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Inhibition of Metmyoglobin/H_2O_2-Dependent Low Density Lipoprotein Lipid Peroxidation by Naturally Occurring Phenolic Acids

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ABSTRACT. The ferrylmyoglobin ⇔ metmyoglobin redox transitions promoted by hydrogen peroxide and dietary phenolic acids and their potential role in the oxidation of LDL were studied. The use of parinaric acid incorporated in LDL as a probe for radicals (detected by fluorescence quenching of the probe) revealed an oxidative stress inside LDL shortly (<1 min) after addition of hydrogen peroxide to metmyoglobin in the aqueous phase outside the particle, reflecting an efficient access of the oxidant to LDL lipids. However, the propagation step of peroxidation only occurs after a lag phase, as detected by the kinetics of oxygen consumption. Triton X-100 decreases but does not suppress the lag phase of oxidation. Addition of metmyoglobin (without peroxide) to LDL was not followed by significant oxidation during the time of the experiment, unless Triton X-100 was present in the medium. When dietary phenolic acids were present in the medium before peroxide addition, an inhibition of parinaric acid fluorescence quenching and oxygen consumption was recorded as a function of concentration and substitution pattern on the phenol ring of the phenolic acids. This was associated with a conversion of ferrylmyoglobin to metmyoglobin. The results indicate that the naturally occurring phenolic acids prevent ferrylmyoglobin-dependent LDL oxidation in a way strongly dependent on the substitution pattern on the phenol ring. Among the phenolic compounds studied, the *o*-dihydroxy derivatives of cinnamic and benzoic acids (caffeic, chlorogenic, and protocatechuic acids), in a molar ratio of 1 to metmyoglobin, efficiently blocked LDL oxidation initiated by ferrylmyoglobin. Replacement of one OH group from catecholic structure with an H (*p*-coumaric acid) or methoxy group (ferulic acid) decreased the antioxidant activity. Also, the catechol structure fused in heterocyclic rings with adjacent carbonyl groups (ellagic acid) resulted in decreased antioxidant activity. These observations correlate with the efficiency of phenolic acids to reduce ferrylmyoglobin to metmyoglobin. Therefore, the protection of LDL against oxidation is assigned to the reduction of the oxoferryl moiety of the hemoprotein to the ferric form. Additionally, it is suggested that an access constraint of oxidants plays a minor role in the ferrylmyoglobin-induced oxidation against LDL. BIOCHEM PHARMACOL 51;4:395–402, 1996.

KEY WORDS. low-density lipoprotein; lipid peroxidation; antioxidants; ferrylmyoglobin; metmyoglobin; phenolic acids; naturally occurring compounds

The reaction of Mb^{III}[‖] with hydrogen peroxide yields Mb^{IV}, an oxidation product with heme iron in the form of a stable oxoferryl complex (Fe^{IV}=O) and a transient radical in the globin moiety of the hemoprotein [1–6]. It has been shown that ferrylmyoglobin initiates lipid peroxidation in several lipid systems [7–9], including lipoproteins [10], and evidence for its occurrence *in vivo* has been described [11, 12]. In view of these findings, the potential involvement of this hemoprotein, owing to its high oxidation state, in the pathophysiology of ischemia-reperfusion injury has been proposed [13]. Assuming that myoglobin may be released from disrupted cells in atherosclerotic lesions, a significant contribution of the hemeprotein to the development of atherosclerosis has also been proposed [14].

Several compounds have been shown to efficiently reduce the oxoferryl moiety in ferrylmyoglobin to ferric iron in metmyoglobin, namely, thiols [15–17], ascorbate [18–20], trolox [21, 22], and desferrioxamine [19]. It has also been observed that naturally occurring phenolic acids present in human diet, particularly the derivatives of cinnamic acid (Fig. 1), reduce the oxoferryl moiety to the ferric form with an efficiency highly dependent on the chemical nature of substituents on the phenolic ring [39]. Consequently, it was suggested that these phenolic derivatives may counteract deleterious oxida-

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‖ *Abbreviations:* LDL, low density lipoprotein; Mb^{III}, metmyoglobin; Mb^{IV}, ferrylmyoglobin; PnA, (parinaric acid) 9,11,13,15-octadecatetraenoic acid; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Triton, octylphenoxypolyethoxyethanol.

