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REACTIVITY OF DIETARY PHENOLIC ACIDS WITH PEROXYL RADICALS: ANTIOXIDANT ACTIVITY UPON LOW DENSITY LIPOPROTEIN PEROXIDATION

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Abstract—The interaction of four phenolic acids, representative of three chemical groups present in human diet, with peroxyl radicals was studied *in vitro* in a low density lipoprotein (LDL) oxidation model. The controlled oxidation of LDL was initiated by free radicals generated from a hydrophilic azo initiator and followed by monitoring the oxygen consumption and the fluorescence quenching of *cis*-parinaric acid previously incorporated into LDL. The hydroxycinnamic acid derivatives, chlorogenic and caffeic acids, have high stoichiometric numbers and reactivity with peroxyl radicals as compared with trolox, the water-soluble analogue of vitamin E, whereas ellagic acid (a tannic compound) compares with trolox effects. Protocatechuic acid (a hydroxybenzoic acid derivative) exhibits a complex reaction with peroxyl radicals, as indicated by UV spectroscopy, resulting in undefined inhibition periods of LDL oxidation and low reactivity with peroxyl radicals. Presumably, secondary radicals of these compounds are unable to initiate LDL oxidation. The antioxidant activity of the various phenolic compounds is discussed in terms of structure–activity relationships.

Key words: antioxidant; phenolic acids; diet; peroxyl radicals; low density lipoproteins; lipid peroxidation

There is growing interest in understanding the role and mechanisms of phenolic compounds as inhibitors of deleterious oxidative processes, particularly cancer and atherosclerosis [1]. Although scientific interest has hereto focused on flavonoids, phenolic non-flavonoid compounds are also present in human diet in representative amounts [2, 3]. Some of these compounds with a catechol structure, namely CHL and CAF (hydroxycinnamic acid derivatives), PRO (hydroxybenzoic acid derivative) and ELL (derived from fruit ellagitannins) have been shown to exhibit multiple biological and pharmacological properties such as antiinflammatory [4], antimutagenic and anticarcinogenic [5, 6] activities. Recently, particular attention has been given to the antioxidant or oxygen free-radical scavenging action of these phenolic compounds accounting for most physiological activities. Some have already been found *in vivo* [7–9] and additional evidence suggests that they act as antioxidants *in vivo*: (i) increasing levels of reduced glutathione in mice fed with ELL [10]; (ii) suppression of lipid peroxide levels in rats treated with CCl_4 or irradiated with ^{60}Co [11, 12]; (iii) inhibition of serum and liver lipid peroxides in rats fed with peroxidized oil [13] and (iv) protection against Endrin-induced hepatic lipid peroxidation decreasing the excretion of oxidative metabolites of lipids, e.g. malondialdehyde [14]. The general antioxidant capability of these compounds *in vitro* is expressed in the following ways: (i) decreased malondialdehyde formation in several lipid peroxidation systems [10, 15]; (ii) scavenging of $O_2^{\cdot-}$ [16, 17] and (iii) decreased rates of $OH^{\cdot}$ formation [18]. However, the quantitative evaluation of the antioxidant activity is far from complete: the kinetics of CAF (and related compounds) as chain-breaking antioxidants in organic solution has only recently been studied [19] and structure–activity relationships tentatively suggested [17, 20].

It is currently believed that the oxidative modification of LDL is an important initial event in pathogenesis of atherosclerosis [21, 22]. Several agents are competent *in vitro* inducers of LDL oxidation, including cells [23], Cu^{2+} [22], lipoxygenase [24], peroxidases [25], peroxynitrite [26], myoglobin [27] and azo initiators [28]. The latter compounds, namely AAPH, although not biologically relevant, have the advantage of generating localized well-defined radicals at a constant rate [29], permitting kinetic quantitative studies of antioxidant activity in the early stages of LDL oxidation [28, 30–32].

