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Antioxidant activity of hydroxycinnamic acid derivatives in human low density lipoprotein: Mechanism and structure–activity relationship

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Abstract

Hydroxycinnamic acid derivatives, i.e., caffeic acid (CaA), chlorogenic acid (ChA), sinapic acid (SA), ferulic acid (FA) and *p*-coumaric acid (CoA), are widely distributed in plants and are known antioxidants. The *in vitro* peroxidation of human low density lipoprotein (LDL) was used as a model to study the free radical-induced damage of biological membranes and the protective effect of hydroxycinnamic acid derivatives. The peroxidation was initiated either by a water-soluble azo-initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), or by cupric ion (Cu^{2+}). The reaction kinetics were monitored either by the uptake of oxygen or by the formation of thiobarbituric acid reactive substances (TBARS). Kinetic analysis of the antioxidation process demonstrates that these hydroxycinnamic acid derivatives are effective antioxidants against both AAPH- and Cu^{2+}-induced LDL peroxidation with the activity sequence of CaA ~ ChA > SA > FA > CoA, and CaA ~ ChA > SA ~ FA ~ CoA, respectively. Molecules bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl groups possess significantly higher antioxidant activity than those bearing no such functionalities.

Keywords: Hydroxycinnamate; Caffeic acid; Low density lipoprotein; Antioxidant; Lipid peroxidation; Structure–activity relationship

1. Introduction

Increasing evidence from epidemiological and biological studies suggests that free-radical-induced oxidative modification of low density lipoprotein (LDL) is a critical factor in promoting atherosclerosis (Esterbauer & Ramos, 1995; Klatt & Esterbauer, 1996; Mertens & Holvoet, 2001; Pryor, 2000; Upston, Kritharides, & Stocker, 2003). Therefore, inhibition of LDL peroxidation by supplementation of antioxidants has become an attractive therapeutic strategy for preventing atherosclerosis. This has led to a great deal of research devoted to the prevention of lipid peroxidation of LDL by antioxidants (Jialal & Grundy, 1993; Liu, Ma, Zhou, Yang, & Liu, 2000; Thomas & Stocker, 2000; Upston et al., 2003).

Phenolic acids, especially hydroxycinnamic acid derivatives, are widely distributed in plants and present in considerable amounts in fruits, vegetables, and beverages of human diet (Robbins, 2003). The daily uptake of caffeic acid, a hydroxycinnamic acid derivative, has been estimated to be 206 mg in subjects drinking coffee (Radtke, Linseisen, & Wolfram, 1998). These compounds have attracted considerable attention due to its various biological and pharmacological activities, including antioxidative activities (Laranjinha, Vieira, Madeira, & Almeida, 1995; Nardini et al., 1995; Roche, Dufour, Mora, & Dangles, 2005; Taubert et al., 2003). We have recently found that green tea polyphenols (flavanols) (Wei, Zhou, Cai, Yang, & Liu, 2006a; Zhou, Wu, Yang, & Liu, 2005b) and flavonols and their glycosides (Hou, Zhou, Yang, & Liu, 2004a, 2004b; Zhou, Miao, Yang, & Liu, 2005a) are good antioxidants against free radical initiated lipid peroxidation, and that the antioxidant activity of these flavonoids depends significantly on the structure of the molecules and the microenvironment of the reaction medium (Zhou

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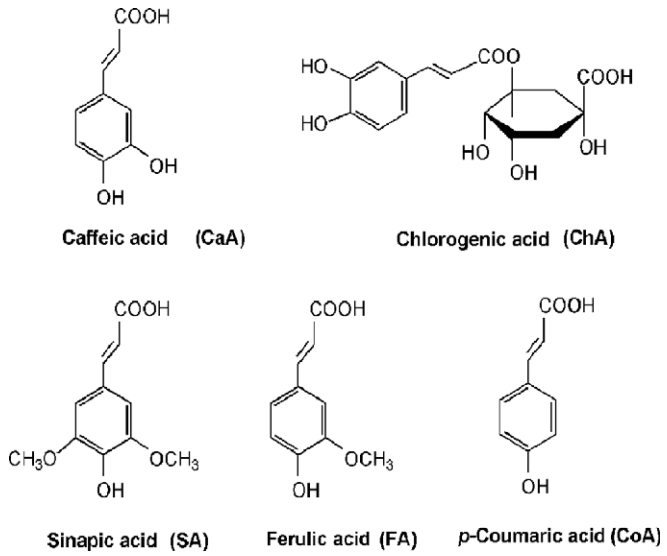


