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Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants

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Abstract In this study the antioxidant activities of the hydroxycinnamic acids, chlorogenic, caffeic, ferulic and *p*-coumaric, have been investigated in peroxidising lipid systems mediated by metmyoglobin. The results show that the order of effectiveness in increasing the resistance of LDL to peroxidation, in protecting LDL cholesterol from oxidation and preventing the oxidative modification of the LDL apoprotein B_{100} is caffeic = chlorogenic > ferulic > *p*-coumaric acid. Assessment of the rates of reaction of the hydroxycinnamates with ferrylmyoglobin, a product of the reductive decomposition of lipid hydroperoxides, reveals that the compounds are more effective as peroxyl radical scavengers than reductants of ferryl myoglobin in peroxidising LDL systems mediated by haem proteins.

Key words: Antioxidant activity; Hydroxycinnamic acid; Phenylpropanoid metabolism; Higher plants

1. Introduction

The evidence for a role for dietary intake of phytochemicals, including the carotenoids, α-tocopherol, ascorbic acid and the flavonoids, in the maintenance of health and protection from disease is accumulating [1–3]. Recent biochemical studies have described the hierarchy of the antioxidant potentials of the polyphenolic flavanoids in relation to those of Trolox, the water-soluble vitamin E analogue, and vitamin C against radicals generated in the aqueous phase [4,5] and there are many reports of their inhibitory effects in lipid systems [4,6–9]. In particular, the catechins and catechin-gallate esters, the polyphenolic constituents of tea, quercetin, kaempferol and the anthocyanidins, constituents of grape skin and red wine, have been shown to be effective antioxidants against radicals in the aqueous and lipophilic phases [3,5,10]. Furthermore, ingestion of these beverages has been reported to enhance the antioxidant levels of plasma [11,12].

Little attention, however, has focused on the antioxidant activity of the simple phenolic acids or phenylpropanoids present in many dietary phytochemicals which are at higher concentrations than the polyphenolic flavonoids and anthocyanidins [13]. Among the most widely distributed phenylpropanoids in plant tissues are the hydroxycinnamic acids, coumaric, caffeic and ferulic produced from the shikimate pathway from L-phenylalanine or L-tyrosine (Fig. 1). Their occurrence is usually in various conjugated forms resulting from enzymic hydroxylation, *O*-methylation, *O*-glycosylation or esterification of *p*-coumaric acid, principally as the quinic acid or carbohydrate esters (reviewed in [14]).

The biological activities [15] of the phenylpropanoids and their role as antimicrobial agents [16,17] is well recognised, as well as their properties as antiallergic and anti-inflammatory agents through lipoxygenase inhibition [18] and their antimutagenic actions [19,20].

In this study we have examined the antioxidant properties of the hydroxycinnamic acids in terms of their abilities to increase the resistance of low density lipoproteins (LDL) to cholesterol oxidation, lipid peroxidation and oxidative modification of the apoprotein B_{100}. The results show that the sequence of the effectiveness against lipid peroxyl radicals generated in the lipophilic phase of LDL and in protecting LDL cholesterol from oxidation is: chlorogenic = caffeic > ferulic > *p*-coumaric acid.

2. Materials and methods

2.1. Experimental

Phosphate-buffered saline 10 mM, pH 7.4 (PBS), was prepared with Analar grade salts from BDH (Merck Ltd., Poole, Dorset BH15 1TD, UK). Equine metmyoglobin (Sigma-Aldrich) was purified prior to use on a 35 × 2.5 cm Sephadex G15–20 column, after oxidation with excess potassium ferricyanide as previously described [21]. Concentration and purity of metmyoglobin were determined spectroscopically applying extinction coefficients. Cinnamic acid derivatives were obtained from Sigma-Aldrich. Stock solutions of the cinnamic acid derivatives were prepared by dissolution in warm water with gentle sonication. Diluted working solutions were prepared daily and concentrations checked spectrophotometrically between 200 and 400 nm in a Beckman DU 7500 diode array spectrophotometer.

