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The Apoprotein is the Preferential Target for Peroxynitrite-induced LDL Damage Protection by Dietary Phenolic Acids

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Peroxynitrite has been shown to modify low-density lipoproteins (LDL) into a form recognized by the macrophage scavenger receptor, suggesting that it may play a significant role in atherogenesis. Considering that the mechanisms underlying LDL modifications by this agent have not been well elucidated, the aim of this study was to characterize the chemical modifications of either the lipid or the protein moieties mediated by synthesized peroxynitrite (preformed) or formed *in situ* by SIN-1, and evaluate the protective effects of some dietary phenolic acids. Preformed peroxynitrite does not induce LDL lipid peroxidation, as assessed either by formation of conjugated diene isomers or degradation of fatty acids and cholesteryl esters, although a rapid loss of α-tocopherol content occurs. Also, peroxynitrite formed *in situ* induces only a slight lipid oxidation. In contrast, under conditions where the LDL lipid moiety is not significantly oxidized, peroxynitrite either preformed or formed *in situ* rapidly elicit significant LDL apoprotein modifications, as evaluated by an increase in carbonyl groups formation and by great decrease in intrinsic tryptophan and thiol groups, in a concentration-dependent manner, that are accompanied by an increase in the LDL net negative charge, leading to an increase in electrophoretic mobility. Phenolic acids, namely caffeic, chlorogenic and ferulic, inhibit all these processes in a concentration dependent way, being the catechols the most efficient. UV spectral analysis of phenols upon interaction with peroxynitrite suggest that, in our assay conditions, such protection is related with the scavenging of this agent by either electron donation for the catechols, caffeic and chlorogenic acids, or nitration for the monophenol ferulic acid. Our data point that in contrast with other physiological oxidants, as ferrylmyoglobin or copper, peroxynitrite triggers the rapid damage to LDL primarily by protein and not lipid oxidation, and that such process is inhibited by dietary phenolic derivatives of cinnamic acids.

Keywords: LDL; Peroxynitrite; Phenolic acids; Apoprotein modification

INTRODUCTION

Peroxynitrite is a relevant oxidant generated *in vivo* from the rapid reaction of superoxide with nitric oxide. The rate constant for this reaction is $1.9 \times 10^{10}\,M^{-1}\,s^{-1}$[1]

$$^{\bullet}NO + O_2^{\bullet -} \rightarrow ONOO^-$$

Although superoxide and nitric oxide are radicals, they are relatively stable and not very reactive *per se*, but peroxynitrite is a much more reactive species.[2] A variety of cells including neutrophils, macrophages, neuronal and endothelial cells are known to produce both superoxide and nitric oxide.[3] Under normal conditions, the amount of superoxide dismutase (SOD) is sufficient to dismutate all the superoxide formed, but when an elevated level of $O_2^{\bullet -}$ prevails or when $^{\bullet}NO$ is produced in excess,

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peroxynitrite is invariably formed.[1] In this regard, pathologies associated with oxidative stress, such as ischemia-reperfusion injury, neurodegenerative diseases, cardiovascular disorders, atherosclerosis and severe inflammation conditions, among others, can lead to increased peroxynitrite formation.[4]

It has been reported that the peroxynitrite causes generalized oxidative damage, reacting with a wide variety of biomolecules including proteins, DNA, lipids and antioxidants. Nevertheless, the reaction rates of peroxynitrite with antioxidants, such as ascorbate and glutathione, are slow as compared with those of peroxynitrite with typical biological targets, particularly metal centers, pointing out that in this case our antioxidant defences are not very efficient.[5] Also, it has been observed that low density lipoproteins (LDL) may be modified by peroxynitrite into a form recognized by the macrophage scavenger receptor, a key event in the development of atherosclerotic lesions.[6,7] Actually, the presence of cells in the artery wall that can produce both $^{\bullet}NO$ and $O_2^{\bullet -}$ is consistent with the formation of $ONOO^-$ which is able to modify LDL. Moreover, 3-nitrotyrosine, a highly specific marker for peroxynitrite-mediated damage to proteins has been detected in LDL isolated from atherosclerotic lesions.[8]

Considering that the mechanisms underlying such LDL alterations have not been well clarified and that the antioxidant defences may not be very efficient, we carried out the present study aiming essentially to elucidate the chemical modifications of both lipid and protein moieties promoted by either preformed peroxynitrite, at various concentrations, or formed *in situ* from auto-oxidation of 3-morpholinosydnonimine (SIN-1). SIN-1 has been used as a model for the continuous release of superoxide and nitric oxide, mimicking $ONOO^-$ formation *in vivo*.[3,9] On the other hand, the potential inhibitory effects of three cinnamic acid derivatives, chlorogenic, caffeic and ferulic acids, ubiquitously present in fruits and vegetables, were evaluated. The lipid and protein oxidations were followed by measuring several parameters that may characterize the extent and/or the rate of LDL oxidation,[10] as well as the protection provided for the phenolic acids under study against the extent of the oxidative degradation.

MATERIALS AND METHODS

SIN-1, phenolic acids, α-tocopherol and cholesteryl esters were from Sigma Chemical Company (St Louis, MO). Monobromobimane was obtained from Fluka Chemie (Neu-Ulm, Switzerland). All the other reagents and chemicals used were of analytical grade or HPLC grade. The solutions were prepared in water purified in a Milli-Q apparatus to minimize the metal contamination. Either SIN-1 or phenolic acids solutions were prepared freshly every day in phosphate buffer (20 mM phosphate, 110 mM NaCl) pH 7.4, containing 100 μM DTPA, and were light protected and maintained in ice during the experiments.

Peroxynitrite Synthesis

Peroxynitrite ($ONOO^-$) was synthesized from nitrite and hydrogen peroxide in a quenched-flow reactor as previously described.[11] Peroxynitrite was concentrated by freeze fractionation and the concentration was determined spectrophotometrically by using $\varepsilon_{302\,nm} = 1670\,M^{-1}\,cm^{-1}$. At all times, the final concentration of peroxynitrite was in the range of 250–400 mM. The alkaline solution of peroxynitrite is relatively stable and was kept in the freezer (−80°C) for some weeks. The peroxynitrite solution concentration was checked at the beginning and end of each study to ascertain that the peroxynitrite had not decomposed during the experiment times.

Isolation of LDL

LDL were isolated from fresh plasma of healthy normolipidemic volunteers by density gradient ultracentrifugation at 15°C by a rapid isolation method (180 min),[12] using a Beckman L 80 ultracentrifuge (Beckman Inst.) equipped with a Beckman 70.1 Ti fixed angle rotor. After isolation, LDL were dialyzed against phosphate buffer (20 mM phosphate, 110 mM NaCl), pH 7.4 and simultaneously concentrated by ultrafiltration through a collodium bag under nitrogen. Then, the LDL sample was filtered through a 0.22 μm pore size filter (Millipore), stored at −80°C after mixture with sucrose 10% (final concentration). Protein concentration was estimated according to Lowry *et al.*[13] using bovine serum albumin (BSA) as standard.

LDL Oxidation

The oxidation of LDL particles was induced by preformed $ONOO^-$ synthesized as described above, or synthesized *in situ* from SIN-1, a peroxynitrite-generating system, in a medium containing 20 mM phosphate, 110 mM NaCl, 100 μM DTPA pH 7.4, at 37°C and under gentle stirring. The buffer solution was always deaerated, by bubbling nitrogen, before use. When the reaction was initiated by pre-synthesized $ONOO^-$, the addition of the oxidant solution was sequential (five additions), to reach a relatively low steady-state concentration during the first 5 min, as recommended by Sies *et al.*[14,15] However, the concentration of $ONOO^-$, referred to in all the assays is the final concentration and not the steady concentration. In contrast, SIN-1, was added

by bolus addition. When present, the phenolic acids were added 3 min before the oxidant.

Blanks were also performed allowing peroxynitrite to degrade for 10 min in the reaction medium, without LDL, at the assay temperature.

Each set of assays was performed with the same LDL preparation and the data represent the mean ± SD of at least three determinations of separated experiments, except for the recordings of the spectral assays which are typical of at least three experiments.

Lipid Oxidation

The extension of lipid modification was evaluated by the measurements of conjugated-diene hydroperoxides formation, the consumption of cholesteryl esters, as well as by fatty acyl chains degradation and the production of thiobarbituric acid reactive substances (TBARS). Also, the consumption of α-tocopherol was determined.

