Differential distribution of ferulic acid to the major plasma constituents in relation to its potential as an antioxidant

Cinzia CASTELLUCCIO*, G. Paul BOLWELL†, Christopher GERRISH† and Catherine RICE-EVANS*‡

*Free Radical Research Group, UMDS–Guy's Hospital, St. Thomas's Street, London SE1 9RT, U.K. and †Division of Biochemistry, School of Biological Sciences, Royal Holloway and Bedford New College, Egham, Surrey TW20 0EX, U.K.

The hydroxycinnamates, intermediates in the phenylpropanoid synthetic pathway, are effective in enhancing the resistance of low-density lipoprotein (LDL) to oxidation in the order caffeic acid > ferulic acid > *p*-coumaric acid. It is unclear whether the mode of action of ferulic acid as an antioxidant is based on its activities in the aqueous or the lipophilic phase. Partitioning of ^{14}C-labelled ferulic acid into plasma and its components, LDL and the albumin-rich fractions, has been studied under conditions of maximum aqueous solubility. The majority of ferulic acid associates with the albumin-rich fraction of the plasma, although a proportion is also found to partition between the LDL and aqueous phases; however, ferulic acid does not associate with the lipid portion of the LDL particle, suggesting that it exerts its antioxidant properties from the aqueous phase. This is of particular interest since the results demonstrate that ferulic acid is a more effective antioxidant against LDL oxidation than the hydrophilic antioxidant ascorbic acid.

INTRODUCTION

There is currently a great deal of interest in the health benefits of the phytochemicals, polyphenolic flavonoids and phenylpropanoids, through their potential antioxidant, anti-inflammatory and anti-aggregatory properties [1–9]. Structure–antioxidant activity relationships in chemical and biological systems have been investigated [4,10], but knowledge of the bioavailability and pharmacokinetics of the polyphenols and phenolic acids is sparse.

Phenolic compounds can act as free radical scavengers by virtue of their hydrogen-donating ability, forming aryloxyl radicals: the stabilization of such radicals by other functional groups in the structure enhances the antioxidant activity. In general, monophenols are less efficient as hydrogen-donating radical scavengers than polyphenols [11,12]. The introduction of a second hydroxy group in the *o* or *p* position is known to increase antioxidant activity in peroxidizing lipid systems [12,13] through the stabilization of the phenoxyl radical formed on hydrogen donation. Several investigators have shown that *o* substitution with electron donor (alkyl or methoxy) groups, e.g. ferulic acid, increases the stability of the aryloxyl radical and thus the antioxidant activity [12–14]. Ferulic acid, due to its phenolic nucleus and unsaturated side chain, readily forms a resonance-stabilized phenoxy radical which accounts for its potent antioxidant activity [15].

Our recent studies have shown the effectiveness of ferulic acid (3-hydroxy-4-methoxycinnamic acid) and other hydroxycinnamates (caffeic acid: 3,4-dihydroxycinnamic acid) and chlorogenic acid (the quinic acid ester of caffeic acid) in increasing the resistance of low-density lipoprotein (LDL) to oxidation [16]. The results suggested that the hydroxycinnamates were acting mainly as peroxyl radical scavengers. However, information on their mode of action in terms of the preferential localization and relative partitioning abilities between aqueous and lipophilic interfaces, as well as their association with proteins in the aqueous phase, is distinctly lacking. The purpose of this study was to investigate the relative propensity of ferulic acid to partition into LDL and its affinity for proteins. The results show that ferulic acid acts from the aqueous phase in protecting LDL from oxidation.

MATERIALS AND METHODS

All chemicals used were of analytical grade (BDH, Sigma, Aldrich). Methoxy[^{14}C]ferulic acid, specific radioactivity 2.11 GBq/mmol, was synthesized and purified at Zeneca Agrochemicals Jealott's Hill Research Station, Bracknell, Berks., U.K., and was a kind gift from Professor Wolfgang Schuch. On HPLC analysis, more than 90% of the radioactivity was recovered as ferulic acid, with no other obvious peaks.

LDLs were isolated from human plasma using the modified method of Chung et al. [17] and dialysed in 150 mM NaCl, 10 mM sodium phosphate-buffered solution (PBS), 10 μM EDTA, pH 7.4. The concentration of LDL protein was estimated according to the method of Markwell et al. [18].