Fig. 1. Molecular structures of hydroxycinnamic acid derivatives.

et al., 2005a). Therefore, it is of interest to extend this research to other dietary non-flavonoid phenolic compounds, such as phenolic acids, to study the structure-activity relationship. We report herein a quantitative kinetic study on the antioxidation effect of a set of typical phenolic acids, hydroxycinnamic acid derivatives, i.e., caffeic acid (CaA), chlorogenic acid (ChA), sinapic acid (SA), ferulic acid (FA) and *p*-coumaric acid (CoA) (Fig. 1), against peroxidation of human low density lipoprotein in phosphate buffered saline (PBS, pH 7.4) under atmospheric oxygen. The peroxidation was initiated either by a water-soluble azo initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) or by cupric ion (Cu^{2+}), and measured by oxygen uptake and formation of thiobarbituric acid reactive substances (TBARS). It was found that hydroxycinnamic acid derivatives, especially CaA and ChA, are good antioxidants for both AAPH- and cupric ion-initiated LDL peroxidation. The mechanistic details and structure-activity relationship of their antioxidative action are discussed.

2. Materials and methods

2.1. Materials

Caffeic acid (CaA, Acros), chlorogenic acid (ChA, Aldrich), sinapic acid (SA, Acros), ferulic acid (FA, Aldrich) and *p*-coumaric acid (CoA, Fluka) were purchased with the highest purity available and used as received. DL-α-Tocopherol (Merck, Biochemical reagent, >99.9%) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Aldrich) were kept under nitrogen in a refrigerator and used as received.

The LDL was isolated from human plasma of a healthy donor by the discontinuous density gradient centrifugation procedure as described in the literature (Chung, Wilkinson, Geer, & Segrest, 1980) at 45,000 rpm (140,000*g*) for 6 h using a HITACHI 55P-72 ultracentrifuge at 4 °C. The isolated LDL fraction was then dialyzed with phosphate buffered saline (PBS, composed of 137 mM of NaCl, 2.7 mM of KCl, 8.1 mM of Na_2HPO_4 and 1.5 mM of KH_2PO_4 in distilled water, pH 7.4) containing 0.1 mM sodium ethylenediaminetetraacetate (EDTA) to prevent oxidation during the isolation. EDTA was removed by dialysis with PBS before the oxidation experiments. The concentration of protein was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.2. Oxygen uptake measurements

The rate of oxygen uptake was measured in a closed glass vessel of ca. 2 ml in volume, at 37 ± 0.1 °C and that was stirred, using a 5946-50 oxygen meter (Cole-Parmer Instruments, USA) which was able to record oxygen concentrations as low as 10^{-8} M. LDL was suspended in PBS (pH 7.4) to the final concentration of 0.4 mg protein/ml equivalent to approximately 0.8 μM of LDL (mass = 2.5×10^6 g/M and protein content of ca. 20%) (Bowry & Stocker, 1993) under air. Hydroxycinnamic acid derivatives were dissolved in DMSO to the concentration of 2 mM as stock solutions. The final concentration of DMSO in the suspension was less than 0.1% (v/v) of the LDL suspension to avoid disturbance of the system. AAPH was directly dissolved in PBS (pH 7.4) and injected into the LDL suspension to initiate the peroxidation. Every experiment was repeated three times and the results were reproducible to within 10% experimental deviation.

2.3. α-Tocopherol (TOH) determination

A Gilson model 702 liquid chromatograph was used to separate α-tocopherol (TOH) with a Sychropack KPP-100 reversed-phase column (4 × 250 mm) and eluted with methanol–*iso*-propanol–formic acid (80:20:1 v/v/v) containing 50 mM of sodium perchlorate as a supporting electrolyte at a flow rate of 1 ml/min. Aliquots of 0.6 ml of reaction mixture were taken out from three identical reaction vessels of 2 ml in volume at appropriate time intervals, and TOH was extracted by hexane-ethanol partitioning (hexane:EtOH:LDL = 12:3:1 v/v/v) which yielded >97% of TOH (Bowry & Stocker, 1993). TOH was electrochemically detected by using a Gilson Model 142 electrochemical detector by setting the oxidation potential at +700 mV.