In view of the potential application of the phenolic acids as preventive agents in human health and the lack of a simple criterion of antioxidant potency [33, 34], a full characterization of antioxidant activities is of obvious interest and systematic studies

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Abbreviations: AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; LDL, low density lipoproteins; PnA, *cis*-parinaric acid, 9,11,13,15-octadecatetraenoic acid; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CHL, chlorogenic acid; CAF, caffeic acid; ELL, ellagic acid; PRO, protocatechuic acid; SDS, sodium dodecyl sulphate.

in simple and reproducible oxidation systems are required in the modelling of antioxidant criteria. Therefore, we studied the interaction of CHL, CAF, PRO and ELL with AAPH-derived peroxyl radicals in a LDL model system where kinetic parameters of oxidation were established with standardized methods [32]. This involved the use of an oxidation probe, PnA, owing to its highly sensitive fluorescence quenching in the early stages of the oxidation process [35].

MATERIALS AND METHODS

Isolation of LDL. LDL particles (density 1.030–1.053 g/mL) were isolated from healthy human plasma by differential density ultracentrifugation [36] as previously described [32]. LDL samples were stored at 4° under N_2 atmosphere until use for a maximum of 7 days. Protein was determined by the Lowry method [37] and purity of the LDL preparation was checked by SDS–polyacrylamide gradient (3–20%) gel electrophoresis [38]. The same LDL preparation was used in all experiments described.

Oxidation procedures. Experimental conditions for PnA oxidation were set up as previously described [32] to ensure that the fluorescence intensity of incorporated PnA was linear with its concentration and that LDL concentration was high enough to incorporate most of the probe, thus preventing PnA from interfering in the water phase.

The assays were performed at 37° in the thermostated cuvette of a Perkin–Elmer LS 50 spectrofluorometer in a standard mixture (2 mL of buffer) containing 110 mM NaCl, 20 mM phosphate, pH 7.4 (referred to as phosphate buffer), 60 μg LDL protein and 2 nmol PnA added from an ethanolic solution (10 μL maximum volume). The incorporation of the probe was allowed to proceed for 5 min with gentle stirring and the oxidation reaction initiated by addition of a small volume of AAPH solution (5 mM final concentration). The fluorescence of the probe was recorded in time using excitation and emission wavelengths of 324 and 413 nm, respectively (slit widths 3.5 nm).

Measurements of O_2 consumption were carried out to follow the oxidation of LDL native lipids initiated by a peroxyl radical steady flux similar to that used for PnA oxidation. Accordingly, the reaction was initiated by addition of AAPH (5 mM final concentration) to 1 mL phosphate buffer containing 60 μg of LDL protein in a closed glass chamber protected from light, thermostated at 37° and provided with a stirrer and changes in oxygen tension were recorded with a Clark-type oxygen electrode (YSI Model 5331, Yellow Springs Inst.).

Measurement of antioxidant activities. Stock solutions of phenolic acids were prepared in phosphate buffer and added to the reaction mixture 1 min before initiation of the oxidative reaction with AAPH. The capacity of phenolic acids to protect either LDL lipids or PnA incorporated into LDL from peroxyl radical oxidative damage was determined by the increase in the initial inhibition period of O_2 consumption or of probe fluorescence decay, respectively, caused by the added compounds. The inhibition periods (t_i) were determined graphically from the profiles of O_2 consumption and PnA fluorescence decay; t_i values were taken from the intersections of tangents to the inhibited and uninhibited rates of oxidation. Unlike O_2 consumption, PnA fluorescence decay kinetics is not a zero order event and, therefore, the trace of the tangent to the uninhibited rate lacks precision. To minimize uncertainties, the fluorescence control record of LDL oxidation without antioxidant was computer subtracted from experiment traces in the presence of phenolic acids. The tangents were then drawn to the obtained traces. For the calculation of the oxidation rates during the inhibition periods, the amount of oxygen consumed in the oxidation reaction was corrected for the amount of oxygen consumed by the decomposition of AAPH in buffer at the same temperature.