Equine metmyoglobin was purified on a 35 cm × 2.5 cm Sephadex column, after oxidation with excess potassium ferricyanide as previously described [19], and the concentration of metmyoglobin was determined spectrophotometrically.

LDL oxidation

LDL oxidation was performed by incubating 0.25 mg/ml LDL with 10 μM purified metmyoglobin for 6 h at 37 °C in the presence or absence of the various phenylpropanoids or vitamin C [16]. The reaction was stopped with 20 μM butylated hydroxytoluene (final concentration). The extent of oxidation was measured by monitoring the formation of lipid hydroperoxides applying ferrous ion oxidation in the Xylenol Orange assay [20] and by measuring the relative electrophoretic mobility, which

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; PBS, sodium phosphate-buffered solution.

‡ To whom correspondence should be addressed.

indicates a modification in the surface charge of apolipoprotein B_{100}, using the Beckman LIPO electrophoresis kit.

Preparation of LDL from plasma in the presence of radiolabelled ferulate

To 15 ml of plasma, [^{14}C]ferulic acid corresponding to 5×10^5 d.p.m. was added, as well as non-radioactive ferulic acid (200 μg/ml). After shaking for 30 min the density of the plasma was modified by NaBr and plasma underlayered in centrifuge tubes filled with 0.9 % NaCl solution, according to the method of Chung et al. [17]. After the first centrifugation (100000 ***g*** for 2 h) LDL was separated on a discontinuous equilibrium gradient; three different fractions were collected: L_1, aqueous phase (top layer); L_2, 'crude' LDL (middle layer); and L_3, high-density lipoprotein (HDL) + albumin + other plasma proteins (bottom layer). The radioactivity was determined in all the different fractions.

The LDL fraction (L_2), obtained with the first centrifugation, was then purified from HDL and albumin contamination by centrifuging at 100000 ***g*** for 20 h, after diluting LDL with 1.154 g/ml NaBr density solution. Purified LDL, the upper layer, was collected and the radioactivity measured in comparison with the solution.

Studies on the partitioning of [^{14}C]ferulic acid

To investigate the interaction of [^{14}C]ferulic acid with a non-protein system, liposomes were prepared using L-α-phosphatidylcholine (the main phospholipid on the LDL surface) from frozen egg yolk (type VII-E) in chloroform solution (Sigma-Aldrich). An aliquot (1 ml) of a chloroform solution of phosphatidylcholine (100 mg/ml) was evaporated under nitrogen. Next 6.7 ml of 10 mM PBS, pH 7.4, was added to give a concentration of 15 mg/ml. The solution was sonicated for 20 min (4 times for 5 min with 2 min breaks) in an ultrasonic disintegrator Soniprep 150 MSE, using a small probe [21].

[^{14}C]Ferulic acid (5×10^5 d.p.m.) as well as non-radioactive ferulic acid (400 μg/ml) were added to 0.5, 1 and 2 ml of liposome solution and then each sample was diluted up to 2 ml with 10 mM PBS, pH 7.4. The samples were incubated for 30 min while shaking and then centrifuged at 100000 ***g*** for 30 min. The radioactivity was determined in the pellet and in the solution by liquid scintillation counting and the percentage partition calculated.

Extraction of lipids

Lipids were extracted from both LDL (0.250 mg) and plasma (0.250 ml), to which radiolabelled ferulic acid (1×10^5 d.p.m. per ml of plasma or LDL solution) and non-radioactive ferulic acid (400 μg/ml) were previously added, with a mixture of methanol/cyclohexane (1:1, v/v). The samples were mixed for 1 min and then centrifuged at 3000 rev./min for 10 min; the upper layer was removed and the methanol phase was centrifuged again at 12000 rev./min for 5 min, in order to precipitate the proteins. The pellet was then suspended in 10 mM PBS and the radioactivity measured in all the different fractions.

Statistics

Multiple groups were analysed by ANOVA. Dunnett's or the Tukey test were applied to determine significance of differences.

