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The Effect of the Phenolic Antioxidant Ferulic Acid on the Oxidation of Low Density Lipoprotein Depends on the Pro-oxidant Used

LOUISE CLARE BOURNE and CATHERINE A. RICE-EVANS*

International Antioxidant Research Centre, UMDS-Guy's Hospital, St. Thomas's Street, London SE1 9RT

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The action of ferulic acid during the oxidation of LDL has been investigated using both copper ions and the haem protein metmyoglobin as pro-oxidants. The results demonstrate the ability of ferulic acid to act as a pro-oxidant when LDL oxidation is induced by copper at concentrations of the phenolic acid which are protective when the LDL oxidation is mediated by metmyoglobin. The suggested mechanism involves the reduction of Cu^{2+} to Cu^{+} by ferulic acid resulting in the production of the ferulic phenoxyl radical.

Keywords: Ferulic acid, oxidized low density lipoprotein, copper, metmyoglobin

INTRODUCTION

The health benefits of fresh fruit and vegetables, in the context of antioxidant properties, have largely been attributed to the antioxidant vitamins and the carotenoids. More recently the polyphenolic components of higher plants have been shown to be highly efficacious antioxidants *in vitro* and thus much consideration is currently being given to their potential contributions to these properties *in vivo*. Among the most widely distributed phenylpropanoids in plant tissues are the hydroxycinnamic acids, coumaric (p-hydroxycinnamic) acid, caffeic (3,4-dihydroxycinnamic) acid and its quinic acid ester chlorogenic acid, and ferulic (4-hydroxy-3-methoxycinnamic) acid produced from the shikimate pathway from phenylalanine or L-tyrosine.

The antioxidant properties of the hydroxycinnamates have been demonstrated against peroxidising polyunsaturated fatty acids in lipid systems and in low density lipoproteins.[1] In addition to scavenging alkoxyl and peroxyl radicals, they have also been demonstrated to scavenge initiating free radical species such as ferryl myoglobin.[2] Furthermore, the ability of ferulic acid to increase the resistance of LDL to oxidation mediated by metmyoglobin has been demonstrated to be operative through the scavenging of peroxyl

* Corresponding author. Tel.: +44-171-955-4240. Fax: +44-171-955-4983. E-mail: c.rice-evans@umds.ac.uk.

radicals and to be effective from the aqueous phase.[3] The antioxidant properties of such phenolic compounds as α-tocopherol and butylated hydroxytoluene have long being characterised. However, although α-tocopherol is considered to be a strong hydrogen donor there are conditions under which α-tocopheroxyl radicals may act as chain initiators in lipophilic media.[4–6]

Copper- and haem-dependent oxidation of both lipids and LDL are thought to be dependent on the presence of preformed lipid hydroperoxides.[7–9] In this study the reaction of ferulic acid during the oxidation of LDL has been investigated using both copper ions and the haem protein metmyoglobin as pro-oxidant. This investigation demonstrates the ability of ferulic acid to act as a pro-oxidant when LDL oxidation is induced by copper at concentrations of the phenolic acid which are protective when LDL oxidation is mediated by metmyoglobin.

MATERIALS AND METHODS

All chemicals used were of analytical grade.

Isolation and Purification of LDL

Low density lipoproteins were isolated from human plasma using the modified method of Chung *et al.*[10] The LDL thus obtained was dialysed against PBS containing 10μM EDTA, sterilised by filtration through a 0.2μm filter and stored up to 4 days at 4°C before use. The concentration of LDL protein was estimated according to Markwell *et al.*[11] LDL was diluted to final concentration of 62.5μg LDL protein/ml for oxidation, levels of EDTA being less than 0.25μM.

Preparation of Metmyoglobin

Equine myoglobin was purified on a Sephadex G-25 column, after oxidation with excess of potassium ferricyanide, and the concentration of metmyoglobin determined spectrophotometrically.[12]

Oxidation of LDL

Oxidation of LDL was assessed by measuring conjugated diene formation. The formation of conjugated diene hydroperoxides was followed by continuously monitoring the change in the absorbance at 234nm using a Beckman DU7500 spectrophotometer with Peltier temperature control and equipped with an automatic sample changer (allowing the determination of six samples at the same time). The duration of the lag phase was calculated by extrapolating the propagation phase as defined by Esterbauer *et al.*[8] LDL oxidation was performed by incubating LDL at 62.5μg LDL protein/ml (final concentration) with 5μM of purified metyoglobin or 1.66μM copper sulphate for up to 10hrs in the presence or absence of ferulic acid. Copper-mediated oxidation studies were carried out at 30°C in line with other published work and metmyoglobin at 37°C due to the slow rate of oxidation at 30°C.