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tive processes mediated by metmyoglobin/H_2O_2 [39]. However, a direct causal relationship between ferrylmyoglobin reduction by the phenols and protection against oxidation cannot be easily drawn. Indeed, upon electron transfer to the hemeprotein, the phenols yield, in a first step, phenoxyl radicals that may cause deleterious oxidations in spite of the stabilization by resonance. In the case of LDL, the lipids, protein, and endogenous antioxidants, namely α-tocopherol, are possible targets for the radicals. It has recently been shown [40] that simple phenoxyl radicals are highly reactive towards α-tocopherol, oxidizing it. Therefore, clarification of the role of phenolic acids in metmyoglobin/H_2O_2-dependent oxidations is required.

The study of chemical reactivity of naturally occurring phenolic acids has acquired major physiological significance, since they have been shown to exhibit anti-inflammatory [41, 42], antimutagenic, and anticarcinogenic [43, 44] properties. Moreover, evidence has been reported for *in vivo* antioxidant activity [45–48] and, in the case of caffeic acid, for its presence in human plasma [49].

In view of the crucial role of oxidized LDL in the development of atherosclerosis [23] and the suggested role of ferrylmyoglobin as a contributing factor in this process [14], we studied the role of the pseudo-peroxidatic activity of myoglobin, encompassing reduction of H_2O_2 and oxidation of phenolic compounds, in LDL oxidation. It is shown that the dietary phenolic compounds block lipid peroxidation of LDL as a function of concentration and chemical structure. Oxidizing species inside LDL particles were sensitively probed by the fluorescence quenching of parinaric acid (9,11,13,15-octadecatetraenoic acid, PnA) (reflecting the efficiency of radical attack) and the propagation reaction of lipid peroxidation was measured by the consumption of O_2. The use of parinaric acid incorporated in LDL as a probe for radicals is convenient to study the accessibility of the oxidant to LDL lipids, since it is readily oxidized by oxidants coming from outside the particle without significant lag phases.

MATERIALS AND METHODS

Reagents

Horse heart metmyoglobin and phenolic acids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydrogen peroxide was purchased from Merck (Darmstadt, Germany) as a 30% solution. Metmyoglobin was dialyzed against phosphate buffer (20 mM phosphate, 110 mM NaCl), pH 7.4, containing 50 μM EDTA (referred to as phosphate buffer) and previously passed through Cuelex 100.

LDL Isolation

LDL particles were isolated from fresh human plasma by density gradient ultracentrifugation [modification of 24] in a Beckman L-80 ultracentrifuge (Beckman Inst. Inc., Palo Alto, CA, U.S.A.) equipped with a Beckman 70.1 Ti fixed angle rotor (12 × 10 mL) for 3.25 hr at 65000 rpm at 15°C. LDL fraction was then concentrated and simultaneously dialyzed for 45 min by ultrafiltration under nitrogen. Protein was determined using the Lowry method [25], and purity of the LDL preparation was checked by SDS-polyacrylamide gradient (3–20%) gel electrophoresis [26].

Myoglobin Redox Transitions

Redox transitions of myoglobin were monitored with a Perkin Elmer lambda 6 spectrophotometer thermostatted at 37°C in a standard mixture consisting of 2 mL phosphate buffer, 10 μM metmyoglobin, 50 μM EDTA, and 15 μM hydrogen peroxide; concentrations of LDL and phenolic acids are indicated in the legends to figures. At the concentrations used, phenolic acids had no measurable effect on the metmyoglobin spectrum.