RESULTS AND DISCUSSION

The antioxidant activities of cinnamic acid and ferulic acid against LDL oxidation have been investigated in comparison with ascorbic acid, which is the major water-soluble antioxidant in plasma. LDL was incubated in the presence and absence of both the cinnamic acid derivatives and ascorbic acid and a hydroperoxide-dependent oxidative stress, metmyoglobin, applied [22]. Metmyoglobin catalyses the oxidative and reductive decomposition of lipid hydroperoxides through its ability to redox cycle, according to the following equations:

$$\mathrm{LOOH + HX - Fe^{III} \rightarrow LOO^{\bullet} + HX - Fe^{II}}$$

$$\mathrm{LOOH + HX - Fe^{III} \rightarrow LO^{\bullet} + HX - [Fe^{IV} = O]}$$

$$\mathrm{LOOH + HX\text{-}Fe^{II} \rightarrow LO^{\bullet} + HX\text{-}Fe^{III}}$$

The antioxidant activities were assessed by comparing their abilities to increase the resistance of LDL to oxidation by suppressing the formation of lipid hydroperoxides and by inhibiting the alteration of the surface charge on apolipoprotein B_{100}. Table 1 demonstrates the relative efficacies as antioxidants of ferulic acid compared with cinnamic and ascorbic acid at the identical concentrations of 1 μM. This concentration was selected as close to that giving approx. 50 % inhibition of the increased relative electrophoretic mobility from previous studies [16]. Thus, whereas ferulic acid at a concentration of 1 μM inhibits peroxide formation in LDL to the extent of about 50 %, ascorbic acid, a major aqueous-phase antioxidant in plasma, at this concentration shows no significant effect. As predicted from the structure, cinnamic acid does not show radical scavenging properties, compared with its 3-hydroxy-4-methoxy derivative.

In order to clarify the localization of ferulic acid in plasma and in LDL, the partitioning of this compound was examined between the different layers during LDL preparation after addition to plasma (Table 2A). The concentration of ferulic acid applied was set so that the maximum amount of ferulic acid would be available for partitioning in the region of maximum aqueous solubility. The percentage of ferulic acid incorporated into the LDL fraction after addition to plasma during the early stage of LDL preparation corresponded to 3.2 % in the 'crude' fraction. Even though this percentage seems not particularly relevant in comparison with the amount found in plasma, the purified LDL fraction, obtained from the crude fraction with a further centrifugation, still shows 14 % of radioactivity with respect to the aqueous solution, suggesting that an amount of ferulic acid is still associated with LDL (Table 2B). Moreover, the partitioning of radioactivity between the LDL fraction and the aqueous phase per unit of volume shows a distribution of

Table 1 The inhibition of LDL oxidation by ferulic, cinnamic and ascorbic acids

*$P < 0.05$ with respect to oxidized LDL, according to Dunett's test. Results are means ± S.D. (at least $n = 3$; [a]$n = 8$; [b]$n = 11$).

	Lipid hydroperoxides (nmol/mg of LDL protein)	Relative electrophoretic mobility (% inhibition)
LDL (native)	21 ± 8	
Oxidized LDL	524 ± 78[a]	
Ferulic acid (1 μM)	243 ± 109*[a]	46 ± 13[b]
Cinnamic acid (1 μM)	540 ± 13	0
Ascorbic acid (1 μM)	469 ± 30	17 ± 5

Table 2 The partitioning of ferulic acid in plasma and in purified LDL under conditions of maximum aqueous solubility

Plasma was separated in a NaBr density gradient. Upper layer L_1, density solution. Lower layer L_3, HDL, albumin and other plasma proteins. Purified LDL, LDL fraction after further centrifugation (see the Materials and methods section). The results are expressed as mean ± S.D. ($n = 3$).

	$10^{-3} \times$ Radioactivity (d.p.m./fraction)	Percentage labelled
(A) Plasma		
L_1 (top layer)	11.5 ± 1.8	2.3 ± 0.4
L_2 (LDL fraction)	16.1 ± 1.4	3.2 ± 0.3
L_3 (bottom layer)	472.4 ± 2.0	94.5 ± 0.4
(B) Purified LDL		
LDL	2.2 ± 0.1	14.0 ± 0.5
Aqueous solution	13.8 ± 0.1	86.0 ± 0.5

Table 3 The distribution of radiolabelled ferulic acid in plasma and in LDL

Results are means ± S.D. ($n = 3$). Specific activity of ferulic acid was calculated after dilution with the non-radioactive compound, according to the percentage of incorporation during the first centrifugation (Table 2A); the value for the purified fraction of LDL was extrapolated from the others, in proportion to the radioactivity detected in each fraction per unit of protein. Same superscript (* or †) indicates values significantly different from each other ($P < 0.05$, according to Tukey's test).