In order to assess the efficacy of the hydroxycinnamates to inhibit lipid peroxidation their effects on the oxidative modification of low density lipoproteins were assessed. Low density lipoproteins were isolated from plasma using the modified method of Chung et al. [22]. The samples were stored at 4°C in phosphate-buffered saline, pH 7.4, in the presence of 100 μM EDTA; for each series of experiments small aliquots of the samples were dialysed with PBS in 10 μM EDTA and used within 4 days. Concentration of LDL protein was estimated using the method of Markwell et al. [23].

In order to evaluate the relative abilities of the hydroxycinnamates to inhibit LDL oxidation, 0.25 mg LDL protein/ml were incubated with 10 μM metmyoglobin for 6 h at 37°C. The reaction was stopped by adding 20 μM butylated hydroxytoluene (BHT). LDL oxidation was assessed by monitoring changes both in the surface charge of apolipoprotein B_{100} and in lipid peroxidation. Relative electrophoretic mobility (REM), which is an index of the surface charge of $apoB_{100}$, was measured using the Beckman Paragon LIPO electrophoresis kit and calculated as the ratio of electrophoretic mobility of the treated samples to that of native untreated LDL incubated for the same duration in buffer.

Lipid peroxidation was measured by determining aldehydic decomposition products of lipid peroxides using the thiobarbituric acid assay [24] applying appropriate controls [25]: 0.5 ml of TCA and 0.5 ml of a freshly prepared solution of thiobarbituric acid in 0.1 N hydrochloric

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acid (0.75%) were added to 0.5 ml of the samples. The mixture was heated for 20 min at 95°C and, after cooling, centrifuged at 3000 rpm for 15 min. The absorbance was measured at 532 nm and corrected for any minor contribution at 580 nm.

2.2. Cholesterol oxidation

Cholesterol was measured by the cholesterol esterase/cholesterol oxidase method (Boehringer-Mannheim CHOL-PAD kit) [26]. LDL (0.5 mg LDL protein/ml) was incubated with 20 μM metmyoglobin, retaining the same LDL/haem protein ratio as in the other investigations. For the assay 10 μl aliquots of the sample were incubated with 1 ml of the reagent at room temperature for 10 min. The absorbance of the final product of the enzymatic reaction was measured at 550 nm.

2.3. Suppression of ferryl myoglobin

The changes in the oxidative profile of activated myoglobin were investigated by visible spectroscopy using a Beckman DU7500 diode array spectrophotometer. MetMb (20 μM) without and with the appropriate concentration of the cinnamic acid derivative was incubated in the presence of H_2O_2 (1:1.25 mol ratio) at a final volume of 1 ml, in PBS, pH 7.4. Spectra were run at 2 min intervals from zero time (point of H_2O_2 addition) for 90 min. The oxidation states of Mb were determined using the Whitburn algorithms based on the extinction coefficients for ferryl, met- and oxymyoglobin [27]. Rate constants for the decay of ferryl myoglobin in the presence of the hydroxycinnamates were calculated applying a least-square fitting program.

3. Results

In order to assess the relative efficacies of the hydroxycinnamates as antioxidants against lipid peroxidation, their abilities to enhance the resistance of LDL to oxidation was investigated. An oxidative stress was applied which was not dependent on an initiating free radical species, in order to avoid the confounding effects of direct interaction between the initiating species and the phenolic antioxidant. Rather a pro-oxidant was applied, the mechanism of action of which was dependent on the oxidative and reductive decomposition of lipid hydroperoxides, namely, metmyoglobin [28,29]. Copper-induced LDL oxidation was avoided in order to eliminate the potential inhibitory contribution from copper chelation by the ortho dihydroxyphenolic structures.