2.4. MDA formation measurements

The formation of malondialdehyde (MDA) was determined by thiobarbituric acid assay as a thiobarbituacid reactive substances (TBARS) to monitor LDL peroxidation (Buege & Aust, 1978). The LDL was incubated at 37 °C in 0.1 M potassium phosphate buffer, pH 7.5, and made up to a final protein concentration of 0.4 mg/ml. The peroxidation was initiated by 10 μM of $CuSO_4$ and

inhibited by 2 μM of hydroxycinnamic acid derivatives which was added as a DMSO solution and the final concentration of DMSO in the suspension was less than 0.1% v/v that did not show appreciable interference to the reaction as shown by control experiments. The reaction mixture was gently shaken at 37 °C and aliquots of the reaction mixture were taken out at specific intervals and a TCA–TBA–HCl stock solution (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N HCl) was added to the reaction mixture, together with 0.02% w/v butylated hydroxytoluene (BHT). This amount of BHT completely prevented the formation of any nonspecific TBARS (Palozza & Krinsky, 1992). The solution was then heated in a boiling water bath for 15 min. After cooling the precipitate was removed by centrifugation. MDA in the supernatant was determined at 532 nm using the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Buege & Aust, 1978).

3. Results

3.1. The antioxidant activity of hydroxycinnamic acid derivatives in AAPH-induced LDL peroxidation

Fig. 2 shows representative oxygen uptake curves recorded during the water-soluble azo-initiator AAPH-induced LDL peroxidation in the absence and in the presence of exogenous CaA. In the absence of CaA the oxygen uptake did not take place immediately as in the case of lipid peroxidation conducted in model membranes (Barclay, 1993; Fang et al., 2002; Zhou et al., 2005a), but was still inhibited for ca. 22 min (Fig. 2, line a). This demonstrates the presence of endogenous antioxidants in LDL, such as α-tocopherol (TOH), ubiquinol-10 and carotenoids (Esterbauer & Ramos, 1995), which can trap the propagating radicals to inhibit the peroxidation. The oxygen uptake rate during the inhibition period is designated as R_{inh}. After the inhibition period the oxygen uptake became faster, indicating depletion of the endogenous antioxidants. The turning point from the inhibition period to the restoration of oxygen uptake refers to the inhibition time, t_{inh}. The slope of the oxygen uptake curve after the inhibition period represents the intrinsic peroxidation rate, R_p, of the LDL in the absence of antioxidants. After a short time of the inhibition period, different amounts of CaA were added. It was found that addition of CaA completely inhibits the LDL peroxidation, produces a new inhibition period and the inhibition time is proportional to the concentration of CaA (Fig. 2, lines b–d and the inset). This demonstrates that the peroxidation of LDL is inhibited dose-dependently by CaA in the absence of endogenous antioxidants. Other hydroxycinnamic acid derivatives also show the dose-dependent inhibition for the LDL peroxidation (figures not shown).

Fig. 3 shows representative oxygen uptake curves recorded during the AAPH-induced LDL peroxidation in the presence of the same concentration (4 μM) of hydroxycinnamic acid derivatives which were added after the depletion of endogenous antioxidants. It is seen that after depletion of the endogenous antioxidants, addition of all of these tested compounds produces a new inhibition period, indicating that LDL peroxidation could be inhibited by these compounds in the absence of endogenous antioxidants. The inhibition time, t_{inh}, is significantly different for different hydroxycinnamic acid derivatives and follows the efficacy sequence of CaA ~ ChA > SA > FA > CoA (Table 1). The observed inhibition time of LDL peroxidation are indicative of chain-breaking antioxidant activities of these compounds.

When hydroxycinnamic acid derivatives were added before the AAPH-initiation the intrinsic inhibition period of the native LDL was remarkably prolonged (Fig. 4) and the overall inhibition time was approximately equal to the sum of the intrinsic inhibition time of the native

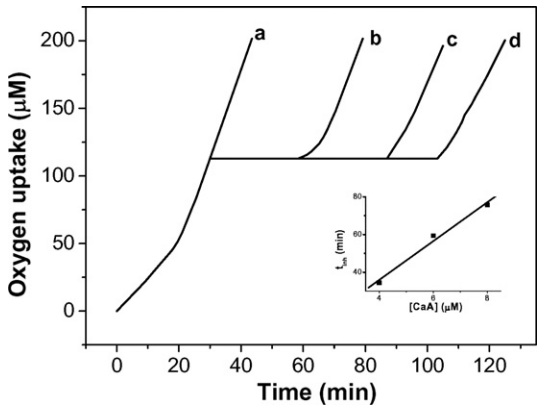


Fig. 2. Representative oxygen uptake curves recorded during the AAPH-initiated and CaA inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.4 mg/ml; $[AAPH]_0 = 10.0$ mM. (a) Native LDL containing 3.37 μM of TOH; (b) $[CaA]_0 = 4$ μM; (c) $[CaA]_0 = 6$ μM; (d) $[CaA]_0 = 8$ μM. The inset shows the concentration dependence of the inhibition time (t_{inh}).