UV spectral changes of phenolic acids. Spectral changes of phenolic molecules induced by AAPH were followed in time using selected wavelengths from the UV spectra. A solution of 5 mM AAPH in phosphate buffer was prepared and aliquots of 2 mL were immediately transferred to reference and sample thermostated cuvettes of a Perkin–Elmer Lambda 6 spectrometer at 37°. An autozero was immediately run, the phenolic solution was added at a final concentration of 1.5 μM and the absorbance recorded in time. The same procedure was used to record UV spectra changes with time but the phenolic acid solutions were 10 μM. The spectra were recorded at 4 min intervals.

RESULTS

Phenolic acids as antioxidants upon LDL peroxidation

The chemical structures in Fig. 1 represent three main chemical groups present in human diet, namely hydroxycinnamic (CHL and CAF) and hydroxybenzoic acid derivatives (PRO) and tannins (ELL). A common feature to all structures is the presence of catechol (*o*-dihydroxy) moiety to which most chemical properties of these compounds can be ascribed.

AAPH generates free radicals at a constant rate in the aqueous phase inducing the chain oxidation of human LDL by a free radical-mediated chain mechanism [28, 32, 30]. As previously shown [32], PnA incorporated into LDL particles is very sensitive to peroxyl radicals coming from the aqueous medium and detects the initial events of oxidative damage to LDL providing a calibration curve has been established to correlate the loss of fluorescence with the concentration of PnA. Figure 2 shows an example of the PnA fluorescence-based assay for peroxyl radical scavenging activity. In the absence of phenolic compounds, PnA rapidly underwent oxidative modification as indicated by its fluorescence decay, with no significant delay (≈1 min) after AAPH addition (trace 1). The phenolic compounds, as shown for CHL (Fig. 2A), effectively suppressed the oxidation of PnA, producing concentration dependent inhibition periods (t_{inh}) and, with the exception of PRO (Fig. 2B), the fluorescence decay rate following the inhibition period was resumed at a rate identical to the control.

Figure 3 shows an example of the effect of these

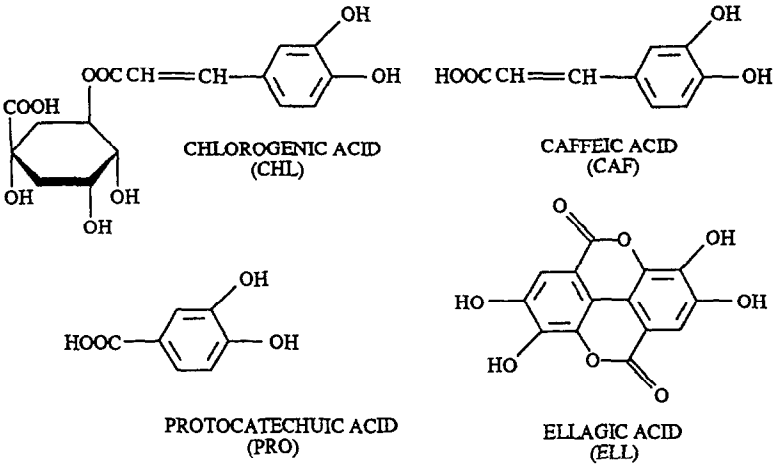

Fig. 1. Chemical structure of phenolic acids used in this study.

