Fruit Hydroxycinnamic Acids Inhibit Human Low-Density Lipoprotein Oxidation in Vitro

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Oxidative modification of low-density lipoprotein (LDL) is believed to play a crucial role in atherogenesis. The antioxidant activity of hydroxycinnamic acids typically present in fruits was investigated in vitro using a human LDL oxidation assay. The *o*-dihydroxy compounds caffeic, caftaric, chlorogenic, and neochlorogenic acids had highest antioxidant activities and inhibited LDL oxidation from 86 to 97% at 5 μM. Ferulic and fertaric acids had lower antioxidant activities, and *p*-coumaric and *p*-coutaric acids had the lowest. Differences in antioxidant activity were thus primarily related to the hydroxylation and methylation pattern. The esterification to tartaric acid slightly enhanced the antioxidant activity of *p*-coumaric and ferulic acids, but esterification of caffeic acid to quinic acid (as in chlorogenic and neochlorogenic acid) had no effect on or slightly decreased antioxidant activity. The observed differences in activities are discussed in terms of structural dissimilarities of the compounds. It is proposed that for the less active hydroxycinnamic acids, *p*-coumaric and ferulic acids, esterification to tartaric acid may enhance their ability to inhibit LDL oxidation by binding to apolipoprotein B in the LDL particle.

Keywords: *Antioxidant; LDL; p-coumaric, ferulic, caffeic, hydroxycinnamoyltartaric acids; hydroxycinnamoylquinic acids*

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) is believed to be implicated in atherogenic plaque formation, which is one of the main causes of coronary heart disease (Steinberg, 1988). Dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis and prevent coronary heart disease (Esterbauer et al.,1992; Frankel et al., 1993; Kinsella et al., 1993). There is strong evidence from epidemiological data that consumption of fresh fruits is associated with a lowered risk of coronary heart disease mortality (Criqui and Ringel, 1994). This apparent cardioprotective action of fruits is currently believed to be at least partly attributable to the antioxidant activity of ascorbic acid, β-carotene, tocopherols, and flavonoids abundant in fruits and other plant foods (Gey, 1995; Hertog et al., 1993, 1995; Kinsella et al., 1993). Comparatively little is known about the antioxidant and potential biological activities of hydroxycinnamic acid compounds. Hydroxycinnamic acids are phenolic compounds ubiquitously present in plant foods. Their chemical structures consist of a phenolic ring with a lateral three-carbon chain (C_6-C_3) (Figure 1) (Macheix et al., 1990).

In the diet, hydroxycinnamic acids are largely derived from fruits, grains, and coffee. In fruits, hydroxycinnamic acids are mainly derived from *p*-coumaric, caffeic, and ferulic acids and predominantly occur in esterified form with quinic acid or glucose (Herrmann, 1989).

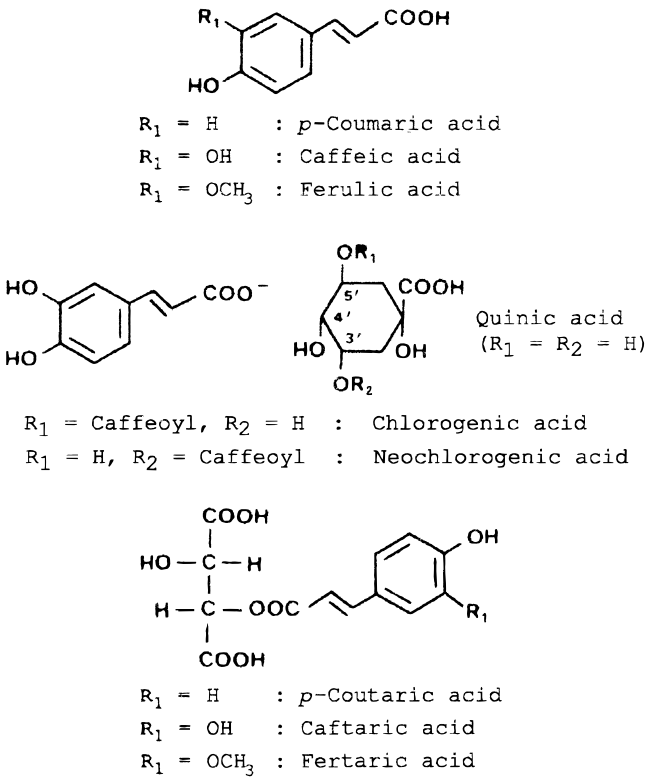

Figure 1. Structures of the hydroxycinnamic acids and their derivatives used in this study.