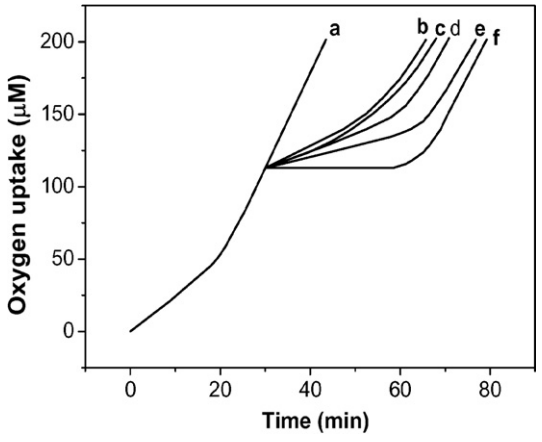


Fig. 3. Representative oxygen uptake curves recorded during the AAPH-initiated and hydroxycinnamic acid derivatives (AOH) inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.4 mg/ml; [AAPH] = 10 mM; $[AOH]_0 = 4$ μM. The AOHs were added after the intrinsic inhibition period. (a) Native LDL containing 3.37 μM of TOH; (b) FA; (c) CoA; (d) SA; (e) ChA; (f) CaA.

Table 1
Kinetic parameters for the AAPH-initiated peroxidation of human LDL and its inhibition by hydroxycinnamic acid derivatives (AOH)[a]

AOH	R_{inh}[b] (10^{-8} M s^{-1})	R_p[b] (10^{-8} M s^{-1})	t_{inh} (min)	kcl_{inh}[g]	kcl_p[g]	k_{inh} (10^5 M^{-1} s^{-1})	n[h]
Native[c]	4.0 ± 0.3	10.4 ± 0.4	22.2 ± 1.2	8.0	20.8	4.1	2[i]
CaA[d]	[f]	9.5 ± 0.5	34.5 ± 1.8	[f]	19.0	[f]	2.6
ChA[d]	0.8 ± 0.1	8.0 ± 0.3	35.1 ± 2.0	1.6	16.0	10.2	2.6
SA[d]	1.5 ± 0.2	8.0 ± 0.2	30.9 ± 1.2	3.0	16.0	6.2	2.3
FA[d]	1.7 ± 0.2	7.0 ± 0.2	25.2 ± 1.4	3.4	14.0	6.9	1.9
CoA[d]	1.4 ± 0.2	7.0 ± 0.3	22.2 ± 1.1	2.8	14.0	7.9	1.7
CaA[e]	1.5 ± 0.1	8.5 ± 0.4	53.4 ± 1.6	3.0	17.0	3.7	2.2
ChA[e]	1.9 ± 0.2	8.5 ± 0.6	51.0 ± 2.6	3.8	17.0	3.1	2.1
SA[e]	1.7 ± 0.1	8.0 ± 0.4	49.2 ± 2.3	3.4	16.0	3.6	2.0
FA[e]	2.5 ± 0.3	10.7 ± 0.6	37.5 ± 1.7	5.0	21.4	3.1	1.5
CoA[e]	2.5 ± 0.2	10.0 ± 0.8	37.8 ± 1.1	5.0	20.0	3.0	1.5

[a] Determined in PBS (pH 7.4) at 37 °C; [LDL] = 0.4 mg/ml; [AAPH] = 10 mM; $[AOH]_0$ = 4 μM. Every experiment was repeated three times and the SD are shown in the table. R_i is taken as 5.0 nM/s by using Eq. (2). k_{inh} is calculated by using Eq. (4).
[b] The decomposition of AAPH at physiological temperature generates alkyl radicals, which subsequently add oxygen and this results in the background oxygen consumption. The background oxygen consumption (R_i) has been subtracted to accurately identify LDL peroxidation.
[c] Intrinsic TOH (3.37 μM) and other antioxidants in native LDL.
[d] AOH added after the depletion of the intrinsic antioxidants (see Fig. 3).
[e] AOH added before the initiation (see Fig. 4).
[f] Could not be calculated, see text.
[g] $kcl_{inh} = R_{inh}/R_i$; $kcl_p = R_p/R_i$.
[h] $n = R_i t_{inh}/([AOH]_0 + [TOH]_0)$.
[i] Assuming that TOH is the major antioxidants in the native LDL and each TOH molecule traps two peroxyl radicals.