Measurement of Cu(I)

Reduced copper was quantitated using bathocuproinedisulphonic acid (2,9-dimethyl-4, 7-diphenyl-1,10-phenanthrolinedisulphonic acid) (BC). This indicator molecule binds the reduced form of copper,[13] the copper-bathocuproine sulphonic acid complex formed exhibiting an absorbance maximum at 480nm (ε = 12540M^{-1} cm^{-1}).[14]

RESULTS

LDL (62.5μg LDL protein/ml) was incubated with 5μM metmyoglobin in the presence and in the absence of ferulic acid (1μM) for 10 hours (Fig. 1). The lag phase for the metmyoglobin mediated oxidation was approximately 60mins

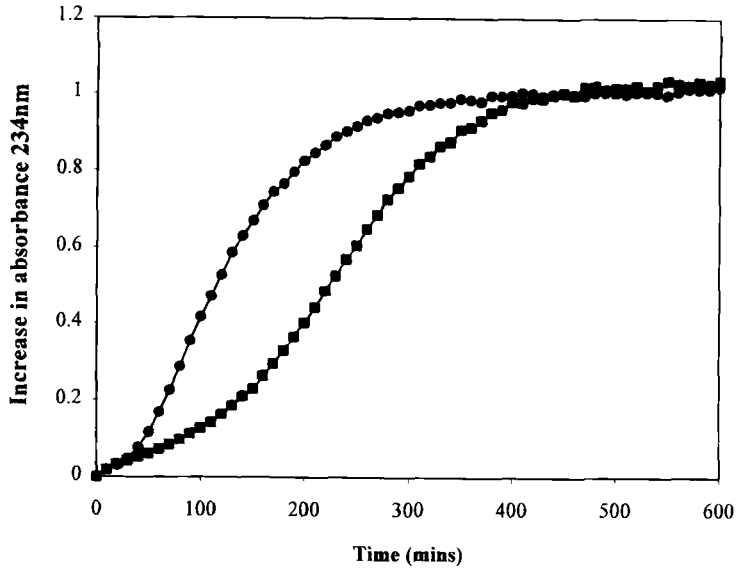

FIGURE 1 The formation of conjugated dienes. LDL 62.5mg protein/ml was incubated with 5μM metmyoglobin (●) and the formation of conjugated dienes measured at 234nm at 37°C. Ferulic acid was added at a final concentration of 1μM (■).

whereas in the presence of ferulic acid it was 137 ± 9mins (n = 3). The incorporation of ferulic acid extended the lag phase to oxidation and hence indicated an antioxidant activity of ferulic as shown previously.[1] Oxidation was also induced using copper as the pro-oxidant under the same conditions. LDL (62.5μg LDL protein/ml) was incubated with 1.66μM copper sulphate in the presence and absence of ferulic acid (1μM) and in this case a highly significant ($p < 0.01$) shortening of the lag phase was observed (Fig. 2). The lag phase for the control was 87 ± 3mins whereas in the presence of ferulic acid it was 57 ± 5mins.

Further investigations using a range of ferulic acid concentrations (0.5μM–2.0μM) were undertaken in the copper system and a significant shortening of the lag phase was observed at all concentrations (Fig. 2), suggesting a pro-oxidant activity. Further experiments showed that the situation pertained up to 12.5μM, when a change in the rate of propagation was observed. The propagation rate for the control, in this case LDL and copper, was 0.0198 ± 0.0008 min^{-1} (n = 3) whereas in the presence of ferulic acid (12.5μM) the rate of propagation was reduced to 0.0128 ± 0.002 min^{-1} (n = 3). The change in the rate of propagation was highly significant $p < 0.01$.