Hydroxycinnamoylquinic acids are the quantitatively most dominant form in pome and stone fruits, with chlorogenic acid (5′-caffeoylquinic acid) being predominant in apples, pears, and peaches and neochlorogenic

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acid (3′-caffeoylquinic acid) dominating in, for example, plums (Herrmann, 1989). Hydroxycinnamoylquinic acids are important for the development of color and flavor in foods and also play a prominent role in polyphenol oxidase catalyzed browning in fruit processing (Macheix et al., 1990; Nicolas et al., 1994).

In grapes (genus *Vitis*), the quinic acid esters are uniquely replaced by tartaric acid esters such as caftaric acid [caffeoyl-L-(+)-tartaric acid], coutaric acid [*p*-coumaroyl-L-(+) tartaric acid], and fertaric acid [feruloyl-L-(+)-tartaric acid] (Herrmann, 1989). Of these, *trans*-caftaric acid is considered a major substrate for coupled oxidation and enzymatic browning reactions when grapes are processed for wine making (Cheynier et al., 1989; Singleton, 1987).

In plants, hydroxycinnamic acids can also be covalently linked to cell wall polysaccharides (Fry, 1982; Herrmann, 1989) or esterified with lignin (Macheix et al., 1990), but little is known about the precise positions, abundance, or chemical reactivity of these bound derivatives in fruits and other plant foods.

Information on human metabolism of hydroxycinnamic acid compounds is limited. In a preliminary investigation with fewer than 10 volunteers, metabolites of caffeic and ferulic acids were detected in the urine, indicating some absorption of these compounds (Jacobson et al., 1983). However, at present there is no direct evidence that hydroxycinnamic acids exert a cardioprotective antioxidant activity in vivo.

Pure caffeic acid was demonstrated to act as a potent inhibitor of LDL oxidation in various in vitro systems (Abu-Amsha et al., 1996; Chen and Ho, 1997; Laranjinha et al., 1994; Nardini et al., 1995; Teissedre et al., 1996; Vinson et al., 1995a). In analogy to other natural phenolic antioxidants (Hudson and Lewis, 1983; Rice-Evans et al., 1996) apparently the presence of the *o*-dihydroxy group (Figure 1) generally enhances the antioxidant activity of hydroxycinnamates toward LDL (Abu-Amsha et al., 1996; Chen and Ho, 1997; Nardini et al., 1995). The antioxidant activities of ferulic acid and other hydroxycinnamic acids reported in the literature vary (Chen and Ho, 1997; Cuvelier et al., 1992; Graf, 1992; Scott et al., 1993). In a high-temperature accelerated methyl linoleate autoxidation assay the order of antioxidant activity was caffeic > chlorogenic > ferulic > *p*-coumaric acid (Cuvelier et al., 1992). In general, the results are difficult to interpret because different test systems were employed to assess antioxidant activity. It appears that there are no data for the antioxidant activity of hydroxycinnamoyltartrates or for neochlorogenic acid.

The purpose of this study was to evaluate systematically the antioxidant activity toward human LDL oxidation in vitro of hydroxycinnamic acids typically found in fruits. The specific aim was to investigate the effect on activity of (1) the number of hydroxy groups and (2) the degree and type of esterification.

MATERIALS AND METHODS

Materials. Caffeic acid, catechin, *p*-coumaric acid, ferulic acid, and quinic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorogenic acid and tartaric acid were from Aldrich (Steinheim, Germany). Neochlorogenic acid was a gift from Dr. Murray Isman (University of British Columbia, Vancouver, Canada). Hydroxycinnamoyltartrates were purified from fresh grapes as described below.