LDL and the inhibition time induced by hydroxycinnamic acid derivatives when it was used after depletion of the endogenous antioxidants (compare Figs. 3 and 4). The efficacy sequence is CaA > ChA > SA > FA ~ CoA (Table 1), similar to that obtained when these compounds were used after depletion of the antioxidants in the native LDL. This suggests that hydroxycinnamic acid derivatives serve as chain-breaking antioxidants independently and they do not have synergistic interaction with the intrinsic antioxidants, e.g., α-tocopherol (TOH), in the native LDL. It has been reported previously that if an exogenous antioxidant, such as vitamin C or green tea polyphenols, could react with TOH in a synergistic fashion in the native LDL, the overall inhibition time would significantly longer than the sum of the intrinsic inhibition time of the native LDL and the inhibition time induced by the exogenous antioxidant (Liu et al., 2000; Niki, Saito, Kawakami, & Kamiya, 1984).

3.2. Inhibition of Cu^{2+}-induced LDL peroxidation

Cu^{2+} was also used to induce LDL peroxidation and the formation of malondialdehyde (MDA), which is the final oxidation product of lipid peroxidation, was used to monitor LDL peroxidation (Buege & Aust, 1978). Fig. 5 shows the MDA formation when LDL peroxidation challenged with Cu^{2+}. Similar to the oxygen uptake experiments

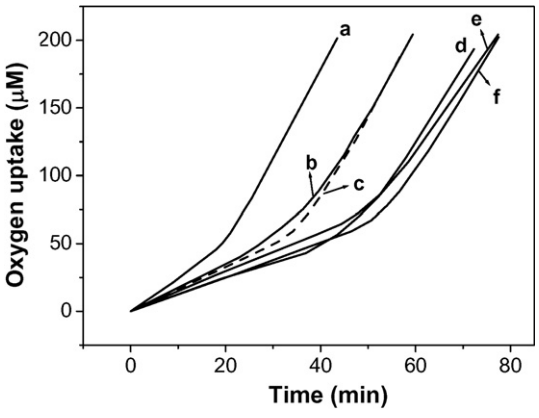


Fig. 4. Representative oxygen uptake curves recorded during the AAPH-initiated and hydroxycinnamic acid derivatives (AOH) inhibited LDL peroxidation in PBS (pH 7.4) at 37 °C under atmospheric oxygen. [LDL] = 0.4 mg/ml; $[AAPH]_0$ = 10 mM; $[AOH]_0$ = 4 μM. The AOHs were added before the initiation. (a) Native LDL containing 3.37 μM of TOH; (b) CoA; (c) FA; (d) ChA; (e) SA; (f) CaA.

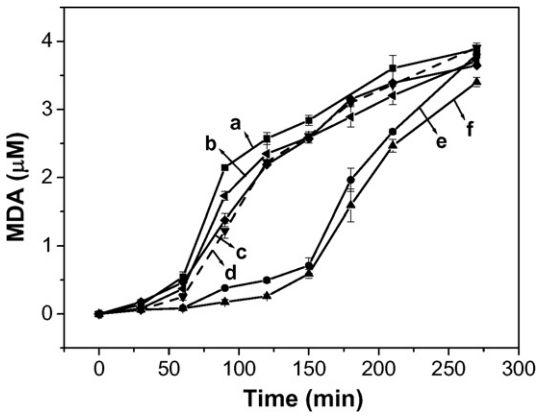


Fig. 5. Inhibition of MDA formtion during the Cu^{2+}-induced peroxidation of LDL by hydroxycinnamic acid derivatives (AOH) at 37 °C. The LDL was suspended in PBS (pH 7.4) at a protein concentration of 0.4 mg/ml. $[CuSO_4]$ = 10 μM; $[AOH]_0$ = 4 μM. (a) Native LDL; (b) FA; (c) CoA; (d) SA; (e) CaA; (f) ChA. Every experiment was repeated three times.