To explore this paradox further the interactions between ferulic acid and Cu^{2+} were examined. Incubation of Cu^{2+} (10μM) with equimolar ferulic acid was performed at 30°C in the presence of excess BC (360μM), an indicator molecule for the reduced form of copper. Incubation of Cu^{2+} with ferulic acid for one minute resulted in reduction to Cu^{+} as indicated by formation of a peak with maximal absorbance at 480nm characteristic of the copper-bathocuproine sulphonic acid ($BC{\cdot}Cu^{+}$) complex (Fig. 4) which did not increase in intensity on further incubation. The chemistry of the complex defines a 1:1 stoichimetry. Thus, under the experimental conditions, the applied ferulic acid is capable of reducing Cu^{2+} to Cu^{+}.

Related hydroxycinnamates such as chlorogenic, caffeic and para-coumaric acids were also examined for their copper reducing potentials. The most effective compounds in reducing Cu^{2+} to Cu^{+} were caffeic, chlorogenic and ferulic acid

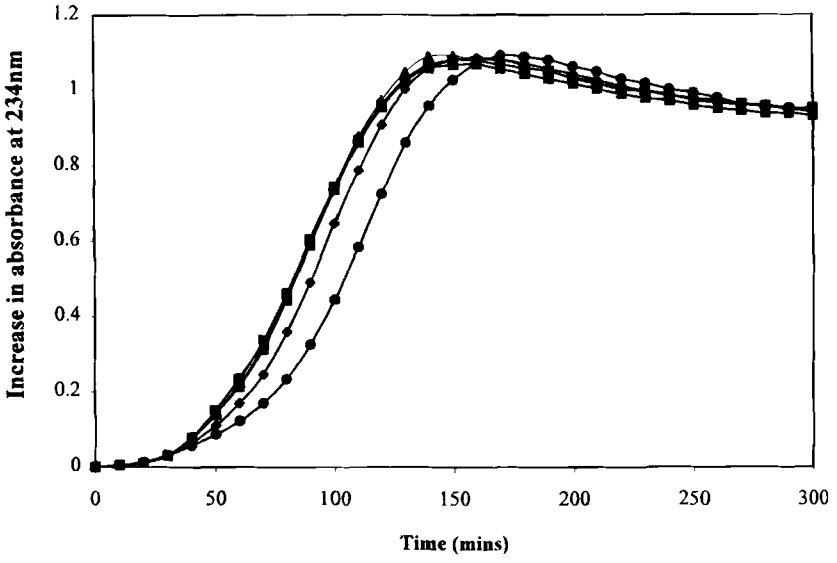

FIGURE 2 Concentration -dependent promotion of the oxidation of LDL by ferulic acid. LDL 62.5mg protein/ml was incubated with 1.66μM copper (●) and the formation of conjugated dienes measured at 234nm at 37°C. Ferulic acid was added at a final concentration of 0.5μM (◆), 1μM(▲), 1.5μM(■), 2.0μM(■).

followed by para-coumaric acid with the final concentration of Cu^{+} being 10μM, 9.8μM and 10μM and 3.7μM respectively, as indicated in Figure 3.

DISCUSSION

The oxidation of LDL mediated by Cu^{2+} or metmyoglobin occurs through the oxidative and reductive decomposition of peroxides and the redox cycling of the free- or haem-bound transition metal respectively (scheme 1).[9]

The redox cycling of copper involves the formation of lipid alkoxyl radicals $LO^{\cdot}$ which are reported to rapidly rearrange to the epoxyallylic radical and ultimately form peroxyl radical and which may, in turn, cause more peroxidation or under go β-scission to form aldehydes.[16] Antioxidants can inhibit LDL oxidation in a number of ways, through scavenging peroxyl or alkoxyl radicals, through reducing ferryl myoglobin (in the case of haem protein-mediated oxidation) or chelating copper ions (in the case of copper-mediated oxidation). When considering metmyoglobin-mediated LDL oxidation the rate of scavenging of peroxyl radicals is more significant than that of ferryl myoglobin reduction as can be seen from the rate constant 8 $M^{-1}s^{-1}$ for ferulic acid.[1] However, reduction of ferulic acid by copper ions and the formation of phenoxyl radicals and Cu^{+} ions are feasible candidates for promoting pro-oxidant mechanisms at low concentrations.