Conjugated-dienes were detected by second derivative spectrophotometry, in a Perkin–Elmer Lambda 6 spectrophotometer, according to Corangiu *et al.*,[16] directly in the LDL preparation (90 μg protein/ml) without need of lipid extraction as previously described.[17]

Cholesteryl esters in LDL samples were determined by HPLC in a Beckman-System Gold with UV detection at 210 nm, as described by Vieira *et al.*[18]

Fatty acyl chains were analyzed by gas–liquid chromatography (GLC) in a Varian-Series 1400 gas chromatograph equipped with a BP 20 (bonded polyoxyethylene) column aluminum clad (25 m × 0.53 mm). The temperature program started at 180°C, kept for 5 min, and then went up to 230°C at 4°C/min. The injection temperature was 210°C. Peaks were identified by comparison of retention times with those of authentic standards and quantified by suitable program computer (Axiom Chromatography model 717). Fatty acid methylesters from LDL lipid extracts were prepared as previously described by Dinis *et al.*[19]

Thiobarbituric acid reactive products were measured by fluorescence after butanol extraction, as described elsewhere,[20,21] using a Perkin–Elmer LS 50 spectrometer (the emission and excitation wavelength were 553 and 515 nm, respectively). Butylated hydroxytoluene 0.01% (final concentration) was added to the reagent to prevent additional chromophore formation during the assay procedure.

The consumption of α-tocopherol was followed by HPLC quantification (Beckman System Gold, UV detector model 166), on a Lichrospher 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany) after extraction as described elsewhere.[22] α-Tocopherol was eluted at a flow rate of 1.5 ml/min, with a solvent mixture consisting of 65% methanol and 35% ethanol/isopropanol (95/5 v/v) and UV detection at 292 nm.

Apoprotein Modifications

Protein modifications were evaluated in terms of surface charge alterations, tryptophan and thiol groups oxidation and carbonyl groups formation.

Agarose gel electrophoresis was used to assess surface charge alterations of the apolipoprotein B of the LDL after its reaction with peroxynitrite in phosphate buffer pH 7.4, at 37°C, during 1 h in the absence and presence of phenolic acids. Aliquots of the samples were subsequently applied to the gels in barbital buffer pH 8.6, at 100 V (Beckman Paragon Lipo Gel electrophoresis system). The electrophoretic mobility of the peroxynitrite-treated LDL samples in the absence or presence of phenolic acids was measured relative to that of untreated (native) LDL (R.E.M.), i.e. as the ratio of the distances migrated by the treated and native LDL samples. Lipoproteins were visualized by staining with Sudan Black B.

The tryptophan loss due to oxidation by $ONOO^-$ was followed by its intrinsic fluorescence, as previously described.[23] Fluorescence, measurements were performed at 37°C in a Perkin–Elmer LS 50 spectrometer. The excitation wavelength was 265 nm (slit width 5 nm) and the emission wavelength window was 300–400 nm (slit width 5 nm). The tryptophan loss was evaluated by the decrease in the emission fluorescence intensity and expressed by the ratio of the initial fluorescence intensity value at 360 nm, I_0, (measured in the absence of $ONOO^-$) to the intensity value after $ONOO^-$ addition, I. In order to minimize the quenching effect of phenolic acids, the fluorescence measurements were carried out at 360 nm, where such effect is not significant in the concentration range tested.

To estimated the apoprotein thiol groups, the fluorescent probe monobromobimane (mBBr) was used. Bimane derivatives, as fluorescent labeling agents for cellular thiols, were developed by Kosower *et al.*[24] The thiol content of LDL apoprotein (100 μg/ml), in the absence and presence of $ONOO^-$, was estimated after incubation with 50 μM mBBr, at 37°C, for 1 h, by measuring the fluorescence intensity in a Perkin–Elmer LS 50 spectrometer. The excitation and emission wavelengths were set at 395 and 470 nm, respectively (slits width 5 nm).

LDL apoprotein carbonyl content was measured according to Levine *et al.*,[25] by the widely used reaction with 2,4-dinitrophenylhydrazine (DNPH). DNPH reacts with protein carbonyls to form protein hydrazones that were quantified spectrophotometrically, on the basis of the molar absorption coefficient, $\varepsilon_{370\,nm} = 22,000\,M^{-1}\,cm^{-1}$.

In all the experiments, when present, the phenolic acids were always pre-incubated with LDL for 3 min before the $ONOO^-$ addition.

Interaction of Phenolic Acids with Peroxynitrite

Stock solutions of phenolic acids (caffeic, chlorogenic and ferulic) were prepared in phosphate buffer. The chemical modifications of phenols (25 μM) upon reaction with $ONOO^-$ in a concentration range from 25 to 500 μM, and the formation of oxidation and/or nitration products were characterized by absorption spectral changes, between 250 and 500 nm, in a Perkin–Elmer Lambda 6 UV/VIS spectrophotometer. Simultaneously, control experiments were carried out by using either ferrylmyoglobin as a reference oxidant, or sodium nitrite to induce the nitration of phenolic acids, according to Laranjinha *et al.*[26] and Grace *et al.*,[27] respectively.

RESULTS AND DISCUSSION

Stability of Performed Peroxynitrite

As previously reported, the alkaline solution of peroxynitrite is relatively stable, but rapidly decomposes after dilution in buffer solutions, pH about 7.0. In this work, all the experiments were carried out in a phosphate buffer (20 mM phosphate, 110 mM NaCl, 100 μM DTPA, pH 7.4), the best medium tested in terms of peroxynitrite stability (here, the significant decrease in the absorbance due to this oxidant spends about 5 min). DTPA increases the reproducibility of the results because it chelates contaminating transition metals (especially iron) to a poorly active redox form without interfering significantly with peroxynitrite reactivity.[28] Worthy of notice, after addition of peroxynitrite solution, the medium pH does not change significantly for the $ONOO^-$ concentration range used.

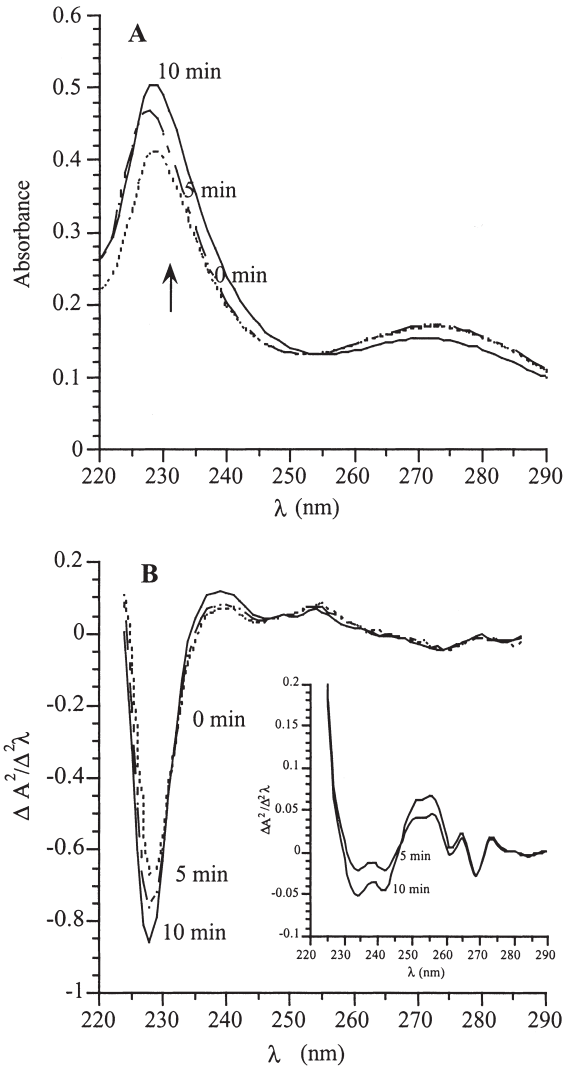

FIGURE 1 (A) Absorption spectra of LDL (90 μg protein/ml) challenged by 250 μM peroxynitrite. Evidence for the slight increase in absorbance at 234 nm, in the first 10 min of reaction, characteristic of conjugated-dienes formation.(B) Second derivative of the spectra presented in (A) showing a negative peak below 230 nm. The inset shows comparatively the second-derivative spectra of LDL challenged by the well known oxidant ferrylmyoglobin, with two negative peaks, at about 233 and 242 nm, the characteristic peaks of *trans*, *trans* and *cis*, *trans* conjugated dienes isomers, respectively.