Table 2
Inhibition times (t_{inh}) of hydroxycinnamic acid derivatives (AOH) in Cu^{2+}-induced peroxidation of human LDL[a]

AOH	TOH[b]	CaA	ChA	SA	FA	CoA
t_{inh} (min)	51 ± 3.0	139 ± 5.0	137 ± 4.6	56 ± 1.2	53 ± 2.6	56 ± 1.4

[a] Determined in PBS (pH 7.4) at 37 °C; [LDL] = 0.4 mg/ml; [$CuSO_4$] = 10 μM; $[AOH]_0$ = 2 μM. Every experiment was repeated three times and the SD are shown in the table.
[b] Intrinsic TOH (3.37 μM) and other antioxidants in the native LDL.

mentioned earlier, formation of MDA is inhibited for a short period of time due to the presence of endogenous antioxidants in the LDL. It is seen that addition of hydroxycinnamic acid derivatives significantly suppresses the rate of MDA formation and increases the inhibition period of the native LDL. The antioxidative activity of hydroxycinnamic acid derivatives is assessed by their inhibition period, t_{inh}, and it follows the sequence similar to that observed in the oxygen uptake assay mentioned above, i.e., CaA ~ ChA > SA ~ FA ~ CoA (Table 2).

4. Discussion

It has been proved that lipid peroxidation in model biomembranes follows the same classical rate law for auto-oxidation as that in homogeneous solutions (Barclay, 1993). The kinetic parameters deduced from Figs. 2–4 are listed in Table 1. The kinetic of LDL peroxidation initiated by AAPH and its inhibition by the chain-breaking antioxidant (AH) have been discussed in detail in our previous paper (Liu et al., 2000). In Table 1, k_{inh} is the rate constant of inhibition (Eq. (1)) that represents the activity of the antioxidant, n is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule, and the kinetic chain length (kcl) defines the number of chain propagations by each initiating radical and is expressed by kcl_{inh} and kcl_p for inhibited and uninhibited peroxidations, respectively. By taking the n value of TOH as 2 (Bowry & Stocker, 1993; Burton & Ingold, 1986), R_i, the apparent rate of initiation, is generally determined from the inhibition period (Eq. (2)) and/or from the decay rate of TOH (Eq. (3)). In the present experiment the R_i value calculated from the inhibition period is 5.0 nM/s. This value is in good agreement with the value of 5.2 nM/s obtained from the decay of TOH (figure not shown).