Using metmyoglobin and copper ions as pro-oxidants for LDL, for the purposes of studying the efficacy of antioxidants in preventing LDL oxidation *in vitro*, has the advantage that there is no initiating radical species eliminating the possibility of confounding effects of direct interaction between the initiating species and the phenolic antioxidant. [Redox cycling of metmyoglobin on interaction with lipid hydroperoxides produces the compound II ferryl state not compound I (ferryl myoglobin tyrosyl radical), which would involve two oxidising equivalents.] The advan-

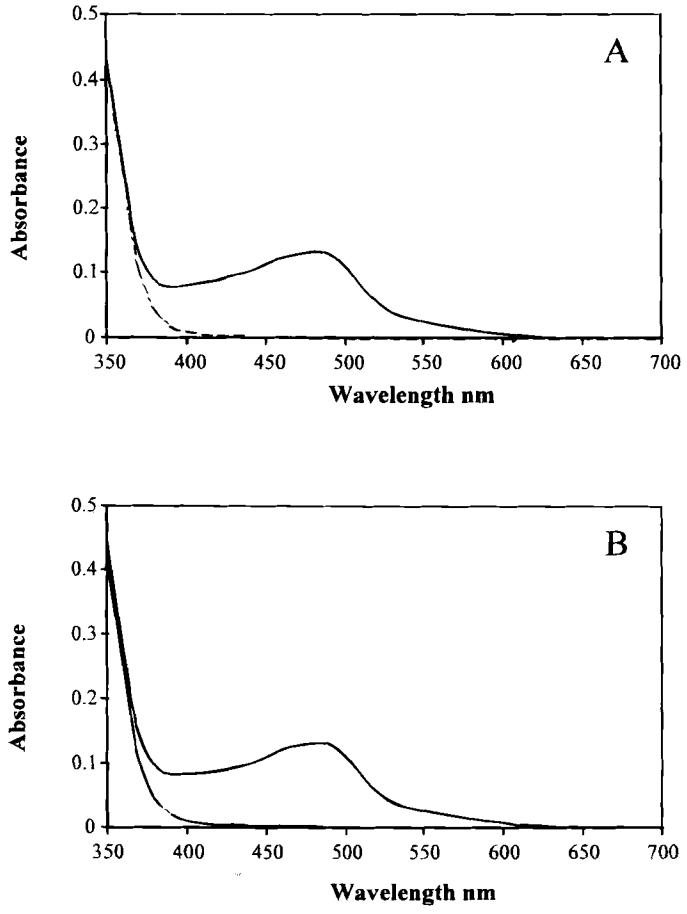

FIGURE 3 Formation of BC-Cu (I) during the reaction between Cu (II) and the hydroxycinnamic acids. Absorbance spectra were recorded of incubations containing BC (360μM) and Cu^{2}+ (10μM) in PBS [shown here as a dotted line -·-·-·-], and 1 minute after the addition of 6μM caffeic acid **(A)**, chlorogenic acid **(B)**, para-coumaric **(C)** and ferulic acid **(D)** [shown here as a solid line ———].

tage of the haem protein over the transition metal ion system is that inhibition of copper-induced LDL oxidation by phenolic compounds might also include the potential for copper chelation or redox cycling by the phenolic compound in addition to the latter's peroxyl scavenging properties. While it has been reported that ferulic acid does not chelate iron,[17] these studies demonstrate the ability of ferulic acid and other hydroxycinnamates to reduce Cu^{2+} to Cu^{+}.

The studies of Bowry and Stocker[18] and Bowry *et al.*[19] have shown that at low radical flux α-tocopherol, an endogenous phenolic antioxidant within the LDL particle, can exert pro-oxidant effects on LDL in the presence of copper.[20,21] In particular, the propensity of this antioxidant to promote lipid peroxidation arises when the flux of peroxyl radicals is low.[18,22,23] The suggested mechanism for the pro-oxidant reaction of the α-tocopheroxyl radical is the initiation of lipid peroxidation through hydrogen abstraction from a polyunsaturated fatty acid.

There are two potential mechanisms which may account for this pro-oxidant activity. Firstly, in the case of copper-mediated LDL oxidation, redox cycling of Cu^{2+} to Cu^{+} by ferulic acid results in the production of the ferulic phenoxyl radical. This may then be capable of initiating