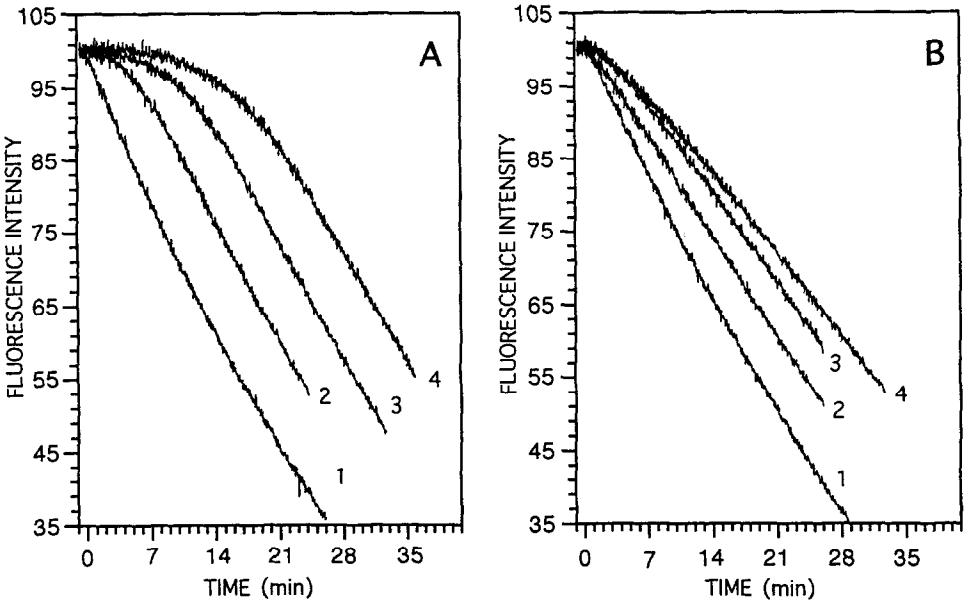

Fig. 2. PnA fluorescence decay in LDL, in the absence (lines 1) and presence of 0.5 (2), 1 (3) and 1.5 μM (4) of CHL (A) and PRO (B). The phenol compounds were added 1 min before AAPH addition (5 mM) at time zero. Appropriate controls concerning the effect of each phenol (no AAPH) on PnA fluorescence signal were always performed. The results of a single representative kinetic experiment are presented. The experimental variations for the same LDL preparation are negligible.

compounds on peroxyl radical initiated peroxidation of LDL native lipids, as monitored by O_2 consumption. Again, all the compounds, except PRO, induced a clear concentration dependent inhibition period of O_2 consumption, increasing the initial lag-phase, followed by a constant rate similar to the propagation rate of the control without the phenolic compounds. The rate (R_{inh}) during the inhibition periods of PnA oxidation and O_2 consumption was markedly low as compared with the respective rates after the inhibition periods. However, the R_{inh} range of O_2 consumption is narrower than that of PnA oxidation (Table 1), although in the same sequence; in the presence of ELL, PnA was oxidized 7.8 times faster than in the presence of chlorogenic acid, while O_2 consumption increased only by a factor of 1.5.

As shown in Fig. 4, over the concentration range used, a linear relationship ($R^2 \geq 0.98$) between antioxidant concentration and t_{inh} was observed and, although t_{inh} values of probe degradation are slightly shorter than those of LDL lipid oxidation, as

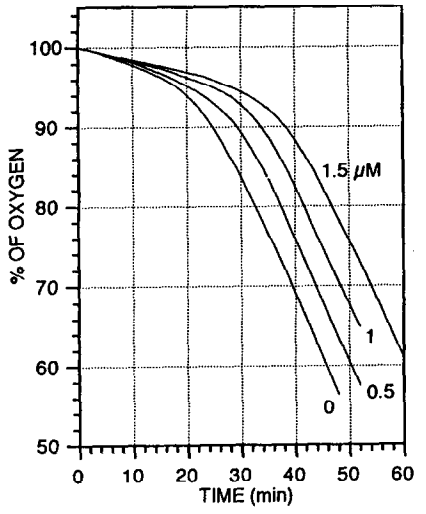

Fig. 3. Rate of oxygen consumption during oxidation of LDL induced by AAPH, added at time zero to LDL preparation in the absence (0) and presence of 0.5, 1 and 1.5 μM of CHL.

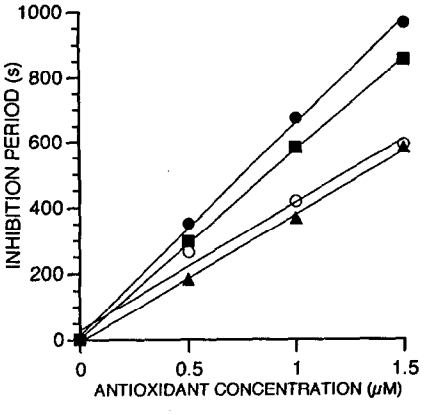

Fig. 4. Inhibition periods of PnA fluorescence decay as a function of phenol concentration. (●) CHL, (■) CAF, (▲) ELL and (○) trolox. The data are representative of several reproducible separate experiments with $R^2 \geq 0.97$.