Lipid Peroxidation Induction and Assay

Parinaric acid incorporation and oxidation were performed under conditions ascertaining a linear relation between fluorescence intensity and probe concentration, as previously described [27]. Fluorescence measurements were monitored with a Perkin-Elmer LS 50B luminescence spectrometer in 2 mL phosphate buffer containing 2 nmol PnA, 50 μM EDTA, and 60 μg LDL protein, at 37°C with gentle stirring. The oxidation reaction was initiated by addition of metmyoglobin (1 μM) followed 2 min later by hydrogen peroxide (1.5 μM). Phenolic acids were added 1 min before the peroxide in a concentration range between 0.1 and 5 μM, as indicated in the figure legends. To minimize PnA fluorescence quenching due to the presence of hemoprotein, excitation and emission wavelengths were 310 and 425 nm, respectively. The effect of myoglobulin redox transitions on PnA signal was checked.

Oxygen consumption was measured with a Clark-type oxygen electrode in 1 mL phosphate buffer at 37°C containing 180 μg LDL protein and 50 μM EDTA. Lipid peroxidation was initiated by metmyoglobin (6 μM) followed 2 min later by hydrogen peroxide (12 μM). Phenolic acids in a concentration range between 1 and 24 μM were added 1 min before the peroxide.

RESULTS

Myoglobin Redox Transition

The oxoferryl complex in ferrylmyoglobin is reduced to metmyoglobin [39] by the phenolic acids used here (Fig. 1), with the efficiency depending on the chemical structure. The catechol derivatives of cinnamic acid, chlorogenic, and caffeic acids were the most efficient reductants.

Figure 2 shows the myoglobin redox transitions promoted by H_2O_2 (1.5-fold the Mb^{III} concentration) and phenolic acids in the absence and presence of LDL. Addition of H_2O_2 to Mb^{III} solution promoted the formation of a species with spectral properties of Mb^{IV} characterized by peaks at 548 and 582 nm (Fig. 2A), as described in the introduction. In the presence of LDL (Fig. 2B), the spectrum of Mb^{IV}, with its characteristic peaks at 548 and 582 nm, was also obtained following peroxide addition to Mb^{III}. These spectral changes were accompanied in the Soret region by a shift of the 408 nm peak of Mb^{III} to the

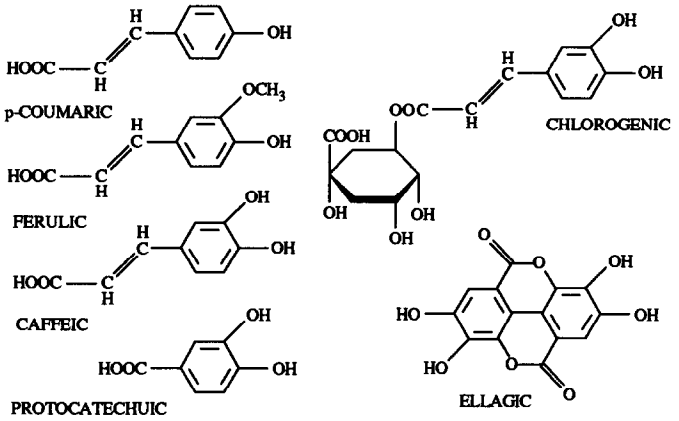

FIG. 1. Chemical structure of phenolic acids.

421 nm characteristic of Mb^{IV} (not shown). If the phenolic acids were present in the medium, a transient modification of the Mb^{III} spectrum was observed, as shown for *p*-coumaric acid (2-fold the Mb^{III} concentration) in Fig. 2C; the extent and time of this modification were shortened with increasing concentrations of the phenolic acids. Figure 2B also shows that the LDL spectrum exhibits a broad absorption band around 450–500 nm as a consequence of endogenous carotenoids [28]. Therefore, the spectrum of Mb^{III} and particularly the 505 nm peak suffered an increase in absorbance intensity when LDL was added to the Mb^{III} solution. The spectrum of Mb^{III} plus LDL was stable (verified over 90 min), but that of Mb^{IV} in the presence of LDL exhibited a decrease in intensity with time, in agreement with previous studies [14] showing destabilisation of the heme chromophore and iron release induced by LDL upon oxidation mediated by Mb^{IV}. Under our conditions, there was a loss of 12% total heme for 1 hr as estimated from the 421 nm peak of Mb^{IV} in the Soret region.

