to be capable of extracting a hydrogen atom from unsaturated fatty acids, causing lipid peroxidation and initiating free radical chain reactions. As a result, free radical chain reactions can cause massive damage to biological molecules such as DNA, cellular membranes, and lipoproteins. Therefore, scavenging $\cdot\text{OH}$ should prevent lipid peroxidation and may reduce serum cholesterol levels as well as protect LDL against oxidation. Because CA scavenges $\cdot\text{OH}$ at a near-diffusion control rate, it can be expected to prevent lipid peroxidation and reduce serum cholesterol levels. As shown in Figs. 1 and 2, the administration of CA lowered the level of the lipid peroxidation by-product, 8-EPI, by 60% and reduced the LDL cholesterol level by 33% in rat serum of animals treated with 317 mg CA/day for 30 days. Thus our results provide a plausible explanation for the previously proposed ability of CA to prevent lipid peroxidation, reduce serum cholesterol levels, and enhance the resistance of LDL to oxidation (3, 12, 23).

Atherosclerosis resulting in coronary heart disease is the leading cause of death in men after age 35. In atherosclerosis, increases in cholesterol are closely associated with circulating levels of LDL cholesterol and increasing morbidity. Circulating monocytes are capable of releasing superoxide anion radicals, which can be dismutated by superoxide dismutase and converted to $\cdot\text{OH}$, via participation of transition metals such as iron (21). Oxidation of LDL by ROS is currently considered to be an important pathobiological pathway by which incremental changes occur in the endothelial cells lining the intima in blood vessels. Repeated or continuing insults from ROS ultimately result in their injury and development of disease (20). Although cells contain multiple antioxidant defenses to protect themselves against free radicals, these protective mechanisms are kept at equilibrium and are not present in excess. Exogenous sources of antioxidants are needed to compensate the oxidative injury resulting from pollution, cigarette smoke, and other toxic biochemical reactions that result in lipid peroxidation. The water and lipid solubilities of CA, together with its substantial efficiency to scavenge $\cdot\text{OH}$ and to inhibit lipid peroxidation and reduce LDL cholesterol levels in vivo, provide important insights for future investigations. Dietary supplements may provide a useful approach for the prevention of injury to vascular cell membranes, thus preventing or delaying the onset of cardiovascular disease.

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