Table 1. Rates of PnA oxidation and O_2 consumption during the inhibition periods induced by the phenolic acids in the oxidation of LDL initiated by AAPH and stoichiometric numbers of peroxyl radicals trapped by each phenolic acid

	R_{inh} (M/s)		
Antioxidant	PnA oxidation	Oxygen consumption	N*
CHL	4.1×10^{-8}	1.4×10^{-9}	3.1 ± 0.16
CAF	1.1×10^{-7}	1.7×10^{-9}	2.7 ± 0.2
ELL	3.2×10^{-7}	2.1×10^{-9}	1.7 ± 0.16
Trolox	5.8×10^{-7}	2.1×10^{-9}	(2)†

* Mean ± SEM of three experiments.

† It is taken as standard and the other values of N are calculated relative to this value.

measured by the inhibition of O_2 consumption, the results from the two methodologies correlate reasonably ($R^2 = 0.96$) (Fig. 5). PRO was not included since inhibition periods could not be accurately established (Fig. 2B) indicating a kinetically diverse reaction with peroxyl radicals as will be discussed later. From Fig. 4 it is clear that CHL and CAF induce longer inhibition periods than trolox, the water-soluble analogue of vitamin E. Considering that trolox traps 2 × ROO• per molecule (i.e. the stoichiometric number, N = 2) [39], our estimation of molar trapping capacities (Table 1) based on the duration of t_{inh} for PnA as compared with trolox is as follows: CHL, 3.1; CAF, 2.7; ELL, 1.7. Non-ideal inhibition periods induced by PRO do not permit a reliable estimation of molar trapping capacity for this compound. Therefore, the relative order of antioxidant protection measured as the time they protect LDL from ROO• damage follows the order: CHL > CAF > ELL.

Spectral UV changes of phenolic compounds induced by AAPH

The phenolic compounds suffered changes in UV spectra upon oxidation mediated by ROO• (Fig. 6). The kinetics of spectral changes at selected wavelengths is shown in insets. Fast modifications of spectral characteristics were observed as rapid initial absorbance decrease, followed by steady state low rates. The rapid initial change was synchronous with the inhibition period of PnA fluorescence decay induced by the same phenol concentration and ROO• flux as depicted in insets to Fig. 6.

DISCUSSION

The phenolic acids under study, representing three significant chemical groups present in human diet, inhibit the lipid peroxidation of LDL initiated by aqueous peroxyl radicals to different extents and efficiencies. All the compounds possess in their structure an *o*-dihydroxy (catechol) group which is the putative radical target site. Data from the inhibition profiles of PnA oxidation and O_2 consumption (Figs 2 and 3) indicate that the compounds under study, with the exception of PRO, rapidly scavenge peroxyl radicals in the aqueous phase (e.g. donation of an H atom), thereby preventing the chain initiation of LDL peroxidation. The antioxidant is consumed at a constant rate (producing inhibition periods) and, when depleted, the propagation rate of the reaction is resumed, indicating that the putative antioxidant phenoxyl radical formed is devoid of activity to initiate the peroxidation reaction, i.e.

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (1)$$

$$A^{\bullet} \rightarrow \text{stable combination and disproportionation products} \quad (2)$$

where ROO• is the AAPH-derived peroxyl radical and AH the phenolic compound.

The inhibition profile induced by PRO shows a decrease of PnA oxidation rate and O_2 consumption that failed to return to the propagation rate.