LDL Oxidation

Evidence for ferrylmyoglobin-initiated LDL lipid peroxidation has already been reported [10]. In this study, we probed the initial oxidative reactions inside LDL by the fluorescence quenching of parinaric acid (PnA) incorporated into LDL, in addition to the propagation phase of LDL lipid peroxidation monitored by O_2 consumption. PnA methodology proved very sensitive in detecting reactive oxidant species inside LDL originating either outside (e.g. peroxyl radicals [27]) or inside the particle [29]. Therefore, this probe is very useful to assess the reactive efficiency of antioxidant compounds with radical species, events that underlie the oxidative prevention of biological substrates. PnA incorporated in LDL is rapidly oxidized by ferrylmyoglobin in the absence of phenolic acids, as shown in Fig. 3, indicating an oxidative stress inside LDL ca. 1 min after addition of H_2O_2 to Mb^{III}. Since the quantum yield of PnA fluorescence decreases following slight modifications in the probe chemical structure, it might be assumed that the initial events of peroxidation are monitored. Therefore, PnA oxidation (Fig. 3) lag-phases are short (<1 min) as compared with those of O_2 consumption (Fig. 4). The rate of probe degradation decreased in the presence of phenolic acids as a function of concentration, illustrated for chlorogenic acid in Fig. 3.

Protection of LDL lipids from oxidation followed a similar profile to that observed for the protection of PnA, as exemplified for chlorogenic acid in Fig. 4. The appearance of initial lag phases (ca. 24 min) of LDL oxidation is most likely related to consumption of endogenous LDL antioxidants as reported extensively elsewhere [for review see ref. 28]. During the oxidation reaction, carotenoid bleaching is expected to occur. Therefore, the 505 nm peak of the recovered Mb^{III} spectrum in Fig. 2C (line + H_2O_2 17.5 min) is lower than the original spectrum of Mb^{III} plus LDL (arrow). PnA, a preferential substrate for oxidation in LDL challenged with exogenous oxidant species, is not efficiently protected by endogenous antioxidants and, therefore, no significant lag phases of fluorescence quenching are recorded, as reported previously [27].

The extent of LDL protection from Mb^{III}/H_2O_2-initiated oxidation depends on the type of phenolic acid. Figure 5 shows the effect of all the phenolic compounds on the oxidation of PnA (Fig. 5A) and LDL unsaturated lipids (Fig. 5B). The pattern is similar in both cases. The catechols were the more potent compounds and, among them, the derivatives of cinnamic acid (caffeic and chlorogenic) were the most efficient in blocking Mb^{III}/H_2O_2-initiated LDL oxidation, whilst the monohydroxy derivative of cinnamic acid (*p*-coumaric) was the least efficient, and ferulic and ellagic acids exhibited an intermediate degree of protection. This concurs well with the efficiency in reducing Mb^{IV} to Mb^{III}, since the rates obtained for the reduction of Mb^{IV} to Mb^{III} ranged from 15.6 $\mu M \times min^{-1}$ for caffeic acid to 2.4 $\mu M \times min^{-1}$ for *p*-coumaric acid [39]. As shown in Fig. 5, chlorogenic, caffeic, and protocatechuic acids in molar ratios of 1 relative to Mb^{III} almost completely blocked the oxidation reaction, whereas *p*-coumaric acid was far less efficient.

In complementary experiments, the effect of Triton X-100 on O_2 consumption rates promoted by interacting ferryl and metmyoglobin with LDL was studied. Clearly, Triton X-100 decreases the lag period of LDL resistance to oxidation and increases the rate of peroxidation in the case of Mb^{IV} (Fig. 6); additionally, in its presence, Mb^{III} induced a fast peroxidation reaction. Since it is supposed that Triton X-100 increases the physical access to LDL components, an interaction of metmyoglobin with endogenous LDL peroxides may explain this latter observation. Spectra of met- and ferrylmyoglobin were stable during the time scale of this experiment in the presence of Triton. Therefore, the steep increase in O_2 consumption in the presence of Triton cannot be assigned to putative contaminating peroxides in Triton solution.