$$LOO^{\cdot} + AH \xrightarrow{k_{inh}} LOOH + A^{\cdot} \quad (1)$$

$$R_i = (n[AH]_0)/t_{inh} \quad (2)$$

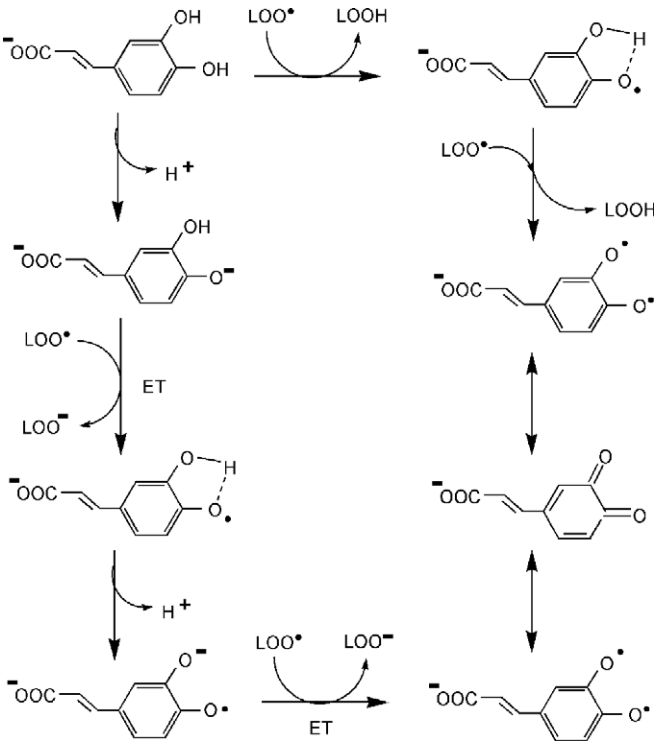
$$R_i = -n(d[AH]/dt) \quad (3)$$

$$-d[O_2]/dt = R_{inh} = k_p R_i[LH]/(nk_{inh}[AH]). \quad (4)$$

It can be seen from Figs. 2–4 and Table 1 that in the present experimental conditions the endogenous TOH which contributes >95% of endogenous antioxidants in native LDL (Esterbauer & Ramos, 1995; Nokuchi et al., 1993) acts as a chain-breaking antioxidant, in accordance with previous reports (Culbertson, Antunes, Havrilla, Milne, & Porter, 2002; Liu et al., 2000; Niki, Noguchi, & Gotoh, 1993; Noguchi, Gotoh, & Niki, 1993). The inhibition rate constant, k_{inh}, of α-tocopherol is calculated to be $4.1 \times 10^5\ M^{-1}\ s^{-1}$ according to Eq. (4), taking the rate constants for the chain propagation, k_p, in phospholipid membranes as $16.6\ M^{-1}\ s^{-1}$ (Barclay, Vinqvist, Antunes, & Pinto, 1997) and the concentration of polyunsaturated fatty acids, [LH], in LDL as 0.55 M (Esterbauer & Ramos, 1995).

All these compounds produce clear inhibition periods and decrease significantly kinetic chain lengths in the absence of endogenous antioxidants, demonstrating that they are good antioxidants in LDL. The inhibition rate constant, k_{inh}, of these compounds is comparable to that of TOH and the stoichiometric factor, n, of CaA and ChA is 2.6, implying that each molecule of CaA and ChA might be able to trap about two peroxyl radicals, hence more than one hydroxyl group must be involved in the antioxidation process. These facts make CaA and ChA the most active antioxidant among these compounds. Based on the inhibition time and/or stoichiometric factor the antioxidant efficacy of hydroxycinnamic acid derivatives follows the sequence of CaA ~ ChA > SA > FA > CoA.

The important event of lipid peroxidation is oxygen absorption and the final product of the peroxidation is MDA (Buege & Aust, 1978). In the present work, both indexes were used to evaluate the antioxidative activity of hydroxycinnamic acid derivatives to find the structural determinant responsible for the antioxidant activity *in vitro*. Comparison of the data of Tables 1 and 2 demonstrates that the antioxidant activity of hydroxycinnamic acid derivatives follows the similar sequence in spite of the peroxidation being initiated by AAPH or by Cu^{2+}, and in spite of the activity being monitored by oxygen uptake or by MDA formation. It is clearly seen that the antioxidative activity of CaA and ChA is significantly higher than the other compounds. That is, molecules bearing *ortho*-dihydroxyls are remarkably more active than those bearing no such functionalities. It was known that the *ortho*-hydroxyl substitution on phenol would make the oxidation intermediate, *ortho*-hydroxyphenoxyl radical, more stable due to the intramolecular hydrogen bonding interaction as reported recently from both experiment (Foti & Ruberto, 2001) and theoretical calculations (Wright, Johnson, & Dilabio, 2001) (Scheme 1). In addition, *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion shall be easily further oxidized to form the final product *ortho*-quinone as exemplified in Scheme 1 (Foti & Ruberto, 2001). The fact that the stoichiometric factor, n, of CaA and ChA is larger than two (Table 1) suggests that the second peroxyl radical must be involved in the



Scheme 1. Mechanism of CaA inhibited LDL peroxidation.

antioxidation reaction leading to the formation of the corresponding *ortho*-quinone as shown in Scheme 1. In AAPH-induced LDL peroxidation, SA and FA bearing 4-hydroxy-3-methoxyl functionalities possess significantly higher antioxidant activity than CoA bearing no such functionalities. It has also been proved that the *ortho*-methoxyl group can form an intramolecular hydrogen bond with the phenolic hydrogen, making the H-atom abstraction from the *ortho*-methoxyphenols surprisingly easy (de Heer, Mulder, Korth, Ingold, & Lusztyk, 2000). The high reactivity of *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl functionalities was also observed in flavonols and their glycosides (Dai, Miao, Zhou, Yang, & Liu, 2006; Hou et al., 2004a, Hou, Zhou, Yang, & Liu, 2004b; Zhou et al., 2005a), resveratrol analogues (Cai, Fang, Ma, Yang, & Liu, 2003; Fang et al., 2002) and curcumin analogues (Chen, Deng, Zhou, Yang, & Liu, 2006; Wei, Chen, Zhou, Yang, & Liu, 2006b).