Extraction and Isolation of Hydroxycinnamoyltartrates from Grapes. *trans*-Caftaric acid, *trans*-*p*-coutaric acid, and *trans*-fertaric acid were extracted from unripe Grenache grapes (8 kg, 10 °Brix) according to the procedure of Singleton et al. (1985). Grapes were harvested from the experimental vineyards of the Department of Viticulture and Enology at the University of California, Davis, in the early fall of 1995. The methanolic extracts obtained from the grapes were pooled and concentrated by rotary evaporation under vacuum (<35 °C) to a total concentration of hydroxycinnamic acids of ≈20 mg L^{-1}. The hydroxycinnamoyltartrates were then purified by semipreparative high-performance liquid chromatography (HPLC) using a Waters 510 pump (Milford, MA) and a Hewlett-Packard model 1050 (Palo Alto, CA) UV−vis detector set at 360 nm. The eluent used was 8% methanol in 3% acetic acid in water delivered isocratically at a flow rate of 5.0 mL min^{-1} through an RCM Novapak C_{18}, 25 × 100 mm, 4 μm particle size column (Waters Associates, Milford, MA).

HPLC Analysis. The purified hydroxycinnamoyltartrates were identified by their spectra and retention times using an HPLC procedure described earlier (Lamuela-Raventos and Waterhouse, 1994). The retention times and spectral properties of the three purified cinnamic acid tartrates are shown in Figure 2.

Isolation of Human LDL. LDL was prepared from the blood of three normolipidemic males as described previously (Frankel et al., 1992). Prior to oxidation, isolated LDL was dialyzed with deoxygenated, phosphate-buffered (10 mM, pH 7.4) saline (100 mM) at 4 °C. After determination of the protein concentration according to the method of Lowry et al. (1951), LDL was diluted to a standard protein concentration of 1.0 mg mL^{-1} for the antioxidant assay.

Antioxidant Activity toward LDL Oxidation. Antioxidant activity of hydroxycinnamic acids was determined by monitoring hexanal production by static headspace chromatography from copper-catalyzed oxidation of human LDL (2 h at 37 °C, 80 μM $CuSO_4$) as previously described (Frankel et al., 1992). Immediately prior to assay, the individual compounds were dissolved in doubly distilled water and tested at concentrations of 5, 10, and 20 μM. Known dilutions of caffeic acid were used to quantify the molar concentration of caftaric acid, *p*-coumaric acid was used to quantify *p*-coutaric acid, and ferulic acid was used to quantify fertaric acid, assuming similar extinction coefficients at 316 nm for each compound and its tartrate ester. The different concentrations of all compounds tested were calibrated to add equal sample sizes of 10 μL to 0.25 mL of LDL reaction mixture. Antioxidant activities were calculated after replicate analyses and expressed as percent relative inhibition of LDL oxidation: (%In) = $[(C - S)/C] \times 100$, where C was the average amount of hexanal formed in the control and S was the average amount of hexanal formed in the sample.

Statistical Analyses. Differences in antioxidant activities were tested by one-way analyses of variance using Minitab Statistical software (Addison-Wesley, Reading, MA).

RESULTS

The hydroxycinnamates were effective in inhibiting the in vitro copper-catalyzed oxidation of human LDL. Caffeic acid almost blocked LDL oxidation at all micromolar levels tested. The other hydroxycinnamates showed a dose-dependent antioxidant activity (Table 1). Pure tartaric and quinic acids showed very low antioxidant activity (4−13% inhibition) and exhibited no dose−response relationships across the different levels of addition. The most significant differences in antioxidant activities were found when the hydroxycinnamic acids were evaluated at 5 μM. When compared at this concentration, the inhibition of LDL oxidation ranged from 24.3 to 97.6% with *p*-coumaric and ferulic acids exerting the lowest (24.3−24.5%) and caffeic, caftaric, chlorogenic, and neochlorogenic acids the highest activity with 86.5−97.6% inhibition (Table 1). In comparison, catechin, which is known to be a potent antioxidant