	Specific radioactivity (d.p.m./mg of protein)	Ferulic acid content (nmol of ferulic acid/mg of protein)
Crude LDL	1308 ± 104*	41.5 ± 6.6†
Pure LDL	1040 ± 49*	32.7 ± 2.4
Other plasma proteins	808 ± 42*	25.6 ± 0.1†

about 50%, suggesting that ferulic acid partitions between the two layers as through a homogeneous solution. When the results are expressed as radioactivity per mg of protein in each layer, the distribution of radiolabelled compounds between the residual plasma layer (L_3, containing albumin, HDL and all the other plasma proteins) and in 'crude' or purified LDL seems comparable; these results indicate a possible non-specific binding of ferulic acid to LDL protein and other plasma proteins (Table 3). After filtration of the LDL fraction through centrifugal concentrators (cut-off 300 kDa), 38% of radioactivity is still found in LDL; this result supports the idea of protein-binding, even though non-specific and reversible.

The interaction of ferulic acid with lipids in the absence of proteins was investigated using phosphatidylcholine liposomes. A total of 2% of the radioactivity is found in the liposomal fraction (Table 4). On increasing the concentration of the lipid, no correlation was found between the total amount of phosphatidylcholine and the incorporation of ferulic acid, suggesting maximal incorporation of ferulic acid even at the lowest concentration of liposomes applied.

To obtain further information about the localization of ferulic acid both in plasma and within the LDL particle, lipids were extracted from both plasma and LDL, after adding a known amount of ferulic acid (2×10^5 d.p.m. to 2 ml of plasma or LDL); no radioactivity was found in the lipid phase, while most of the radioactivity (about 80%) was detected in the methanol phase after removing the protein precipitate. Traces of radioactivity

Table 4 The partitioning of ferulic acid in phosphatidylcholine (PC) liposomes

Results are means ± S.D. ($n = 3$). No significant difference between values was observed in each group, according to the multiple comparison Tukey's test.

	Percentage of ferulic acid	Ferulic acid content (nmol of ferulic acid/mg of PC)*
7.5 mg PC	2.62 ± 0.51	15.55
15 mg PC	1.61 ± 0.42	3.60
30 mg PC	1.85 ± 0.23	2.48

*Calculated after dilution with non-radioactive ferulic acid (mean of two experiments).

were measured also in the protein precipitate (about 5% in plasma).

Our previous studies have investigated the relative antioxidant activities of caffeic acid and its methoxy-substituted derivative, ferulic acid, as chain-breaking antioxidants in inhibiting LDL oxidation [16]. The results showed that caffeic acid is relatively more effective than ferulic acid against radicals generated in the lipophilic phase, concentrations required for 50% inhibition of LDL oxidation being 0.3 μM and 0.9 μM respectively. It is of interest to note, from the studies of others on hydroxyl radical-scavenging activities, that methoxylation of the hydroxyl group at the *ortho* position of the dihydroxyphenolics, as in ferulic acid, resulted in a decrease in the rate constant for the reaction with the phenolic antioxidant [23].

The purpose of this study was to investigate the mechanism of the antioxidant action of ferulic acid in inhibiting LDL oxidation, by examining the extent to which it partitions into the lipophilic portion of the LDL and its propensity for binding to proteins. The results obtained demonstrate that ferulic acid is a more effective antioxidant against LDL oxidation than the water-soluble ascorbic acid, but shows no evidence of a partitioning into the lipid phase of plasma or of LDL. The low affinity for lipids is also demonstrated by the minimal extent of partitioning of ferulic acid in phosphatidylcholine liposomes.

Consistent with these results, the partition coefficient of ferulic acid in an octanol/water system is 0.085, clearly showing the higher affinity of ferulic acid for the aqueous phase (C. Rice-Evans and R. C. Hider, unpublished work). On the other hand our observations seem to suggest a possible non-specific binding to protein both in plasma and in LDL: whether this binding is relevant to the antioxidant activity of this compound is still not clear. The antioxidant activity of ferulic acid in scavenging superoxide anion has been reported [24], as has its ability to protect against malondialdehyde generation in peroxidizing membrane systems [25]. Recent work has suggested that chlorogenic acid, the quinic acid ester of caffeic acid, is able to scavenge directly the 1,1-diphenyl-2-picrylhydrazyl radical, inducing N-nitrosation of aromatic amines [26]. Other authors report that cinnamic acid is readily taken up by the gut, and it is highly probable that the same applies to dietary ferulic acid [27].