Lipid Oxidation

Reaction of $ONOO^-$ (250 μM) with LDL (90 μg protein/ml) leads to a slight increase in absorbance at 234 nm, the characteristic absorbance of fatty acid hydroperoxides with conjugated double bonds (dienes), as shown in Fig. 1A, but only in the first 10 min. However, on the basis of a more accurate and reliable method, that correctly permits to discriminate the *trans*, *trans* from the *cis*, *trans* diene isomers,[16] this increase in the absorbance does not seem to correspond to the formation of conjugated diene hydroperoxides. Actually, Fig. 1B shows the second derivative UV spectra of LDL challenged by $ONOO^-$ or ferrylmyoglobin (inset), indicating that the two spectra are completely different. Whereas in the first case ($ONOO^-$), only a negative peak below 230 nm is evident, in the second case (ferrylmyoglobin), the two minima at 233 and 242 nm that characterize the *trans*, *trans* and *cis*, *trans* conjugated diene hydroperoxides, respectively, are observed. So, in our experimental conditions, preformed peroxynitrite does not lead to the conjugated diene hydroperoxides formation, in contrast with that which has been referred to by others in liposomes[29] and frequently reported in literature. However, more recently, Pannala *et al.*[30] obtained similar results to ours, i.e. they observed only a limited formation of conjugated dienes, as evaluated by the increase in the

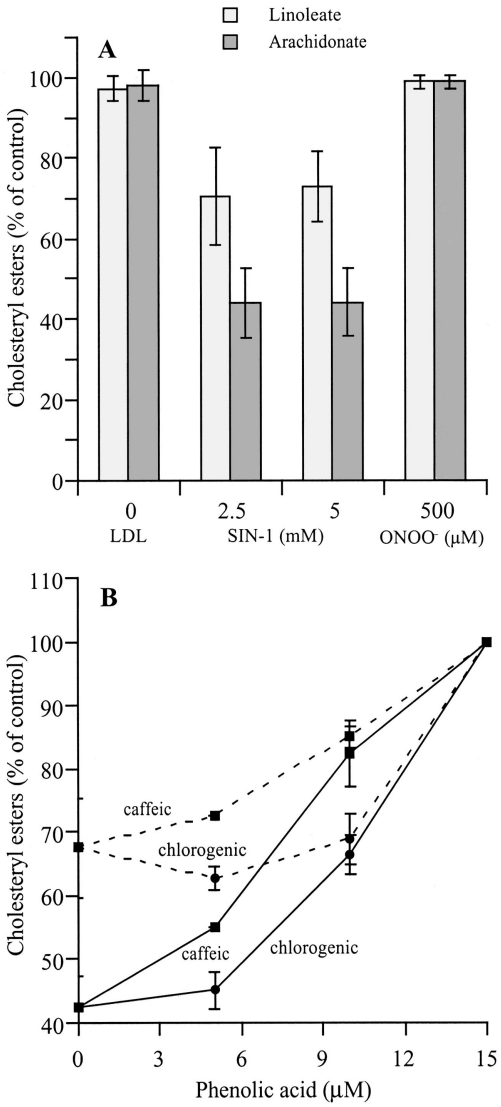

FIGURE 2 (A) Contents of cholesteryl arachidonate and cholesteryl linoleate in LDL after incubation during 24 h at 37°C in the absence and presence of SIN-1 or preformed peroxynitrite. LDL (200 μg protein/ml) were incubated with preformed peroxynitrite (500 μM) or peroxynitrite formed *in situ* from SIN-1 (2.5 or 5 mM) as described in "Materials and Methods" section. The contents of cholesteryl esters were calculated by the ratio of the respective HPLC peak areas to that of the free cholesterol and such ratio was expressed in terms of percentage of the control LDL sample. (B) Effects of caffeic and chlorogenic acids on the cholesteryl arachidonate (solid line) and cholesteryl linoleate (dashed line) contents of LDL under the action of 2.5 mM SIN-1, in the experimental conditions referred to (A). Phenolic acids were added before SIN-1.

absorbance at 234 nm, in peroxynitrite-induced LDL oxidation.

To clarify the $ONOO^-$—induced lipid oxidation in LDL, we evaluated also the cholesteryl esters oxidation. Although these main human LDL lipid components form together with the triacylglycerols, the central lipophilic core of these particles,[31] they are prone to oxidative degradation since LDL have a structure that facilitates the core oxidation.[32] As shown in Fig. 2A, under the experimental conditions used, no significant degradation of cholesteryl esters is detected 24 h after starting the interaction of LDL with performed $ONOO^-$. However, in contrast, in the same conditions, α-tocopherol is promptly depleted (data not shown).

α-Tocopherol represents one of the most important antioxidant in biological systems, including LDL. On a molar basis, α-tocopherol is by far the major antioxidant in LDL.[31] Preformed $ONOO^-$ promptly depleted LDL α-tocopherol under our assay conditions, according to the results reported by others, either in LDL[33] or in synaptosomes and mitochondria.[34]. Also, in a homogenous solution, peroxynitrite rapidly reacts with α-tocopherol and the results predominantly suggest a two-electron mechanism of α-tocopherol oxidation.[35] These data may have important biological consequences because ascorbate is unable to reduce α-tocopherylquinone to α-tocopherol, suggesting an irreversible oxidation of α-tocopherol by peroxynitrite.

Thus, in contrast with some reports in literature that pointed to preformed $ONOO^-$ as a strong lipid oxidant, we have only observed α-tocopherol depletion without significant consequences on fatty acids degradation. Attempting to clarify and to confirm this observation, we carried out similar studies using SIN-1, instead of preformed $ONOO^-$, that has been used as a model for the continuous formation of $ONOO^-$,[3,9] since it generates superoxide anion and nitric oxide simulating the *in vivo* small fluxes of this oxidant. As shown in Fig. 2A, cholesteryl arachidonate and linoleate decreased by about 50 and 30%, respectively, under the action of SIN-1, in a concentration-independent way for the tested range. To the contrary, as referred above and shown in the same figure, 500 μM preformed $ONOO^-$ (total concentration) does not induce any detectable effect.

Under similar experimental conditions, the presence of caffeic or chlorogenic acids before addition of 2.5 mM SIN-1 to LDL solution, lead to the inhibition of cholesteryl arachidonate and linoleate consumption as a function of phenolic acid concentration (Fig. 2B). Caffeic is more efficient than chlorogenic acid at the lowest used concentrations (5–10 μM) but at 15 μM, either caffeic or chlorogenic acids fully protect the esters from degradation. An efficient protection provided by caffeic acid on the contents of cholesteryl linoleate or cholesteryl arachidonate has been already reported by Vieira *et al.*,[18] in LDL under the action of ferrylmyoglobin as a lipid oxidation-inducer.

In parallel to the decrease in the cholesteryl esters, SIN-1 also leads to the formation of the aldehydic decomposition products of lipid hydroperoxides, as analyzed by the TBARS assay, although in a much lesser extension than that for the reference oxidant, ferrylmyoglobin, in similar

TABLE I MDA production from peroxynitrite-induced LDL lipid oxidation. LDL (200 μg protein/ml) were incubated with 500 μM $ONOO^-$ preformed or with 2.5 mM SIN-1, during 24 h at 37°C in the absence or presence of 15 μM caffeic or chlorogenic acids. Ferrylmyoglobin was used as a reference LDL lipid oxidation initiator, according to Laranjinha *et al.*[37]

LDL in different incubation systems	TBARS nmol MDA/mg protein	TBARS inhibition (%)
Native LDL	n.d.	
$ONOO^-$	n.d.	
SIN-1	2.0 ± 0.04	
Caffeic + SIN-1	0.49	75.5
Chlorogenic + SIN-1	0.41	79.5
Ferrylmyoglobin	5.79 ± 0.1	

n.d.—Not detected.

conditions, as shown in Table I. Caffeic and chlorogenic acids (15 μM), added before SIN-1, inhibit in 75.5 and 79.5%, respectively, the TBARS formation. However, in LDL under the action of preformed $ONOO^-$ (500 μM), no formation of TBARS takes place, in agreement with that previously reported by others[30] and with the effects observed on the cholesteryl esters, as referred above.

Also, in LDL under the action of SIN-1, α-tocopherol is consumed along the time (until 10 min) and the phenolic acids, caffeic and chlorogenic, inhibit this consumption (Fig. 3) in a concentration-dependent way. In particular, caffeic acid (15 μM) completely inhibits α-tocopherol consumption during the reaction time. This fact is particularly relevant whether we consider that peroxynitrite may oxidize α-tocopherol by a two-electron process.