DISCUSSION

An oxidative stress inside LDL occurs soon (<1 min) after addition of H_2O_2 to Mb^{III}, as detected by the fluorescence quenching of PnA (Fig. 3) and the reaction propagates (inferred from O_2 consumption) (Fig. 4). Since complete conversion of metmyoglobin to ferrylmyoglobin lasts for more than 5 min (as inferred from the increase in absorbance at 556 nm),

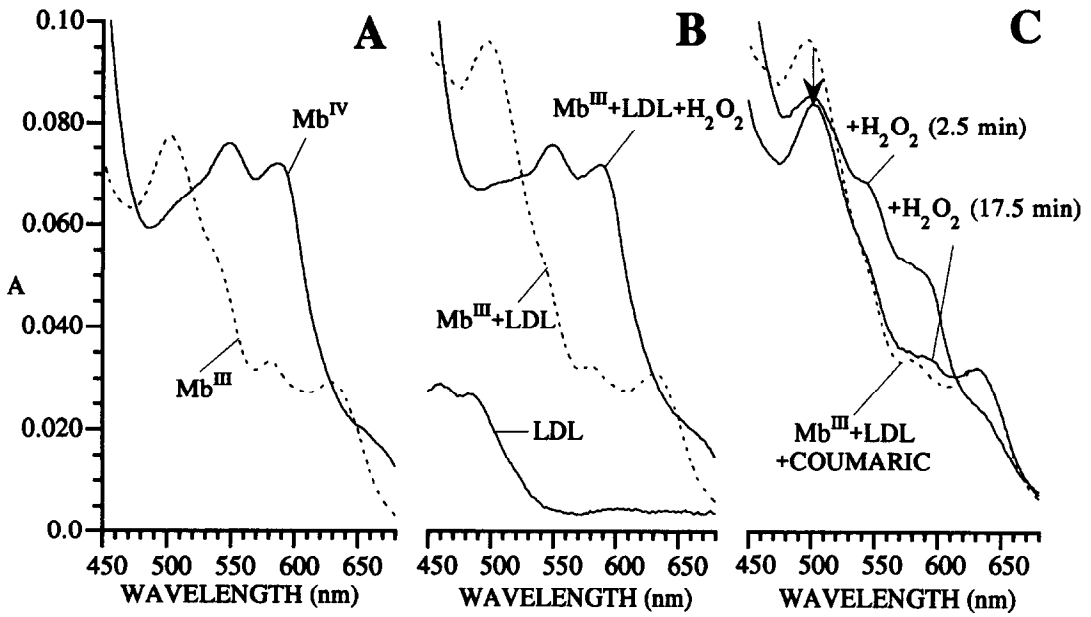

FIG. 2. Metmyoglobin absorption spectral changes induced by H_2O_2 and phenolic acids in the absence and presence of LDL. (A) 2 mL phosphate buffer containing 10 μM metmyoglobin and 50 μM EDTA (Mb^{III}) were supplemented with 15 μM H_2O_2 to form ferrylmyoglobin (Mb^{IV}). Ferrylmyoglobin spectrum was taken 10 min after peroxide addition. (B) As in (A), but 150 μg LDL was present in the metmyoglobin solution before the addition of H_2O_2. The spectrum of 150 μg LDL in 2 mL of phosphate buffer is also shown. (C) As in (B), but 20 μM *p*-coumaric acid was present in solution before the addition of H_2O_2. A transient modification of metmyoglobin spectrum occurred as documented by the spectra taken 2.5 and 17.5 min after peroxide addition. The lower absorption band at 505 nm of recovered Mb^{III} relative to the initial Mb^{III} spectrum (arrow) is ascribed to bleaching of the carotenoid content of LDL.