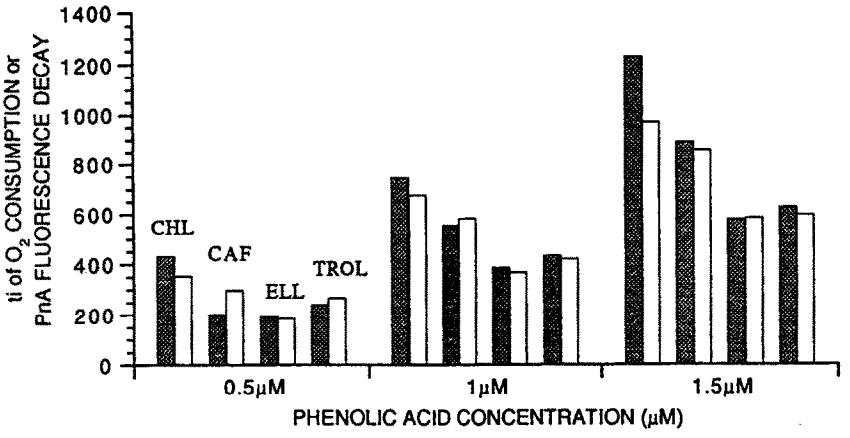

Fig. 5. Relationships between inhibition periods of PnA fluorescence decay (white bars) and O_2 consumption (shaded bars) induced by the same flux of ROO$^\bullet$ in the presence of several phenolic compound concentrations. The sequence of bars indicated for 0.5 μM is maintained for 1 and 1.5 μM. Error bars are not indicated because these values are taken from a single representative set of experiments with the same LDL aliquot and stock solutions of phenolic acids and AAPH (negligible experimental variations).

Therefore, PRO cannot be considered an antioxidant but a retardant [33]; i.e. it reacts with ROO$^\bullet$ to slow down the initiation and propagation steps of lipid peroxidation to a limited extent.

Due to the heterogeneous composition of the LDL particle, the site of initial radical generation must be ascertained in antioxidation studies. The AAPH-derived peroxyl radicals are generated in the aqueous bulk phase; here most of them are quenched by the phenolic acids before oxidizing LDL. However, when the phenolic compounds are added (not shown) to the LDL undergoing oxidation during the propagation step of lipid peroxidation, an inhibition of O_2 consumption and PnA oxidation is observed. This result raises some hypotheses currently being investigated: (1) interaction at the LDL surface (i.e. the site of lipid peroxyl radical migration as a consequence of its polarity [40]) between the phenolic acids and chain-carrier lipid peroxyl radicals; (2) partition to some extent of the phenol molecules into LDL; (3) and regeneration of vitamin E at the LDL surface via a recycling mechanism.

As would be expected from antioxidants exhibiting the chemical behaviour described in equations 1 and 2, a linear relationship was obtained between the inhibition periods (t_{inh}) of oxygen consumption or the oxidative degradation of PnA incorporated into LDL, and the concentration of added phenolic acids, as predicted by the equation $t_{inh} = N/R_i[AH]$ where N is the stoichiometric number of radicals trapped by each antioxidant molecule (AH) and R_i the constant rate of initiation induced by AAPH-derived ROO$^\bullet$ [29].

Oxygen consumption reflects the overall oxidation of LDL substrates in the peroxidation reaction and, therefore, is a very useful but not very sensitive measurement. Clearly, PnA, with four conjugated double bonds highly susceptible to peroxidation, will be a preferential oxidation substrate at the LDL surface. Therefore, the decay of its fluorescence is a very sensitive index of the onset of radical damage to LDL, as reflected by the initial lag-phase of *ca.* 1 min as compared with *ca.* 24 min in oxygen measurements. Consequently, the rates of O_2 consumption during the inhibition periods induced by the phenolic compounds (Table 1) are within a narrow range, between 1.4 and 2.1×10^{-9} M/s, whereas the rates of PnA fluorescence decay during the same periods are higher and better resolved, clearly discriminating the following order of reactivity with peroxyl radicals: CHL > CAF > ELL > trolox, corresponding to inhibition rates varying between 4.1×10^{-8} for CHL and 5.8×10^{-7} M/s for ELL. R_{inh} during the lag-phases of PnA oxidation is, therefore, a sensitive measure of antioxidant efficiency in trapping peroxyl radicals.