Thus, SIN-1 that seems to mimic the *in vivo* $ONOO^-$ production, induces LDL lipid degradation,

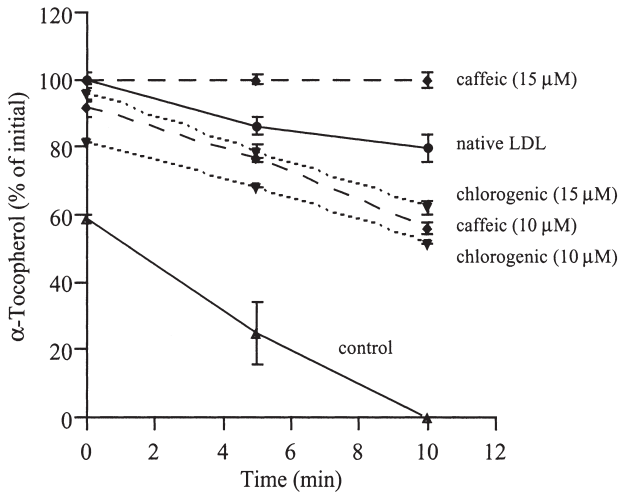

FIGURE 3 Depletion of α-tocopherol in LDL, under the action of SIN-1, along the incubation time and protective effects of caffeic and chlorogenic acids. Human LDL (180 μg protein/ml) were incubated at 37°C with 1 mM SIN-1 in the absence (control) and in presence of two concentrations of caffeic and chlorogenic acids. Samples were withdrawn at 5 and 10 min of incubation and treated as described in "Materials and Methods" section. Caffeic acid, at 15 μM, completely affords protection against peroxynitrite-induced α-tocopherol exhaustion and also protects LDL from the small decrease caused by the exposition to air (native LDL).

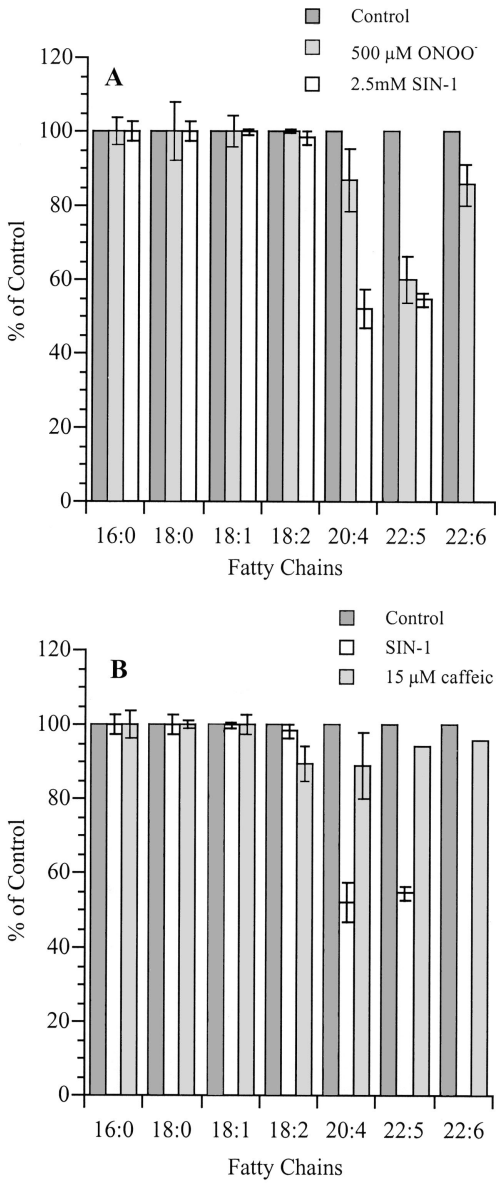

FIGURE 4 (A) Profiles of the more prevalent fatty acyl chains of LDL, as evaluated by gas–liquid chromatography, 24 h after addition of preformed peroxynitrite (500 μM) or SIN-1 (2.5 mM) to LDL (360 μg protein/ml) at 37°C. (B) Protective effect of 15 μM caffeic acid against the fatty acyl chains degradation promoted by 2.5 mM SIN-1 in LDL after 24 h of incubation. Of notice, the protection provided to the more polyunsaturated chains $C_{20:4}$, $C_{22:5}$ and $C_{22:6}$. The acyl chains amounts are expressed relative to those in native LDL (control) submitted to identical assay conditions without peroxidation promoter.

and in all the performed assays, the phenolic acids tested afford protection against such degradation.

The oxidizing effects of $ONOO^-$, either preformed or formed *in situ*, on LDL lipids were also evaluated on the basis of polyunsaturated fatty acids degradation by gas chromatographic analysis, a very specific and sensitive technique for detecting lipid oxidation. Figure 4 shows the effects observed on the more prevalent fatty acyl chains from LDL particles incubated during 24 h with either 500 μM $ONOO^-$ or 2.5 mM SIN-1 (Fig. 4A). The oxidizing effect is only observed on the more polyunsaturated acyl chains, namely $C_{20:4}$, $C_{22:5}$ and $C_{22:6}$. Worthy of notice is that $C_{22:6}$ is completely degraded in the presence of SIN-1. To the contrary, preformed $ONOO^-$ only induces a slight decrease in the lipid substrate, and this effect is not time-dependent, i.e. the observed effects are detected soon after finishing the $ONOO^-$ addition (data not shown). Caffeic acid efficiently protects the loss of fatty chains, particularly the more polyunsaturated $C_{22:6}$, in SIN-1 induced-LDL oxidation (Fig. 4B).

From the results obtained, it is obvious that the LDL lipid oxidation is not easily induced by preformed $ONOO^-$. The only lipid component promptly oxidized is α-tocopherol, suggesting a crucial role of α-tocopherol in protecting LDL from $ONOO^-$ attack. Moreover, the lipoprotein fluidity, a reported index for *in vitro* LDL oxidation, since it is strictly dependent on lipid composition, does not change under the action of $ONOO^-$ (data not shown). Lipoprotein fluidity was evaluated by the fluorescence polarization of two probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and its propionic acid derivative (PA-DPH), which due to their chemical structures present distinct distributions along the particle, preferentially partitioning into the lipoprotein core and at the lipoprotein surface, respectively.[36] In oxidized LDL, a significant increase in polarization with both DPH and its derivative has been observed[36] similarly to that observed in biomembranes.[19]. So, the absence of changes in the LDL lipid fluidity corroborates the data above indicated, pointing that preformed peroxynitrite does not promote significantly LDL lipid oxidation.

These results raise a question about the relatively high concentration of chemically synthesized $ONOO^-$ used in the assays, as well as the different reactivities of $ONOO^-$ when preformed or formed *in situ* from SIN-1. However, the indicated concentrations of preformed peroxynitrite used in the assays are not effective, provided that the oxidant effect depends on both the concentration and the time of exposure to the species. Thus, considering that $ONOO^-$ spontaneously decay at 37°C and at physiological pH (ca $1\,s^{-1}$), the true $ONOO^-$ concentrations are substantially lower. Beckman *et al.*[11] calculated that the exposure to 250 μM peroxynitrite corresponds to the exposure to a steady-state concentration of 1 μM for only 7 min.

On the other hand, SIN-1 promptly decomposes in aqueous medium generating $O_2^{\bullet -}$ and $^{\bullet}NO$ at comparable rates, simulating peroxynitrite formation *in vivo*. Thus, in contrast with preformed $ONOO^-$, the observed SIN-1-induced LDL lipid oxidation may be due to a longer exposure time of LDL to the oxidant. Moreover, in SIN-1-induced LDL oxidation we cannot exclude the possibility of one of the reactive species may be produced in excess relative to the other interfering direct or indirectly on the particle lipid oxidation.

Protein Oxidation

Changes in the Net Surface Charge

It is well known that besides lipid oxidation, the oxidative modification of LDL includes potentially other compositional and structural changes. Actually, during such degradative process, several modifications of the apoprotein may occur contributing to the conversion of native LDL into a form that is taken up by the macrophage scavenger receptor. Therefore, the effects of peroxynitrite, either preformed or formed *in situ* by SIN-1, on chemical modifications of LDL apoprotein were evaluated in terms of changes in the net surface charge, oxidation of tryptophan and thiol groups and formation of carbonyl groups. Such effects on the apoprotein fraction are manifested in a very rapid way and are similar for both preformed $ONOO^-$ and formed *in situ* by SIN-1, in contrast with that observed in lipid medium of LDL, as shown above. Thus, we present only the results obtained with preformed $ONOO^-$, in same experimental conditions referred to above, i.e. in which LDL lipids are not significantly oxidized.