$$\begin{aligned} \text{LOOH} + \text{HX-Fe}^{\text{III}} &\rightarrow \text{LO}^{\bullet} + \text{HX-[Fe}^{\text{IV}} = \text{O]} \\ &\rightarrow \text{LOO}^{\bullet} + \text{HX-Fe}^{\text{II}} \\ \text{LOOH} + \text{HX–Fe}^{\text{II}} &\rightarrow \text{LO}^{\bullet} + \text{HX–Fe}^{\text{III}} \end{aligned}$$

The mode of action of the hydroxycinnamates may be through hydrogen donation to the lipid peroxyl radicals, as chain-breaking antioxidants, reducing them to hydroperoxides; it has been reported that alkoxyl radicals are rearranged through their own reactivity to epoxides [30]. Another possible contribution to the antioxidant effects is the ability of the hydroxycinnamates to reduce ferryl myoglobin produced through the reductive decomposition of lipid hydroperoxides mediated by metmyoglobin.

The concentration-dependent inhibition of lipid peroxidation and oxidative modification of the apoprotein B_{100} after interaction between 10 μM metmyoglobin and 0.250 mg/ml LDL for 6 h is shown in Figs. 2 and 3. The results show that the concentration of hydroxycinnamate for 50% inhibition of LDL oxidation is caffeic acid = chlorogenic acid 0.33 μM, ferulic acid 0.9 μM, *p*-coumaric acid 4 μM, with closely consistent results for the inhibition of LDL polyunsaturated fatty acid peroxidation and for the protection from oxidative modification of the apoprotein B_{100}. The results (Table 1) also show that higher concentrations of the polyphenols are required to achieve 50% inhibition of cholesterol oxidation compared to lipid peroxidation and that the *o*-dihydroxycinnamic acids are more effective than the monohydroxy, monomethoxy substituted derivative, ferulic acid.

The question arises as to whether the suppression of LDL oxidation by the phenolic acids is ascribed to the chain-breaking antioxidant properties in hydrogen donation to the lipid peroxyl radical or whether, in addition, the hydroxycinnamates are capable of reducing the ferryl myoglobin species (formed during the oxidative decomposition of LDL hydroperoxides) to metmyoglobin. In order to determine the relative abilities of hydroxycinnamates to reduce ferryl myoglobin in the absence of lipid peroxides, ferryl myoglobin was generated by the activation of metmyoglobin with hydrogen peroxide (1:1.25 molar ratio) as previously described [31–33] and the reaction monitored for 90 min. The rate constants for the reaction of ferryl myoglobin with the antioxidant compounds were determined. To model the data the equation:

$$N = \frac{\lambda_1}{\lambda_2 - \lambda_1}(e^{-\lambda_1 t} - e^{-\lambda_2 t})$$

has been adopted. In this context no physical significance is attached to the constant λ_1, but λ_2 is taken as a fair description of the exponential decay of the efficacy of the phenolic acid. The equation has been fitted to the data with a least-squares minimization program [31]. The data (Table 2) show that the hydroxycinnamates react with ferryl myoglobin in the order shown: caffeic acid (65 $M^{-1} \cdot s^{-1}$), *p*-coumaric (23 $M^{-1} \cdot s^{-1}$), ferulic acid (8 $M^{-1} \cdot s^{-1}$).

4. Discussion

Considerable interest has focussed recently on the health benefits of phytochemicals in fruits and vegetables (reviewed in [1]), cereals and beverages through their antioxidant properties, not only from their content of antioxidant nutrients – vitamin C, vitamin E and the carotenoids – but also their polyphenolic constituents which have been reported to possess antioxidant (as well as other biological) activities. The antioxidant activity of the polyphenolics is defined principally by the presence of the orthodihydroxy substituents giving maximum radical stabilisation or allowing metal chelation, and an unsaturated 2,3 double bond in combination with a 4-oxo function [34,35]. The antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule, which is strengthened by steric hindrance as well as the electron-donating and -withdrawing capacities of the substituents on the