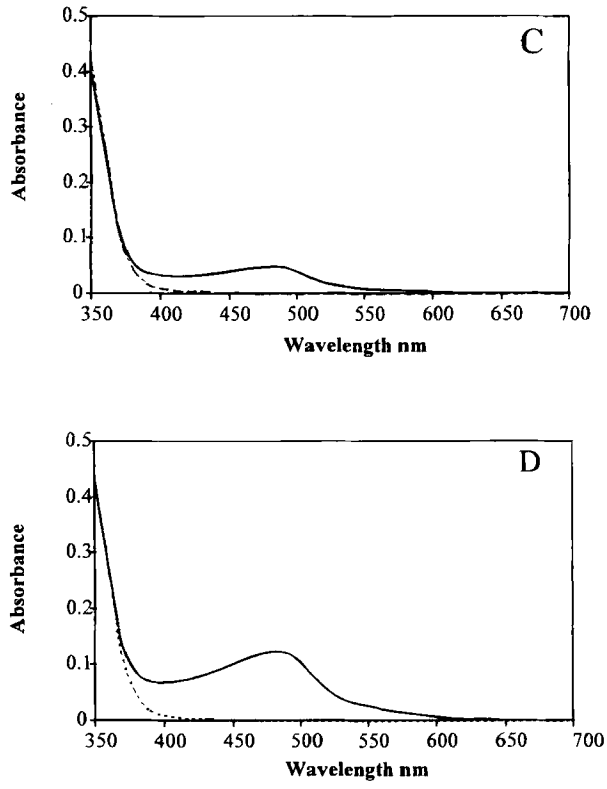

FIGURE 3 (*Continued*)

lipid peroxidation through abstraction of an H atom from a polyunsaturated fatty acid, increasing the rate of lipid peroxidation, and acting by a mechanism analogous to that for the pro-oxidant activity of α-tocopherol. Secondly, as in the equations depicted earlier, since Cu(I) reacts with LOOH much faster than Cu(II), the reaction of Cu(II) with LOOH is the rate-limiting step in the process.[14] The redox cycling of Cu(II) by ferulic acid producing the ferulic phenoxyl radical and Cu(I), might bypass the rate limiting step, hence increasing the rate of oxidation and shortening the lag phase (Fig. 4).

The pro-oxidant activity of ferulic acid has been observed previously in the presence of higher concentrations of iron.[24] Recently it has

$$\mathrm{LOOH + Cu^{II} \longrightarrow LOO\cdot + Cu^{I} + H^{+}}$$

$$\mathrm{LOOH + Cu^{I} \longrightarrow LO\cdot + OH^{-} + Cu^{II}}$$

$$\mathrm{LOOH + HXFe^{III} \longrightarrow LOO\cdot + HX\text{-}Fe^{II} + H^{+}}$$

$$\mathrm{LOOH + HXFe^{III} \longrightarrow LO\cdot + HX\text{-}[Fe^{IV}{=}O] + H^{+}}$$

$$\mathrm{LOOH + HXFe^{II} \longrightarrow LO\cdot + HXFe^{III} + OH^{-}}$$

SCHEME 1 Decomposition of lipid hydroperoxides.

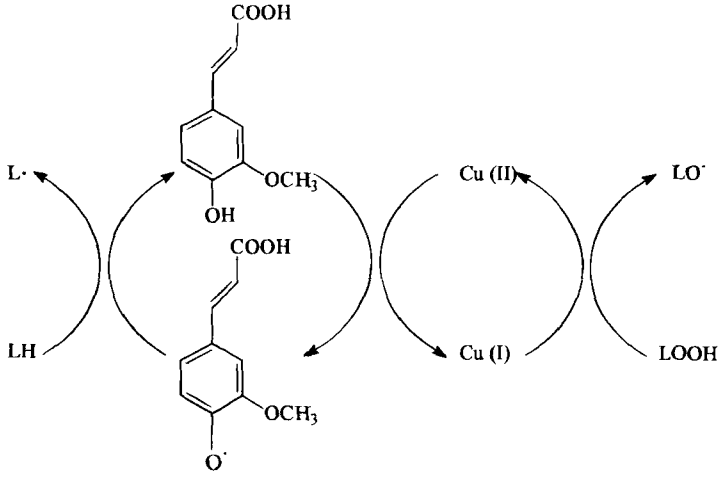

FIGURE 4 The postulated mechanism of the pro-oxidant activity of ferulic acid.

been reported that caffeic acid has pro-oxidant activity when LDL oxidation was induced by copper.[25] From the results presented here it can be seen that the choice of pro-oxidant for LDL is of pivotal importance in the examination of the antioxidant activity of compounds and dietary agents against LDL oxidation.

Acknowledgements

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