When LDL particles were incubated with increasing concentrations of $ONOO^-$ (250–1000 μM), a relative rapid increase in the electrophoretic mobility (R.E.M.) in agarose gel is observed. This increase, owing to an increment in the net negative charge of the lipoprotein particle, depends on the incubation time, particularly during the first 30 min (data not shown), and also on the $ONOO^-$ concentration (Fig. 5A).

It has been reported that the increase in the net negative charge may be due to a decrease in positively charged residues or to the reaction between aldehydes, formed during lipid oxidation, and lysine residues.[37] However, in our assay conditions, derivatization of lysine residues by lipoperoxidation end products is improbable because we did not detect significant LDL lipid oxidation. So, $ONOO^-$ likely attacks directly apoprotein residues involved in the particle net

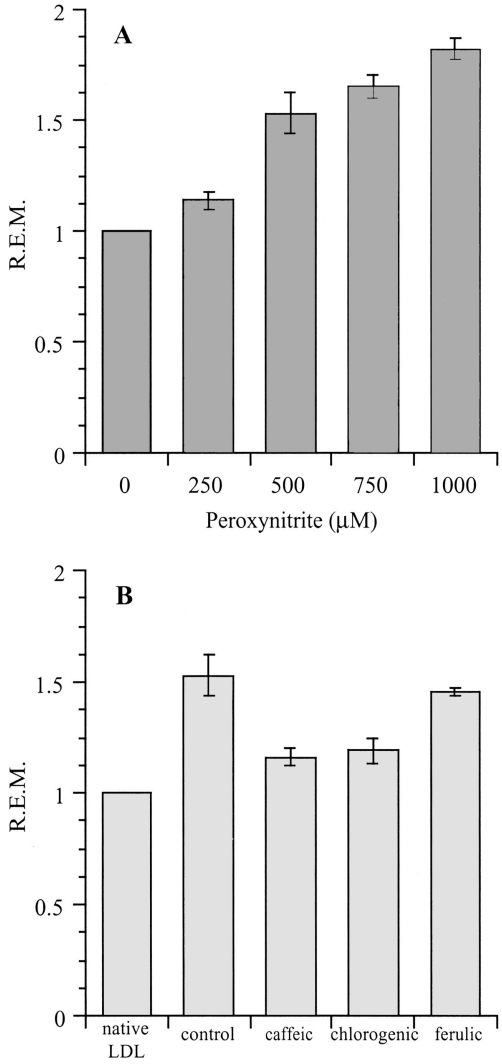

FIGURE 5 (A) Relative electrophoretic mobility (R.E.M.) of LDL particles for different concentrations of preformed peroxynitrite. LDL (360 μg protein/ml), in phosphate buffer pH 7.4, were incubated at 37°C with increasing concentrations of peroxynitrite (250–1000 μM, total concentration) and, after 1 h, the relative electrophoretic mobility of the particles was measured, as indicated in "Materials and Methods" section. (B) Inhibitory effects of phenolic acids on the relative electrophoretic mobility of LDL particles challenged with peroxynitrite. LDL were incubated with 500 μM preformed peroxynitrite in the absence (control) and presence of 10 μM of the indicated phenolic acids, in the conditions referred to (A). The R.E.M. of LDL without peroxynitrite treatment (native LDL) is also indicated.

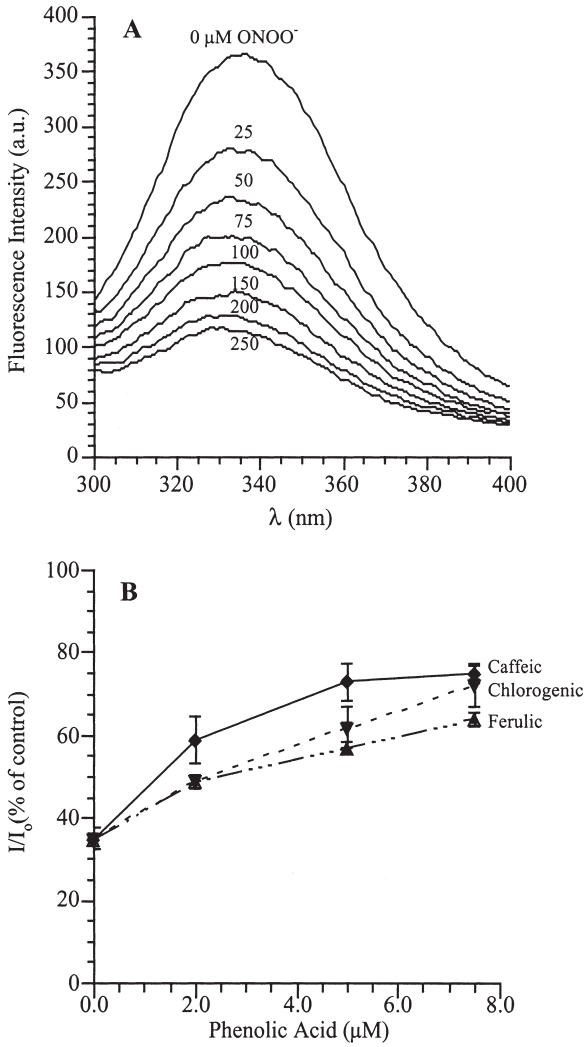

FIGURE 6 (A) Tryptophan fluorescence spectra of LDL (25 μg protein in 2 ml of phosphate buffer) in the presence of increasing concentrations of preformed peroxynitrite after 5 min of incubation. Numbers above or under the lines indicate the final concentration of peroxynitrite. (B) Protection provided by different concentrations of phenolic acids on the fluorescence decrease in LDL induced by 100 μM peroxynitrite. LDL were pre-incubated with phenolic acids and further treated with peroxynitrite in the conditions referred to (A). The results are expressed as referred to in "Materials and Methods" section (I/I_0) and in terms of percentage of native LDL (control).

charge, decreasing the positively charged amino acids, namely lysine, arginine and histidine. The phenolic acids under study decrease such modifications, as reflected in protection against changes in the particle net surface charge, as shown in Fig. 5B. Here, it is evident that caffeic and chlorogenic acids are much more effective than ferulic acid in protecting protein from $ONOO^-$ attack, as assessed by the decrease in the electrophoretic mobility, in agreement with their reported efficiencies as antioxidants against LDL oxidation promoted by other oxidants.[38,39]

Oxidation of Tryptophan

The direct attack of $ONOO^-$ on apoprotein amino acids residues was evidenced by the decrease in the intrinsic tryptophan amount. In fact, the apo B contains 37 tryptophan residues, which are the only amino acids easily monitored continuously due to its intrinsic fluorescence. Thus, the loss of tryptophan in LDL promoted by $ONOO^-$ was followed by the decrease in its typical fluorescence intensity. Actually, the fluorescence of tryptophan is dependent on the integrity of the indole ring and so, disruption of this ring by oxidation or N-nitration may be responsible for the loss of its fluorescence.[40] As shown in Fig. 6A, LDL incubated with increasing

concentrations of $ONOO^-$ present a significant decrease in the characteristic tryptophan fluorescence, in a concentration dependent way, and rapidly after peroxynitrite addition. When LDL (25 μg) were pre-incubated with different concentrations of caffeic, chlorogenic and ferulic acids and challenged with 100 μM $ONOO^-$, some tryptophan residues are preserved. In the absence of phenolic acids, 100 μM $ONOO^-$ induces a loss of 65% in LDL tryptophan, but the pre-incubation of LDL with 5 μM caffeic acid, as an example, leads to a reduction in that loss to only 27% (Fig. 6B). In these assay conditions, although all the phenolic acids under study protect tryptophan residues, the caffeic acid is here, again, the most efficient. So, these data provide evidence that tryptophan destruction, like α-tocopherol consumption, is an early event in $ONOO^-$ induced-LDL oxidation and that those phenolic acids afford protection against such damage.