Table 1
Concentration of hydroxycinnamate for 50% inhibition of LDL lipid peroxidation, apoprotein B_{100} surface charge modification and cholesterol oxidation induced by metmyoglobin (for conditions, see text)

	Cholesterol oxidation (μM)	Lipid peroxidation (μM)	Altered surface charge (μM)
Caffeic acid	1.5	0.33	0.38
Chlorogenic acid	1.5	0.33	0.30
Ferulic acid	2.5	0.86	0.81
p-Coumaric acid	–	4.00	4.40

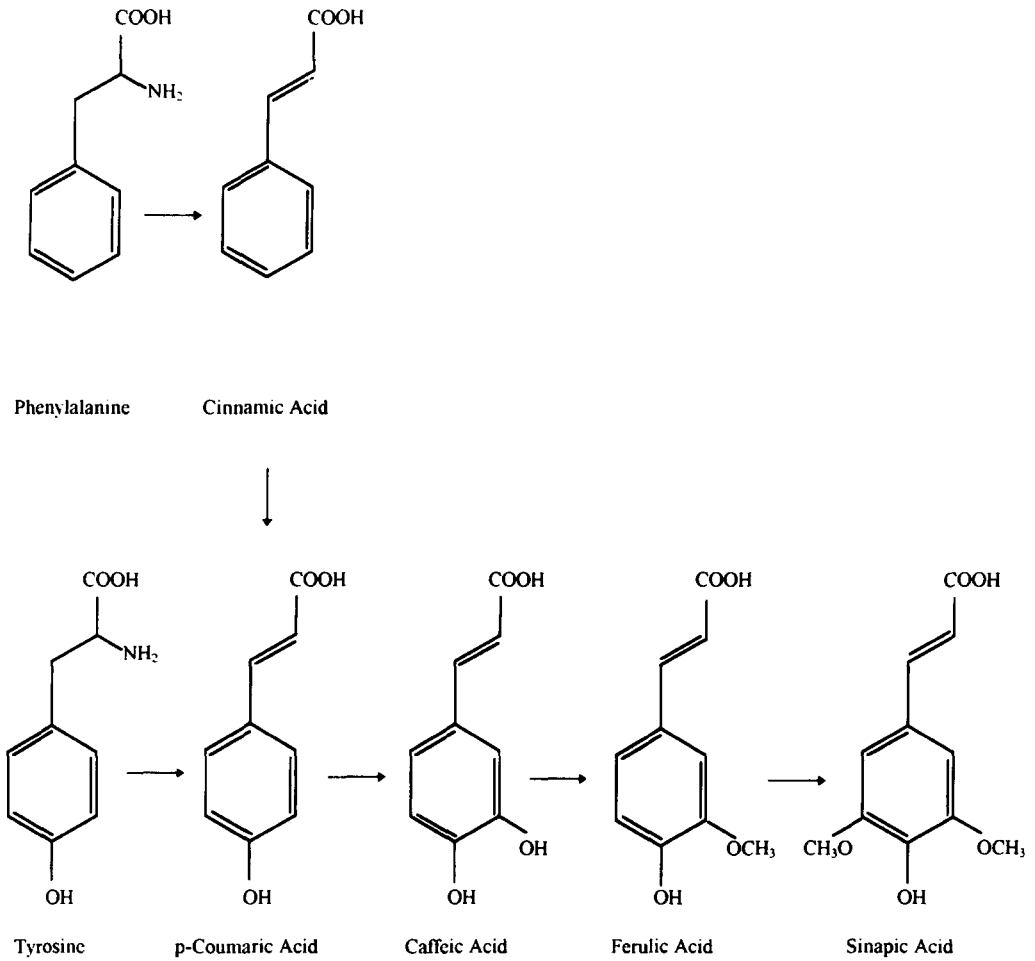

Fig. 1. Synthetic pathway of the hydroxycinnamates in plants.

phenyl ring, hydroxylated cinnamic acids being more effective than their hydroxylated benzoic acid counterparts [36].