The chemical structures of CHL, CAF, PRO and ELL (Fig. 1) are suggestive of chain-breaking (donor) antioxidants since the compounds contain a hydrogen atom on the phenolic OH group prone to donation with subsequent formation of phenoxyl radicals. In fact, as followed by spectral changes with time, these molecules undergo chemical modifications upon their interaction with peroxyl radicals. Spectral data reveal the appearance of well-defined isosbestic points as the reaction proceeds, except for PRO, indicating that, in this case only, intermediates accumulate in the reaction of this compound with peroxyl radicals. Accordingly, clearly defined inhibition periods were not observed with PRO. It is conceivable that the initial rapid absorbance changes in CHL, CAF and ELL reflect structural modifications (antioxidant consumption) occurring in the reaction with ROO$^\bullet$ during the inhibition period (Fig. 6 insets), according to equation 1. In fact, the rapid absorbance change (Fig. 6, insets) fits reasonably with the duration of the inhibition periods.

The stability of the radical formed after scavenging may also be inferred from the fact that the

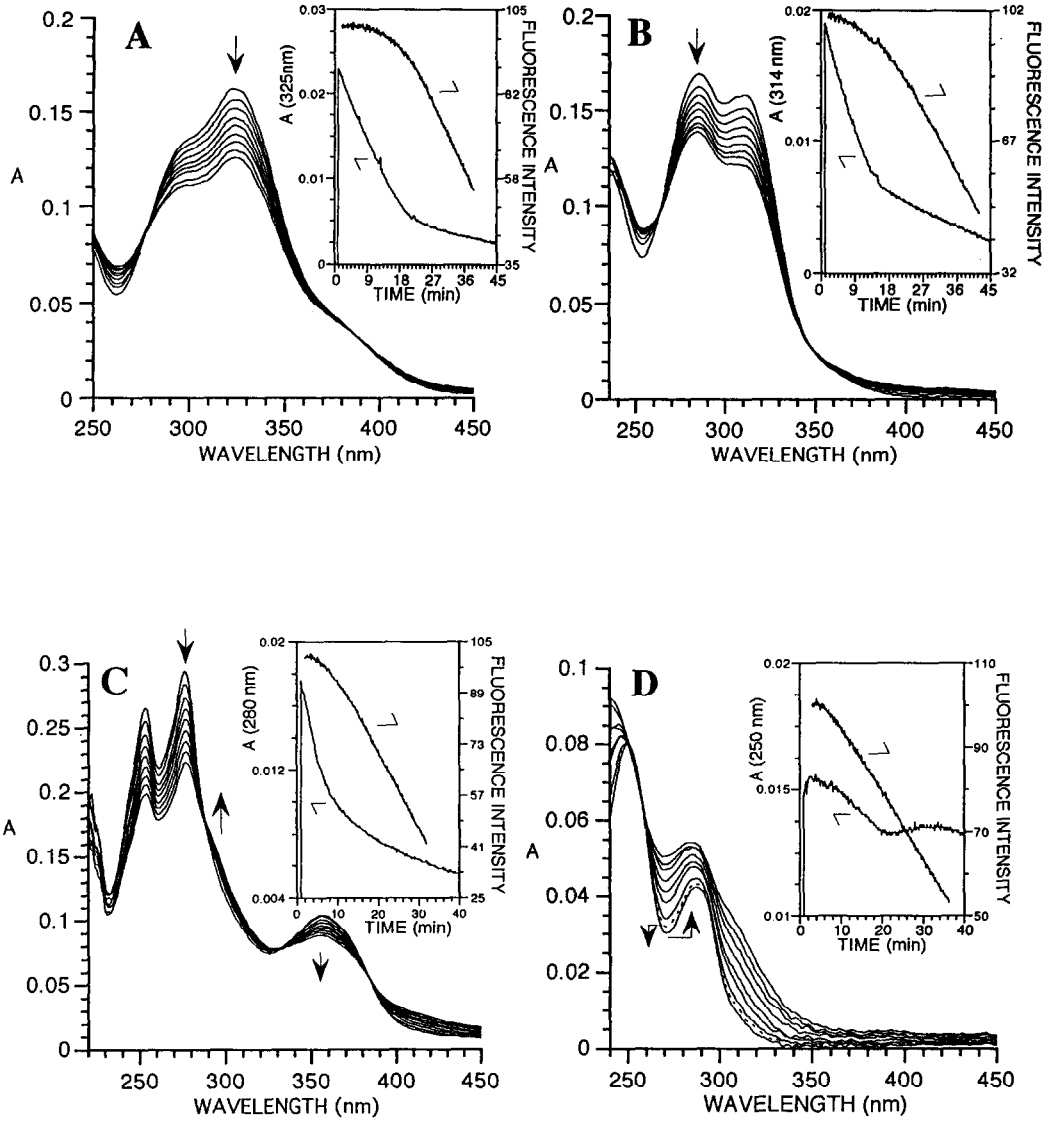

Fig. 6. UV spectral changes of phenolic compounds during the inhibition period of PnA peroxidation. The downward and upward arrows indicate decrease and increase in absorbance as the reaction proceeds. In D, the dotted line represents the spectrum at time zero. Insets depict the time courses of absorbance changes, at selected wavelengths, of 1.5 μM solutions of CHL (A), CAF (B), ELL (C) and PRO (D) (left traces) and the time course of PnA degradation by identical flux of radicals in the presence of 1.5 μM of the phenolic compound (right traces).

propagation rate of peroxidation is restored after the inhibition periods. Furthermore, as the stability of an antioxidant-derived radical increases, annihilation of peroxyl radicals is enhanced (equation 2). Therefore, the greatest antioxidant activity of hydroxycinnamic acid derivatives, CHL and CAF, would be explained if the radical character following the initial reaction with ROO$^{\bullet}$ (most likely an *o*-semiquinone) could be sequentially transferred to the conjugated double bond in the side chain (with putative formation of an alkyl quinone radical) further increasing electron stabilization and