Oxidation of Sulfhydryl Groups

Another early event during the radical-mediated oxidation of proteins is the conversion of SH-groups into disulfides and other oxidized species.[41] Peroxynitrite has also been reported to oxidize protein and nonprotein sulfhydryl groups.[42] In fact, the reaction of LDL with preformed peroxynitrite resulted in a decrease of sulfhydryl groups of apo B, as evaluated by the decrease in the fluorescence intensity of monobromobimane (mBrB), a fluorescent label for protein thiol groups. The use of bimane derivatives as fluorescent labeling agents for cellular thiols was first developed by Kosower *et al.*[24] We used one of these derivatives, mBrB, for probing the thiol status of LDL, in the absence and presence of different concentrations of $ONOO^-$. According to our knowledge, this was the first time that mBrB was used as a label of LDL apoprotein thiols, so the usefulness of mBrB was previously tested, and the experimental conditions were ascertained, namely incubation time, pH and thiol content sensitivity (data not shown).

Figure 7 shows the decrease in the fluorescence intensity of mBrB incorporated in LDL treated with 200 μM $ONOO^-$ (final concentration) relative to native LDL and the protection by either 5 μM caffeic or chlorogenic acids. Whereas peroxynitrite, without phenolic acids, induces a rapid decrease in the apo B thiol content of about 50%, the pre-incubation of LDL with these phenols efficiently protect the sulfhydryl residues against that oxidant, in a similar extension for the same concentrations. As evidenced in the inset of Fig. 7, an inverse relationship between the apoprotein thiol concentration, as measured by the probe fluorescence intensity, and the peroxynitrite concentration added to LDL was always observed.

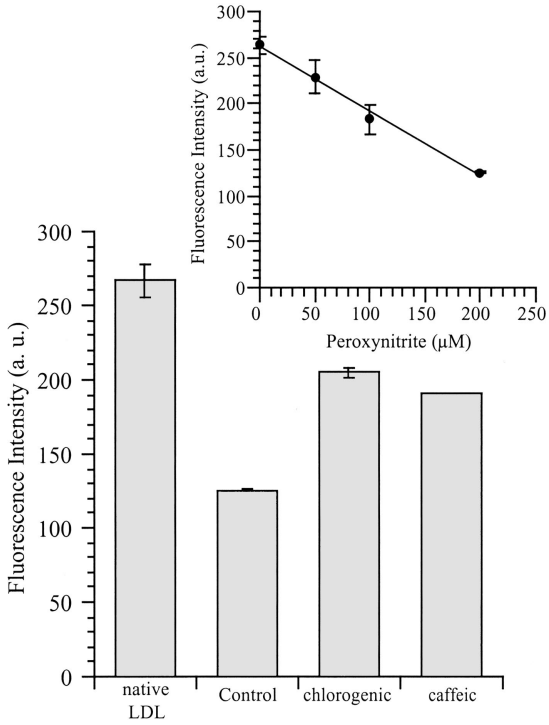

FIGURE 7 Effect of peroxynitrite on the content of LDL sulfhydryl groups, as measured by the fluorescence intensity of incorporated monobromobimane, and protection by phenolic acids. LDL (100 μg protein/ml) were incubated with 200 μM $ONOO^-$ for 5 min, at 37°C, in the absence (control) or presence of 5 μM chlorogenic or caffeic acids. After incubation with 50 μM mBrB for 1 h, the fluorescence intensity was measured. Peroxynitrite significantly decreases the apoprotein sulfhydryl content (about 50%) and both acids strongly afford protection against thiol oxidation. Peroxynitrite oxidizes the apoprotein sulfhydryl groups in a concentration-dependent way (inset).

Thus, these results point to apo B as a very sensitive target for $ONOO^-$ attack. In fact, the reaction between $ONOO^-$ and thiol groups has been shown to be very fast,[1] resulting in their oxidation with the potential formation of the corresponding thiyl free radicals, that may have several biological consequences, namely protein aggregation through the formation of inter and/or intramolecular disulphide bonds.[43]

Formation of Carbonyl Groups

Another typical oxidative modification of proteins is the introduction of carbonyl groups in some residues of amino acids.[25] Thus, in this study, we also examined the ability of peroxynitrite to form carbonyl groups in LDL, as a key feature of the apoprotein oxidative damage, as well as the protection afforded by the phenolic acids, caffeic, chlorogenic and ferulic. The results obtained and shown in Fig. 8 indicate, on one hand, that peroxynitrite efficiently increases the formation of carbonyl groups in LDL in a concentration-dependent way (Fig. 8A) and, on the other hand,

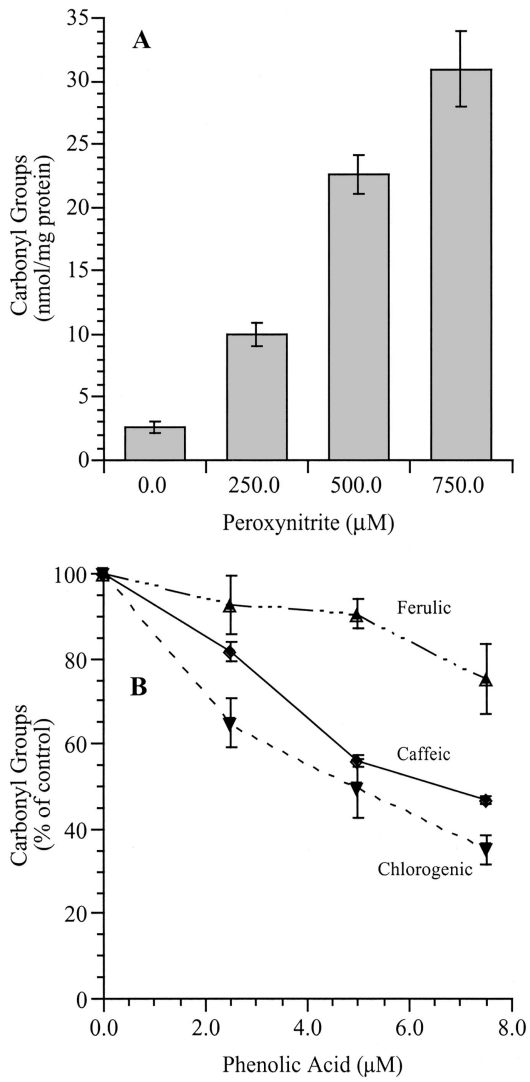

FIGURE 8 (A) DNPH-reactive carbonyl formation in LDL following treatment with several concentrations of preformed peroxynitrite. LDL (750 μg protein/ml) were incubated for 1 h, at 37°C, in phosphate buffer with peroxynitrite and the carbonyl groups formation were evaluated by the reaction with 2,4-dinitrophenylhydrazine (DNPH). (B) Phenolic acids particularly caffeic and chlorogenic acids, added 3 min before peroxynitrite (500 μM) strongly reduce the carbonyl groups formation, in a concentration-dependent way. The results are expressed in percentage of LDL control, obtained in similar experimental conditions, without any phenolic acid.

that the phenolic acids inhibit such formation (Fig. 8B). The efficiencies of the catechols chlorogenic and caffeic acids are much higher than that of ferulic acid, according to the results presented above and to their antioxidant activities.[26,38,39]

The potential formation of carbonyl groups mediated by either transition metal ions or aldehydic products from lipid oxidation is not probable. In fact, DTPA in the medium prevents the presence of free metal ions and in our assay conditions we did not detect significant lipid peroxidation. Thus, the peroxynitrite-mediated carbonyl groups formation in LDL putatively results from the direct interaction of this oxidant, or other oxidation species formed by its decomposition, with apo B amino acids.

Interaction of Phenolic Acids with Peroxynitrite

As pointed out above, one purpose of this study was to evaluate the ability of the cinnamic acid derivatives, caffeic, chlorogenic and ferulic acids, to inhibit peroxynitrite-mediated LDL oxidation. In all the performed assays, caffeic and chlorogenic acids, with an *o*-diphenol structure, were more potent inhibitors than the monophenolic derivative ferulic acid, in the concentrations range used. A similar pattern of protective efficiencies has also been observed against ferrylmyoglobin-dependent LDL oxidation.[39]

In order to understand the mechanisms underlying the referred protective effects, the chemical interactions between phenolic acids and peroxynitrite were studied, in our assay conditions, by spectrophotometric analysis.