In this study we have examined the relative antioxidant activities of the hydroxycinnamates against propagating peroxyl radicals generated in the lipophilic phase. The concentration for 50% inhibition of LDL oxidation is less for chlorogenic acid and caffeic acid (0.33 μM) than ferulic acid (0.9 μM) and *p*-coumaric acid (4 μM). A similar sequence is noted for resistance of LDL cholesterol to oxidation. Thus the diphenolics, chlorogenic and caffeic acids, apparently have a higher radical scavenging ability than monophenolics (*p*-coumaric acid) consistent with the chemical criteria applied to diphenolics [34]. Methoxylation of the hydroxyl group in the ortho position of the diphenolics, as in ferulic acid, results in a decrease in the scavenging reaction, i.e. hydroxylation as in caffeic acid in place of methoxylation is substantially more effective. Ferulic acid is indeed expected to be more effective than *p*-coumaric acid since the electron-donating methoxy group allows increased stabilisation of the resulting aryloxyl radical through electron delocalisation after hydrogen donation by the hydroxyl group.

It is unclear to what extent these effects can be accounted for by peroxyl radical scavenging activities and what contribution is made by their abilities to reduce the ferryl myoglobin formed during the reductive decomposition of lipid peroxides by metmyoglobin. The rate constants for the reactivity with ferryl myoglobin show that caffeic acid is approximately 3 times greater than *p*-coumaric and 8 times greater than ferulic acid. If ferryl myoglobin reduction were the predominant mechanism, the order of effectiveness in inhibiting LDL oxidation would mirror this sequence. Furthermore, the order of scavenging effectiveness observed here in LDL is consistent with the observations of Cuvelier et al. [37], proposing that the antioxidant efficiency of monohydroxycinnamates, such as *p*-coumaric acid, against autoxidation of methyl linoleate under strongly oxidising conditions is increased substantially by methoxy substitution in positions ortho to the hydroxyl group, as in ferulic acid. Other studies on the effects of hydroxycinnamates on the induction period of autoxidising fats have also shown the order of effectiveness caffeic > ferulic > *p*-coumaric acid [38]. Thus the important feature of the antioxidant

Table 2
Rate constants determined for the decay of ferryl myoglobin on interaction with ferulic, caffeic and *p*-coumaric acids

Compound	χ^2	Rate constant (M^{-1} s^{-1})
Caffeic acid	2.4	65
p-Coumaric acid	2.8	22.5
Ferulic acid	2.5	8

χ^2 is an expression of the goodness of fit of the experimentally observed points to the calculated line.

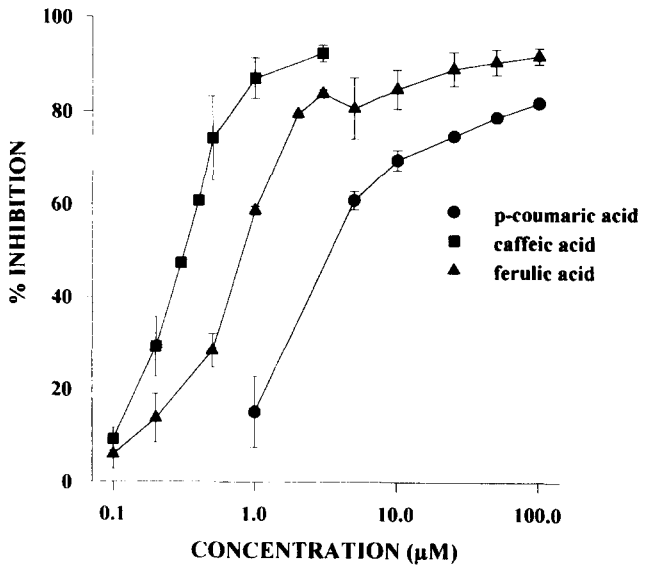

Fig. 2. Concentration-dependent inhibition of LDL peroxidation after interaction between metmyoglobin (10 μM) and LDL (0.25 mg/ml LDL protein). ■ = caffeic acid; ▲ = ferulic acid; ● = *p*-coumaric acid. (Data points expressed as ± S.D. For each point, n = 2–5, each individual experiment performed in triplicate).