It is also noticed that the antioxidant activity of hydroxycinnamic acid derivatives is correlated with the electrochemical behaviour of the molecule. The oxidation potential, $E_{4.7}$ at pH 4.7, was reported to be 0.35, 0.39, 0.57 and 0.73 (V vs. Ag/AgCl) for CaA, ChA, FA and CoA, respectively (Felice, King, & Kissinger, 1976). Therefore, the increase of the oxidation potential is correlated well with the decrease of the antioxidative activity in the sequence of CaA ~ ChA > FA > CoA. This correlation suggests that electron transfer antioxidation might take place simultaneously with the direct hydrogen-abstraction reaction, as exemplified in Scheme 1. It is well known that phenoxides undergo electron transfer oxidation more easily to produce relatively stable phenoxide radical anions in alkaline media. The acid dissociation constants, pKa_1, pKa_2 and pKa_3, of CaA were reported to be 4.36, 8.48 and 11.17, respectively (Silva et al., 2000). It demonstrates that phenolic hydroxyl of CaA can partially dissociate under our experimental conditions (pH 7.4), making the electron-transfer reaction feasible. Cooperation between hydrogen-abstraction and electron-transfer process in antioxidation reactions by phenolic antioxidants has recently been discussed (Cren-Olivé & Rolands, 2003; Wright et al., 2001).

TOH, the most biologically and chemically active form of vitamin E, is considered the major antioxidant within the LDL particle (Esterbauer, Dieber-Rothenneder, Striegl, & Wang, 1991). A meaningful feature of TOH antioxidant activity in LDL is the reduction of α-tocopheroxyl radical (TO$^{\cdot}$) by the co-existent antioxidant (ArOH) (Eq. (5)), such as L-ascorbic acid (vitamin C) (Niki et al., 1984) and green tea polyphenols (Liu et al., 2000; Zhou et al., 2005b), sustaining its antioxidant activity and eliminating the so-called tocopherol mediated peroxidation (TMP) (Bowry & Stocker, 1993). We have proved recently by using stopped-flow electron paramagnetic resonance (EPR) spectroscopy that green tea polyphenols could reduce α-tocopheroxyl radical (TO$^{\cdot}$) to regenerate TOH (Eq. (5)) with bimolecular rate constant ranging from 0.43×10^2 to 1.91×10^2 M^{-1} s^{-1} in sodium dodecyl sulfate (SDS) micelles (Zhou et al., 2005b).

$$TO^{\cdot} + ArOH \rightarrow TOH + ArO^{\cdot} \quad (5)$$

It was also reported that CaA could restore TOH at LDL surface (Eq. (5)), but CoA oxidized it (Laranjinha et al., 1995). Although the reported oxidation potential of CaA (0.54 V vs. a saturated calomel electrode (SCE) at pH 7) (Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994) is slightly higher than that of TOH (0.48 V vs. SCE at pH 7) (Steenkens & Neta, 1982), the oxidation potential of CaA is highly sensitive to pH (decreasing as pH increase) and therefore it is possible CaA can reduce TO$^{\cdot}$ to regenerate TOH (Eq. (5)) at pH 7.4 (Laranjinha et al., 1995). However, it can be seen from Table 1 that the k_{inh} decreases remarkably when hydroxycinnamic acid derivative is added before the initiation, and the overall inhibition time is approximately equal to the sum of the inhibition time of the endogenous antioxidants (principally TOH) in native LDL and that of the hydroxycinnamic acid derivative when it is added after depletion of the endogenous antioxidants. These facts suggest that TOH and the exogenous hydroxycinnamic acid derivative might act independently, i.e., no synergistic antioxidant interaction takes place between the hydroxycinnamic acid derivative and TOH in LDL. This is probably due to the fact that the reaction rate of CaA with peroxyl radicals in LDL, k_{inh}, is too fast (the complete inhibition of LDL peroxidation by CaA implies an infinitely large k_{inh}), while the rate of the TOH regeneration reaction (Eq. (5)) is very slow. Hence the reaction (5) cannot compete with the reaction (1). On the other hand,

the oxidation potential of other hydroxycinnamic acid derivatives (SA, FA and CoA) might be higher than that of TOH at pH 7.4, rendering the TOH regeneration reaction (Eq. (5)) by them thermodynamically unfeasible.

In conclusion, the results reported in this article demonstrate that hydroxycinnamic acid derivatives can inhibit AAPH- and Cu^{2+}-induced LDL peroxidation. The compounds bearing *ortho*-dihydroxyl or 4-hydroxy-3-methoxyl functionality exhibit remarkably higher antioxidative activity than the ones bearing no such functionalities. It gives us useful information for antioxidant drug design. Synergistic antioxidant interaction between hydroxycinnamic acid derivatives and α-tocopherol is not observed in LDL.

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