C. R.-E. and G. P. B. thank the Biotechnology and Biological Sciences Research Council for financial support.

REFERENCES

1 Lands, W. E. M. and Hanel, A. M. (1982) Prostaglandins **24**, 271–275
2 Hudson, B. J. F. and Lewis, J. I. (1983) Food Chem. **10**, 47–55
3 Landolfi, R., Mower, R. L. and Steiner, M. (1984) Biochem. Pharmacol. **33**, 1525–1529
4 Bors, W. and Saran, M. (1987) Free Radical Res. Commun. **2**, 289–294

5 Moroney, M. A., Alcanaz, M. J., Forder, R. A., Carey, F. and Hoult, J. R. S. (1988) J. Pharm. Pharmacol. **40**, 787–791
6 Namiki, M. (1990) Crit. Rev. Food Sci. Nutr. **29**, 273–300
7 Mangiapane, H., Thomson, J., Salter, A., Brown, S., Bell, G. D. and White, D. A. (1992) Biochem. Pharmacol. **43**, 445–450
8 Rice-Evans, C. A., Miller, N. J., Bolwell, G. P., Bramley, P. M. and Pridham, J. B. (1995) Free Radical Res. **22**, 375–383
9 Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P. and Rice-Evans, C. (1995) Arch. Biochem. Biophys. **322**, 339–346
10 Rice-Evans, C., Miller, N. J. and Paganga, G. (1996) Free Radicals Biol. Med., in the press
11 Cuvelier, M.-E., Richard, H. and Berset, C. (1992) Biosci. Biotechnol. Biochem. **56**, 324–325
12 Pokorny, J. (1987) in Autoxidation of Unsaturated Lipids (Chan, H., ed.), pp. 141–206, Academic Press, London
13 Chimi, H., Cillard, J. and Rahmani, M. (1991) J. Am. Oil Chem. Soc. **68**, 307–312
14 Cort, W. (1974) Food Technol. **10**, 60–66
15 Graf, E. (1992) Free Radicals Biol. Med. **13**, 435–448
16 Castelluccio, C., Paganga, G., Melikian, N., Bolwell, G. P., Pridham, J., Sampson, J. and Rice-Evans, C. (1995) FEBS Lett. **368**, 188–192
17 Chung, B. H., Wilkinson, T., Geer, J. C. and Segrest, J. P. (1980) J. Lipid Res. **21**, 284–291
18 Markwell, M. A., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) Anal. Biochem. **87**, 206–210
19 Miller, N. J., Rice-Evans, C. A., Davies, M. J., Gopinathan, V. and Milner, A. (1995) Clin. Sci. **84**, 407–412
20 Nourooz-Zadeh, J., Tajaddini-Sarmad, J. and Wolff, S. (1994) Anal. Biochem. **220**, 403–409
21 Degli Esposti, M., Bertoli, E., Parenti Castelli, G., Fato, R., Mascarello, S. and Lenaz, G. (1981) Arch. Biochem. Biophys. **210**, 21–32
22 Hogg, N., Rice-Evans, C., Darley-Usmar, V., Wilson, M. T., Paganga, G. and Bourne, L. (1994) Arch. Biochem. Biophys. **314**, 39–44
23 Bors, W., Michel, C., Erben-Russ, M., Kreileder, B., Tait, D. and Saran, M. (1984) in Oxygen Radicals in Chemistry and Biology (Bors, W., Saran, M. and Tait, D., eds.), pp. 95–99, Walter de Gruyter, Berlin
24 Toda, S., Kumura, M. and Ohnishi, M. (1991) Planta Medica **57**, 8–10
25 Sharma, O. P. (1976) Biochem. Pharmacol. **25**, 1811–1812
26 Kono, Y., Shibata, H., Kodama, Y. and Sawa, Y. (1995) Biochem. J. **312**, 947–953
27 Wolffram, S., Weber, T., Grenacher, B. and Scharrer, E. (1995) J. Nutr. **125**, 1300–1308

Received 19 December 1995/24 January 1996; accepted 9 February 1996