effects of the hydroxycinnamic acids in lipid systems seems to be the peroxyl radical scavenging abilities. The differential reactivities of the compounds with ferryl myoglobin formed during the redox cycling of metmyoglobin as it oxidises the LDL, as defined by the rate constants, may make a contribution to the antioxidant effects but does not influence this order of antioxidant potentials, suggesting that the predominant mechanism is that of peroxyl radical scavenging.

Studies have shown that phenolic substances are the most effective antioxidants from natural sources including alkoxyl phenols that contain one free and one alkylated hydroxyl

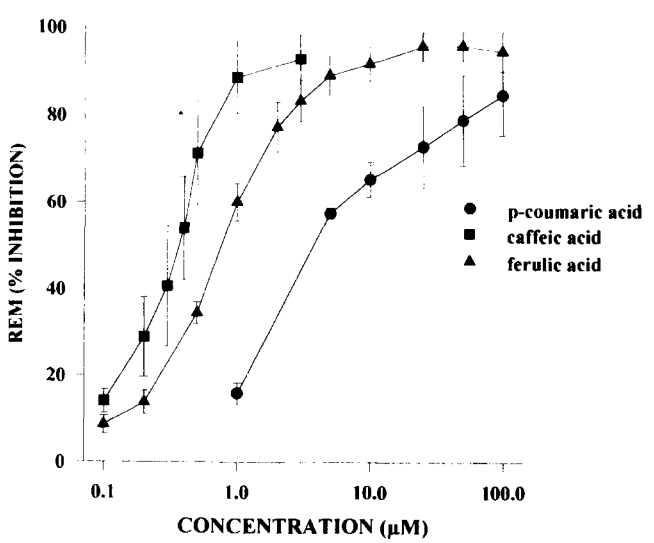

Fig. 3. Concentration-dependent inhibition of the oxidative modification of LDL apoprotein B_{100} expressed as decrease in relative electrophoretic mobility. ■ = caffeic acid; ▲ = ferulic acid; ● = *p*-coumaric acid. (Data points expressed as ± S.D. For each point, n = 3–5, each individual experiment performed in triplicate).

group, usual methoxy, or polyphenols with ortho- or para-dihydroxylic groups, or phenols containing condensed rings, e.g. anthocyans, flavones. Ferulic acid and caffeic, chlorogenic acids fall into the former two categories. The mechanism of action of phenolic acids as antioxidants has been proposed from studies on the autoxidation of linoleic acid micelles [39] through the direct inhibition of *trans,trans*-conjugated diene hydroperoxide isomer formation related to the H-donating ability of the phenol [40]. In addition, ferulic acid (reviewed in [41]) was identified as an antioxidant component of rice bran oil by Fukushi [42] and has been shown to retard the peroxidation of linoleic acid [43]. It has been shown to be an effective antioxidant in the protection of α_1-antiproteinase against oxidation with hypochlorous acid [44]. Ohta et al. [45] have pointed out the importance of *p*-coumaric and ferulic acids as naturally occurring antioxidants in corn bran. They calculate that if the daily human intake of cereal fibre is 10 g/day, then the intake of ferulic acid is 500 mg/day.

Hence the hydroxycinnamates, abundant components of certain common foodstuffs and fruit, are effective antioxidants against aqueous phase radicals and peroxidising LDL and contribute more on a molar basis to the antioxidant status of dietary components than vitamin C.

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