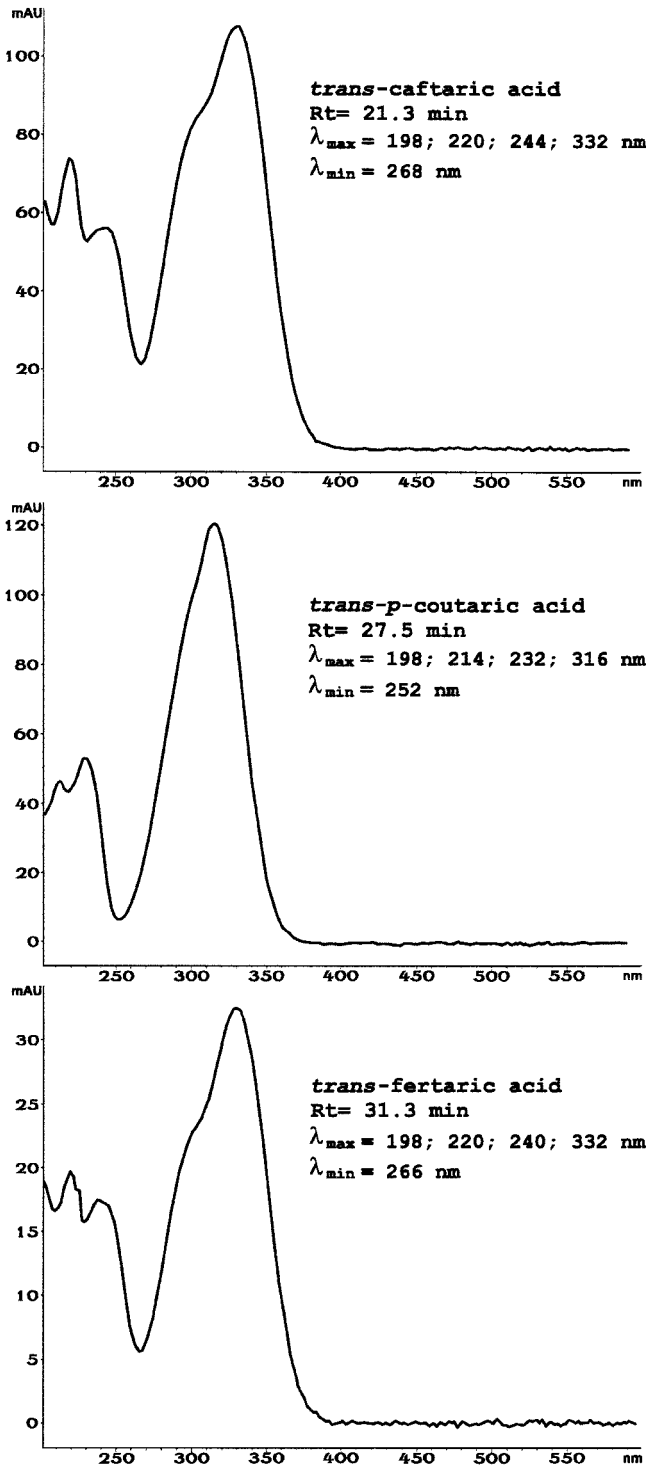

Figure 2. Retention times (RT) and spectra of *trans*-caftaric acid, *trans*-*p*-coutaric acid, and *trans*-fertaric acid separated by HPLC.

in red wine (Frankel et al., 1995) and was included here as a control compound, exerted 67.5% inhibition of LDL oxidation when tested at 5 μM. Ferulic acid and fertaric acid inhibited LDL oxidation by 24 and 39%, respectively, at 5 μM (Table 1). When evaluated at 10 and 20 μM, caffeic acid and its esterified derivatives almost completely inhibited LDL oxidation, with antioxidant activities between 98 and 99%. Ferulic acid and fertaric acid inhibited from 55.7 to 92.6%, and *p*-coumaric and *p*-coutaric acids had somewhat lower activities. Only at 5 μM were the antioxidant activities of *p*-coumaric and ferulic acids not statistically different. Thus, among the free acids the overall order of reactivity was caffeic > ferulic > *p*-coumaric, and similarly among their esterified tartaric acid derivatives the order was caftaric > fertaric > *p*-coutaric (although the differences in reactivity were not statistically significant in all cases) (Table 1).