hence antioxidant activity. On the other hand, it has been suggested that the presence of electron-withdrawing substituents in phenolic molecules decreases reactivity with peroxyl radicals [41, 42]. Therefore, in the case of ELL, in spite of two catechol groups, the presence of carbonyl groups in the lactonic ring may partially explain its lower reactivity as compared with CHL and CAF. Additionally, internal hydrogen bonding between the oxygen atom in the lactonic ring and the closer phenolic OH group reduces the ability of ELL to donate a phenolic hydrogen atom to an attacking peroxyl radical (equation 1). However, in the case of PRO, the lateral COOH group is ionized at the experimental pH (pH 7.4) and since COO^{-} efficiently releases electrons, a stronger antioxidant activity is to be expected.

Therefore, other factors are responsible for the low reactivity of PRO with peroxyl radicals.

Bors *et al.* [43] demonstrated that flavonols are efficient radical scavengers, but only those containing a 3′,4′-catechol structure produce phenoxyl radicals decaying slowly enough to be considered as potent antioxidants; a very reactive secondary radical would propagate rather than interrupt the oxidation reaction. Thus, the catechol moiety confers a higher stability to phenoxyl radicals by participating in electron delocalization. The results shown here for simple molecules demonstrate that catechols are actually efficient quenchers of peroxyl radicals, but the substituents in the side chain are determinants of antioxidant activity. For example, a conjugated double bond in the side chain of a catechol group is likely to have a great effect in stabilizing the putative phenoxyl radical and, therefore, in enhancing antioxidant activity. Accordingly, hydroxycinnamic derivatives exhibit higher stoichiometric numbers and reactivity with peroxyl radicals and, therefore, protect LDL more efficiently against oxidation.

In summary, the data indicate that four plant phenolics, especially CHL and CAF, are efficient quenchers of peroxyl radicals in the aqueous phase, but the substituents in the lateral chain of catechol moiety modulate antioxidant activity. This effect has to be accounted for when plant phenolic compounds are developed for use as inhibitors of deleterious physiological processes.

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