the ready oxidation of the probe indicates that there are no strong physical constraints impairing the access of the oxidant to LDL components. Similarly, the dietary phenolic acids block both events as a function of concentration and chemical structure. An *o*-dihydroxy substitution (e.g., caffeic, chlorogenic, and protocatechuic acids) affords higher antioxidant capacity. Replacing one OH group with an H atom (*p*-coumaric acid) or methoxy group (ferulic acid) decreased the antioxidant activity. Also, the catechol structure fused in heterocyclic rings with adjacent carbonyls (ellagic acid) resulted in diminished antioxidant activity. This is similar to the observed reactivity towards alkyl peroxyl radicals [37], except that caffeic and chlorogenic were much more efficient in quenching the peroxyl radicals than protocatechuic acid. As discussed previously [37, 39], the results may be interpreted in terms of the electron-donating or electron-withdrawing nature of substituents in the phenol ring; in the first case, the antioxidant activity is enhanced, and in the second it is depressed. The concentrations of phenolic compounds that prevent the fluorescence quenching of PnA and oxygen consumption are similarly related (Fig. 5).

These dietary phenolic acids reduce the oxoferryl moiety of the hemoprotein to the ferric form, and the efficiency of peroxidation inhibition relates to the corresponding reduction efficiency of Mb^{IV} to Mb^{III} [39]. These findings strongly suggest that LDL protection from oxidation by these compounds is assigned to their capacity to reduce the oxoferryl moiety of myoglobin to the ferric form. Accordingly, metmyoglobin, in the absence of peroxides, would be expected to be harmless in the oxidation of LDL. A metmyoglobin and hemoglobin(ferrous iron)-mediated propagation of LDL oxidation without addition of exogenous H_2O_2 and without detectable changes in the optical spectrum of metmyoglobin in the presence of LDL was first reported [14, 30]. Later, it was shown [31] that the oxidation of LDL by metmyoglobin requires endogenous LDL lipid hydroperoxides, and the occurrence of ferrylmyoglobin was evidenced by formation of the sulfheme protein derivative peaking at 620 nm, a "fingerprint" reflecting the presence of ferrylmyoglobin [32]. In our system, the spectrum of Mb^{III} was also stable in the presence of LDL, and we did not achieve any clear evidence for an Mb^{III}-mediated LDL oxidation, since the rates of O_2 consumption of LDL in the presence or absence of Mb^{III} were virtually identical during the time of the experiment. However, it was noted that addition of H_2O_2, after the O_2 consumption of LDL had been verified for 1 hr, in the presence of Mb^{III}, promotes a rate of O_2 consumption identical to the propagation rate shown in Fig. 4 (trace 0 μM), but with a lag phase of only 6 min. When Mb^{III} and H_2O_2 were subsequently added, the lag phases were 24 min, as shown in Fig. 4. This observation could reflect a slow Mb^{III}-mediated oxidation reaction in LDL. Recently [33], it was shown that incubation of metmyoglobin with liposomes containing lipid hydroperoxides induced lipid peroxidation accompanied by O_2 consumption, but absorption spectroscopy failed to detect fer-