Table 1. Percent Inhibition of Human LDL Oxidation in Vitro by Hydroxycinnamic Acids from Fruits[a]

compound tested	5 μM	10 μM	20 μM
p-coumaric acid	24.5 ± 0.0^{e}	40.7 ± 0.7^{d}	66.9 ± 0.8^{c}
ferulic acid	24.3 ± 2.2^{e}	55.7 ± 5.9^{bc}	92.6 ± 1.0^{a}
caffeic acid	96.7 ± 1.4^{a}	97.9 ± 0.2^{a}	99.1 ± 0.5^{a}
coutaric acid	ND[b]	50.3 ± 0.1^{cd}	83.2 ± 1.8^{b}
fertaric acid	39.4 ± 0.0^{d}	66.3 ± 3.0^{b}	84.1 ± 1.3^{b}
caftaric acid	97.6 ± 0.1^{a}	99.0 ± 0.5^{a}	99.3 ± 0.1^{a}
chlorogenic acid	90.7 ± 6.2^{ab}	99.1 ± 0.4^{a}	99.0 ± 0.5^{a}
neochlorogenic acid	86.5 ± 2.4^{b}	98.9 ± 0.7^{a}	99.0 ± 0.5^{a}
tartaric acid	9.7 ± 3.2^{f}	12.7 ± 7.1^{e}	11.2 ± 0.0^{d}
quinic acid	4.4 ± 0.0^{f}	11.5 ± 3.6^{e}	13.2 ± 5.3^{d}
catechin	67.5 ± 4.1^{c}	97.8 ± 0.1^{a}	98.7 ± 0.0^{a}

[a] Inhibition data are given as mean percentage values ± standard deviation. Results in the same column followed by the same roman superscript letter are not significantly different at $P < 0.05$. Tartaric acid and quinic acid were included to test the antioxidant activity of the non-hydroxycinnamic parts of the hydroxycinnamic acid esters. Catechin was included as a control compound. [b] ND, not determined.

Table 2. Comparison of Expected Antioxidant Activities (Percent Inhibition) for *p*-Coumaric and Ferulic Acids plus Tartaric Acid with Measured Activities for Corresponding Esterified Acids[a]

compound	5 μM	10 μM	20 μM
p-coumaric + tartaric[b]	34.2 ± 3.2^{a}	53.3 ± 7.7^{ba}	78.1 ± 0.8^{c}
ferulic + tartaric[b]	34.0 ± 5.4^{a}	68.4 ± 1.2^{a}	103.8 ± 1.0^{a}
coutaric acid	ND[c]	50.3 ± 0.1^{b}	83.2 ± 1.8^{b}
fertaric acid	39.4 ± 0.0^{a}	66.3 ± 3.0^{ab}	84.1 ± 1.3^{b}

[a] Based on data from Table 1. Inhibition data are given as mean percentage values ± standard deviation. Results in the same column followed by the same roman superscript letter are not significantly different at $P < 0.05$. [b] Estimated activity based on summation of the compounds' individual antioxidant activities (Table 1). [c] ND, not determined.

Except for ferulic acid at 20 μM, the antioxidant activity of both *p*-coumaric and ferulic acids increased slightly when esterified to tartaric acid. When caffeic acid was esterified to tartrate, there was no change in activity as compared to caffeic acid. This result could be due to the high antioxidant activity of pure caffeic acid. However, when caffeic acid was esterified to quinic acid, as in chlorogenic and neochlorogenic acids, there was a tendency to a slightly lowered antioxidant activity at 5 μM (Table 1). Thus, at 5 μM levels of addition there was a clear grouping of the antioxidant activities of caffeic acid and caftaric acid versus chlorogenic and neochlorogenic acid. However, because the tails of the 95% confidence limits for the antioxidant activities of caffeic and caftaric acids versus chlorogenic acid overlapped slightly, the activity for chlorogenic acid was not significantly lower than that of caffeic acid at this level of significance (Table 1).