When phenolic acids are exposed to preformed peroxynitrite, in phosphate buffer, changes in their characteristics spectra are observed (Fig. 9). In fact, the typical peaks associated with the reduced structure of the acids are rapidly abolished by this oxidant in a concentration-dependent manner (data not shown). To assess the nature of the products formed, spectral changes were also recorded in the presence of either a well-known oxidizing system (ferrylmyoglobin, resulting from the interaction between H_2O_2 and metmyoglobin) or a nitrating system (sodium nitrite, at pH 4.0). Peroxynitrite, in a similar way to ferrylmyoglobin, caused spectral changes in the UV region of caffeic and chlorogenic acids inducing essentially a decline in the major hydroxycinnamate peaks. Moreover, these catechol derivatives do not show any specific change in the visible region, under the action of peroxynitrite, in contrast with ferulic acid (Fig. 9C, line 2) that exhibits an increase in absorbance between 400 and 450 nm, suggesting that nitration of the aromatic ring had occurred. In fact, the spectrum of the nitrated product, resulting from the interaction of phenolic acids with sodium nitrate, shows a new broad peak in the visible region (Fig. 9, line 4). Thus, these results point out that in our assay conditions, in the concentrations range used, peroxynitrite oxidizes, but not nitrate significantly, caffeic and chlorogenic acids, and preferentially nitrates ferulic acid, which are consistent with those obtained previously by Grace *et al.*[27] and Pannala *et al.*[44]

CONCLUSION

As frequently reported, peroxynitrite is rapidly formed *in vivo* from NO and $O_2^{\bullet -}$. It is a short-lived

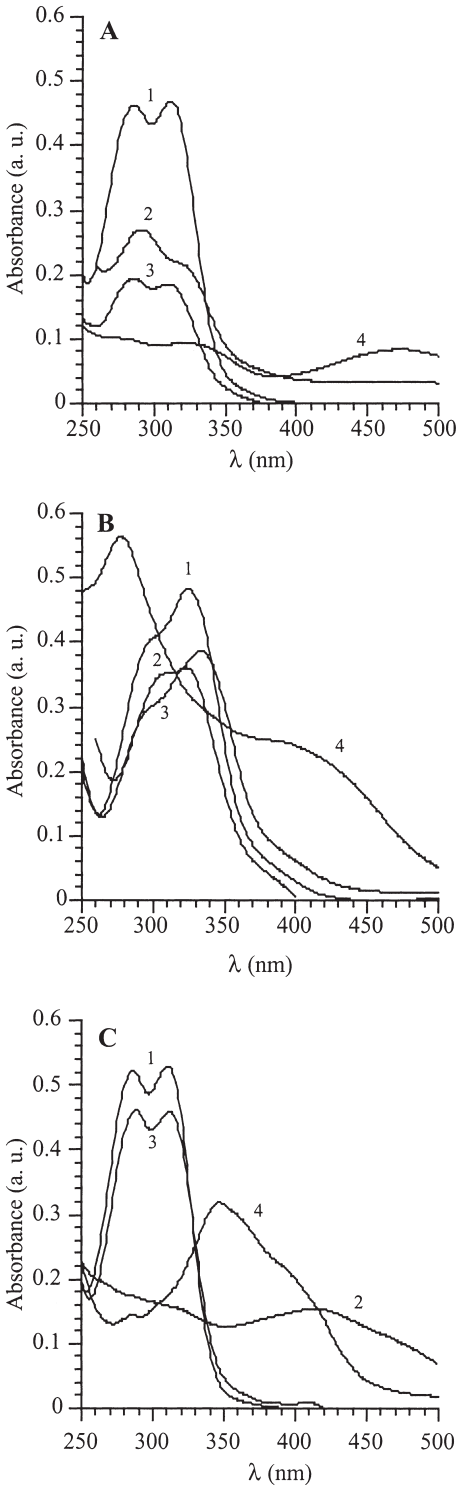

FIGURE 9 Spectra changes of caffeic (A), chlorogenic (B) and ferulic (C) acids caused by peroxynitrite, in comparison with those under the action of ferrylmyoglobin (reference oxidant) and sodium nitrite (nitrating agent). Spectra of phenolic acids (25 μM), in 20 mM phosphate buffer containing 110 mM NaCl, 100 μM DTPA pH 7.4, were recorded in the absence (line 1) or presence of either 50 μM peroxynitrite (line 2) or 10 μM metmyoglobin/15 μM hydrogen peroxide (line 3) or 10 mM sodium nitrite in 50 mM acetate buffer pH 4.0 (line 4).

species and far more reactive than its precursors. Owing to its strong oxidizing character, several biomolecules are potential targets for $ONOO^-$, but *in vivo*, although the multiplicity of targets for this oxidant, only a few are oxidable, according to their reaction rates.[28]

Actually, our results with LDL clearly demonstrate that preformed peroxynitrite reacts much more efficiently with proteins than with lipids. In fact, in contrast with the rapid LDL protein modifications, in terms of all the evaluated parameters, the lipid moiety is not significantly affected, apart from α-tocopherol that is promptly consumed under our assay conditions. The rapidity of oxidation of this main intrinsic antioxidant of LDL may have remarkable consequences, considering that the lipoproteins may become more susceptible to oxidation and that oxidatively modified human LDL plays a crucial role in atherosclerosis.

Nevertheless, the other lipids are resistant to oxidation independently of the localization on the particle, either in the outer layer or in the core. Although peroxynitrite initially localizes in the aqueous phase, the structure of LDL facilitates the oxidation in the core.[32] Moreover, peroxynitrite presents a high permeability coefficient, close to that reported for water, providing an easy diffusibility through biological compartments and interaction with biomolecules located far from its site of production.[45,46] It is the case of carotenoids, located in the hydrophobic core of LDL, that can interact with peroxynitrite, as already demonstrated,[30,47] suggesting that peroxynitrite is capable of penetrating into LDL. However, in our assay conditions, the polyunsaturated fatty acids degradation promoted by $ONOO^-$ is not easy as we could expect, as determined by the formation of conjugated dienes, TBARS and fatty acyl chain analysis.

In contrast, the exposure of LDL to peroxynitrite results in immediate and preferential attack to amino acids residues of apolipoprotein, as assessed by the several assays carried out in the present study. Similar results have been reported using another powerful oxidant, the hypochlorite (OCl^-), produced *in vivo* by a myeloperoxidase-catalyzed reaction, between hydrogen peroxide and chloride anions.[48] Hypochlorite also reacts preferentially with the apoprotein, oxidizing the amino acids residues, similarly to peroxynitrite. Curiously, both hypochlorite and peroxynitrite are strong oxidant agents but they are not free radicals.

On the other hand, the hydroxycinnamic derivatives, caffeic, chlorogenic and ferulic acids, micronutrients present in diets rich in fruit and vegetables, afford protection against the oxidant effect of peroxynitrite on LDL and the order of their efficiencies was always caffeic ≥ chlorogenic >

ferulic, according to the results previously reported with other oxidation promoters.[26,39]

In conclusion, our data strongly suggest that the apoprotein is the primary target of peroxynitrite-induced damage to human LDL, being the extension of oxidation not determined by the localization of the inducer but preferentially by the reactivity between peroxynitrite and the biomolecules. Moreover, the high antioxidant capacity of caffeic acid and chlorogenic acids against peroxynitrite-mediated LDL oxidation confirm once more the high potential of these natural compounds in protecting LDL from oxidative stress induced by physiological oxidants.