None of the activities obtained for coutaric acid and fertaric acid exceeded those expected by adding the antioxidant activities of the individual compounds tartaric acid and *p*-coumaric acid or tartaric acid and ferulic acid, respectively (Table 2). However, it cannot be immediately assumed that activities of different antioxidants are additive. This is because antioxidant

dose−response effects for inhibition of in vitro human LDL oxidation are not necessarily linear, and the shapes of the dose−response curves vary markedly for different plant phenolics (Nardini et al., 1995; Pearson et al., 1997; Vinson et al., 1995b). Moreover, pure tartaric acid did not show any dose−response relationship across the tested levels of addition (Table 1) (analysis of variance across addition levels not shown). The increased antioxidant activity of coutaric acid and fertaric acid as compared to unesterified *p*-coumaric and ferulic acid therefore cannot be ascribed to a radical scavenging or metal chelating capacity of the tartaric acid moiety of these compounds.

DISCUSSION

The pure hydroxycinnamic acids and their esterified derivatives exerted considerable antioxidant potency toward copper-catalyzed human LDL oxidation in vitro, with caffeic acid and its esterified derivatives being the most active compounds. The differences in LDL antioxidant activity between the different hydroxycinnamates can primarily be ascribed to variations in the hydroxylation and methylation pattern of the aromatic ring. The greater antioxidant activity of caffeic acid and its derivatives compared to ferulic and fertaric acids indicates that methylation of one of the *o*-hydroxy groups decreases antioxidant activity toward LDL. In turn, the substitution of the 3-methoxy group for hydrogen as in *p*-coumaric and *p*-coutaric acid further lowers activity. This observed order of antioxidant activity is consistent with the hydrogen-donating mechanism that the number of OH groups, and especially the 3′,4′ *o*-hydroxyls, increase the reactivity (Hudson and Lewis, 1983; Rice-Evans et al., 1996). *o*-Diphenols such as caffeic acid may also be potent metal chelators (Hudson and Lewis, 1983; Nardini et al., 1995). The presence of the *o*-methoxy group in ferulic acid apparently results in the formation of a more stable radical, presumably by electron delocalization, than does *p*-coumaric acid containing only the sole hydroxy group (Figure 1).

The observed differences in antioxidant activity produced by esterification may be ascribed to other structural properties. Decreased antioxidant activity of caffeic acid when esterified to quinic acid as in neochlorogenic and chlorogenic acids is in agreement with data observed in other systems (Chen and Ho, 1997; Cuvelier et al., 1992). The slightly decreased activity may be due to changes in solubility and partition of caffeic acid in the LDL system. However, at present, there are no data available on partition of antioxidants in LDL systems.

Other physicochemical factors may also be considered to explain the increased antioxidant activity with esterification of *p*-coumaric and ferulic acid. It was shown recently that the copper-mediated oxidation of tryptophan residues in LDL−apolipoprotein B plays an important causative role in initiating lipid oxidation in LDL particles (Giessauf et al., 1995). From this, it can be hypothesized that a unique antioxidant mechanism for LDL may be sterical blockage of the copper access to apolipoprotein B tryptophans via binding of antioxidants to apolipoprotein B. Apolipoprotein B constitutes ∼22% of the LDL particle mass (Esterbauer et al., 1992). Thus, as we suggested previously (Teissedre et al., 1996), structural features conferring differences in protein binding may affect the antioxidant activity of phenolic phytochemicals in inhibiting oxidation of LDL. Esterification of *p*-coumaric and ferulic acids to tartaric acid may thus confer better protein binding properties that may affect antioxidant activity. However, more research is needed to explain whether the effect of improved protein binding has a protective effect in reducing oxidation of tryptophan in the apolipoprotein B of LDL. Different antioxidant mechanisms may be in play to inhibit oxidation of LDL. Caffeic acid and its esterified derivatives may work mainly as radical scavengers in the LDL system. The less efficient scavengers *p*-coumaric and ferulic acids and their esterified derivatives may function via other mechanisms, including perhaps binding to apolipoprotein B to block copper access. Further work is clearly needed to establish antioxidant mechanisms effective for the inhibition of human LDL oxidation. The improved understanding of LDL antioxidant mechanisms might also facilitate the transfer of in vitro observations to the effects of hydroxycinnamic acids and other plant phytochemicals in vivo.

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