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References

[1] Koppenol, W.H. (1998) "The basic chemistry of nitrogen monoxide and peroxynitrite", *Free Radic. Biol. Med.* **25**, 385–391.
[2] Squadrito, G.L. and Pryor, W.A. (1998) "Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite and carbon dioxide", *Free Radic. Biol. Med.* **25**, 392–403.
[3] Hogg, N., Darley-Usmar, V.M., Wilson, M.T. and Moncada, S. (1992) "Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide", *Biochem. J.* **281**, 419–424.
[4] Demiryürek, A.T., Çakici, I. and Kanzik, I. (1998) "Peroxynitrite: a putative cytotoxin", *Pharmacol. Toxicol.* **82**, 113–117.
[5] Groves, J.T. (1999) "Peroxynitrite: reactive, invasive and enigmatic", *Curr. Opin. Chem. Biol.* **3**, 226–235.
[6] Hogg, N., Darley-Usmar, V.M., Graham, A. and Moncada, S. (1993) "Peroxynitrite and atherosclerosis", *Biochem. Soc. Trans.* **21**, 358–362.
[7] Steinberg, D., Pathasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) "Modifications of low-density lipoprotein that increase its atherogenicity", *N. Engl. J. Med.* **320**, 915–924.
[8] Leeuwenburg, C., Hardy, M.M., Hazen, S.L., Wagner, P., Oh-ishi, S., Steinbrecher, U.P. and Heinecke, J.W. (1997) "Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima", *J. Biol. Chem.* **272**, 1433–1436.
[9] Hogg, N., Darley-Usmar, V.M., Wilson, M.T. and Moncada, S. (1993) "The oxidation of α-tocopherol in human low-density lipoproteins by the simultaneous generation of superoxide and nitric oxide", *FEBS Lett.* **326**, 199–203.
[10] Puhl, H., Waeg, G. and Esterbauer, H. (1994) "Methods to determine oxidation of low-density lipoproteins", *Methods Enzymol.* **233**, 425–441.
[11] Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) "Oxidative chemistry of peroxynitrite", *Methods Enzymol.* **233**, 229–240.
[12] Vieira, O.V., Laranjinha, J.A.N., Madeira, V.M.C. and Almeida, L.M. (1996) "Rapid isolation of low density lipoproteins in a concentrated fraction free from water soluble plasma antioxidants", *J. Lipid Res.* **37**, 2715–2721.
[13] Lowry, O.H., Rosebrough, A.L., Farr, M.J. and Randall, R.S. (1951) "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.
[14] Sies, H., Sharov, V.S., Klotz, L.O. and Briviba, K. (1997) "Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase", *J. Biol. Chem.* **272**, 27812–27817.
[15] Sies, H. and Arteel, G.E. (2000) "Interaction of peroxynitrite with selenoproteins and glutathione peroxidase mimics", *Free Radic. Biol. Med.* **28**, 1451–1455.
[16] Corongiu, F.P., Banni, S. and Dessi, M.A. (1989) "Conjugated dienes detected in tissue lipid extracts by second derivative spectrophotometry", *Free Radic. Biol. Med.* **7**, 183–186.
[17] Laranjinha, J.A.N., Almeida, L.M. and Madeira, V.M.C. (1992) "Lipid peroxidation and its inhibition in low density lipoproteins: quenching of *cis*-parinaric acid fluorescence", *Arch. Biochem. Biophys.* **297**, 147–154.
[18] Vieira, O., Laranjinha, J., Madeira, V. and Almeida, L. (1998) "Cholesteryl ester hydroperoxide formation in myoglobin-catalyzed low density lipoprotein oxidation. Concerted antioxidant activity of caffeic and *p*-coumaric acids with ascorbate", *Biochem. Pharmacol.* **55**, 333–340.
[19] Dinis, T.C.P., Almeida, L.M. and Madeira, V.M.C. (1993) "Lipid peroxidation in sarcoplasmic reticulum membranes: effect on functional and biophysical properties", *Arch. Biochem. Biophys.* **301**, 256–264.
[20] Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) "Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids", *Proc. Natl Acad. Sci. USA* **81**, 3883–3887.
[21] Parthasarathy, S., Steinbrecher, U.P., Barnett, J., Witztum, J.L. and Steinberg, D. (1985) "Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein", *Proc. Natl Acad. Sci. USA* **82**, 3000–3004.
[22] Gonçalves, E., Almeida, L.M. and Dinis, T.C.P. (1998) "Antioxidant activity of 5-aminosalicylic acid against peroxidation of phosphatidylcholine liposomes in the presence of α-tocopherol: a synergistic interaction?", *Free Radic. Res.* **29**, 53–66.
[23] Santos, A.E., Laranjinha, J.A. and Almeida, L.M. (1998) "Sulfonated chloroaluminum phthalocyanine incorporates into human plasma lipoproteins: photooxidation of low-density lipoproteins", *Photochem. Photobiol.* **67**, 378–385.
[24] Kosower, N.S., Kosower, E.M., Newton, G.L. and Ranney, A.M. (1979) "Bimane fluorescent labels: labeling of normal human red blood cells under physiological conditions", *Proc. Natl Acad. Sci. USA* **76**, 3382.
[25] Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A., Ahn, B., Shaltiel, S. and Stadtman, E.R. (1990) "Determination of carbonyl content in oxidatively modified proteins", *Methods Enzymol.* **186**, 464–478.
[26] Laranjinha, J., Almeida, L. and Madeira, V. (1995) "Reduction of ferrylmioglobin by dietary phenolic acid derivatives of cinnamic acid", *Free Radic. Biol. Med.* **19**, 329–337.
[27] Grace, S.C., Salgo, M.G. and Pryor, W.A. (1998) "Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA", *FEBS Lett.* **426**, 24–28.
[28] Radi, R. (1996) "Kinetic analysis of reactivity of peroxynitrite with biomolecules", *Methods Enzymol.* **269**, 354–366.
[29] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide", *Arch. Biochem. Biophys.* **288**, 481–487.
[30] Pannala, A.S., Rice-Evans, C., Sampson, J. and Singh, S. (1998) "Interaction of peroxynitrite with carotenoids and tocopherols within low density lipoprotein", *FEBS Lett.* **423**, 297–301.
[31] Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) "The role of lipid peroxidation and antioxidants in oxidative modification of LDL", *Free Radic. Biol. Med.* **13**, 341–390.
[32] Noguchi, N., Gotoh, N. and Niki, E. (1993) "Dynamics of the oxidation of low density lipoprotein induced by free radicals", *Biochim. Biophys. Acta* **1168**, 348–357.
[33] Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V. and Moncada, S. (1993) "Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor", *FEBS Lett.* **330**, 181–185.
[34] Vatassery, G.T., Ed Smith, W. and Quach, H.T. (1998) "α-Tocopherol in rat brain subcellular fractions is oxidized

rapidly during incubations with low concentrations of peroxynitrite", *J. Nutr.* **128**, 152–157.
[35] Hogg, N., Joseph, J. and Kalyanaraman, B. (1994) "The oxidation of α-tocopherol and trolox by peroxynitrite", *Arch. Biochem. Biophys.* **314**, 153–158.
[36] Dousset, N., Ferretti, G., Taus, M., Valdiguiè, P. and Curatola, G. (1994) "Fluorescence analysis of lipoprotein peroxidation", *Methods Enzymol.* **233**, 459–469.
[37] Noguchi, N. and Niki, E. (1994) "Apolipoprotein B protein oxidation in low-density lipoproteins", *Methods Enzymol.* **233**, 490–494.
[38] Laranjinha, J.A.N., Almeida, L.M. and Madeira, V.M.C. (1994) "Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low-density lipoprotein peroxidation", *Biochem. Pharmacol.* **48**(1994), 487–494.
[39] Laranjinha, J., Vieira, O., Almeida, L. and Madeira, V. (1996) "Inhibition of metmyoglobin/H2O2-dependent low density lipoprotein lipid peroxidation by naturally occurring phenolic acids", *Biochem. Pharmacol.* **51**, 395–402.
[40] Ischiropoulos, H. and Al-Mehdi, A.B. (1995) "Peroxynitrite-mediated oxidative protein modifications", *FEBS Lett.* **364**, 279–282.
[41] Dean, R.T., Fu, S., Stocker, R. and Davies, M.J. (1997) "Biochemistry and pathology of radical-mediated protein oxidation", *Biochem. J.* **324**, 1–18.
[42] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) "Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide", *J. Biol. Chem.* **266**, 4244–4250.
[43] Gatti, R.M., Radi, R. and Augusto, O. (1994) "Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical", *FEBS Lett.* **348**, 287–290.
[44] Pannala, A., Razaq, R., Halliwell, B., Singh, S. and Rice-Evans, C.A. (1998) "Inhibition of peroxynitrite-dependent tyrosine nitration by hydroxycinnamates: nitration or electron donation?", *Free Radic. Biol. Med.* **24**, 594–606.
[45] Marla, S.S., Lee, J. and Groves, J.T. (1997) "Peroxynitrite rapidly permeates phospholipid membranes", *Proc. Natl Acad. Sci. USA* **94**, 14243–14248.
[46] Denicola, A., Souza, J.M. and Radi, R. (1998) "Diffusion of peroxynitrite across erythrocyte membranes", *Proc. Natl Acad. Sci. USA* **95**, 3566–3571.
[47] Panasenko, O.M., Sharov, V.S., Briviba, K. and Sies, H. (2000) "Interaction of peroxynitrite with carotenoids in human low density lipoproteins", *Arch. Biochem. Biophys.* **373**, 302–305.
[48] Hazell, L.J., van den Berg, J.J.M. and Stocker, R. (1994) "Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation", *Biochem. J.* **302**, 297–304.