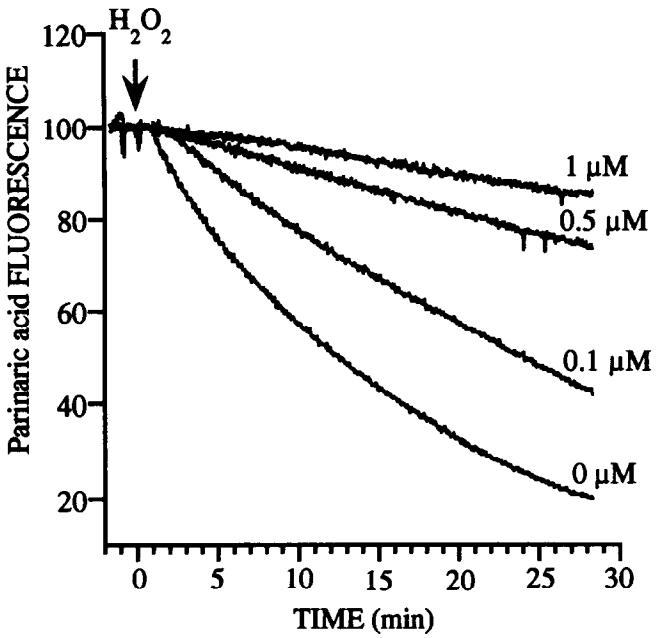

FIG. 3. Effect of chlorogenic acid on metmyoglobin/H_2O_2-induced oxidation of parinaric acid incorporated in LDL. Parinaric acid oxidation rates in LDL particles in the absence and presence of 0.1, 0.5, and 1 μM of chlorogenic acid under conditions described in Materials and Methods. Time 0 represents the normalized fluorescence signal of PnA incorporated in LDL after a slight fluorescence quenching due to metmyoglobin. The reaction was initiated by H_2O_2 at the time indicated by the arrow. Chlorogenic acid was added before H_2O_2. Metmyoglobin or H_2O_2 alone did not exert any significant effect on the time courses of PnA fluorescence decrease during the time of the experiments.

rylmyoglobin formation; it was suggested that H_2O_2 and lipid hydroperoxides oxidize metmyoglobin differently, as indicated by the complexity of molecular products obtained from the reaction of Mb^{III} with lipid hydroperoxides. Hence, it is likely that, depending on the peroxide "tone" of LDL, lipid peroxidation can be unambiguously observed following incubation with Mb^{III} and without detectable changes in the optical spectrum of Mb^{III} [14].

In addition to its efficiency to reduce Mb^{IV}, the concentration of the phenolic compound becomes an important factor in the protection of LDL, since the attacking species is very reactive (Fig. 3 shows that PnA is readily oxidized inside LDL). The results suggest (Fig. 5) that chlorogenic, caffeic, and protocatechuic acids, at a molar ratio of 1 relative to Mb^{III}, maintain Mb^{IV} at low levels, thus minimizing initiation of peroxidation and the subsequent increase in lipid hydroperoxides that could mediate the propagation step of peroxidation by interacting with Mb^{III} arising from Mb^{IV} reduction. With the other compounds, higher ratios are required to achieve similar results (Fig. 5).

From a one-electron transfer reaction between the phenolic acids and ferrylmyoglobin, it may be assumed that in addition to metmyoglobin, a phenoxyl radical will be produced, as previously discussed [39]. Whereas an efficient electron delocalization with consequent radical stability can be expected from the structure of these radicals, harmful effects towards LDL components (either lipids and protein or endogenous antioxidants) remain a possibility. It has recently been demonstrated that the phenoxyl radical derived from phenol is highly reactive towards α-tocopherol [40]. This vitamin is the major antioxidant in LDL and, under mild oxidation conditions, the oxidation of α-tocopherol may cause an α-tocopheroxyl radical-mediated propagation chain of lipid oxidation inside LDL [50]. It is therefore of note that the results suggest no relevant effect of phenolic acid-derived radicals against LDL lipid oxidation.

Several important points remain to be resolved: (a) the relative contribution of the oxoferryl complex and the amino-acid radical at the hemoprotein surface to initiate LDL oxidation and (b) how the oxoferryl complex in the heme crevice of myoglobin gains access to LDL unsaturated lipids to initiate lipid peroxidation. It has been shown recently [34] that the myoglobin-catalysed oxidation of linoleic acid is not mediated by the protein radical, but by reaction (H-abstraction) with the ferryl complex within the heme crevice. On the other hand, evidence for H-abstraction reaction from linoleic and arachidonic acids by globin-peroxyl radical have been provided [6, 35], though controversy regarding the location of the globin radical exists [4, 36]. Our data sustain a significant role for the oxoferryl moiety of Mb^{IV} to initiate LDL lipid peroxidation. As LDL oxidation is catalysed by the oxoferryl moiety, the reduction of the ferryl to the ferric form in the presence of LDL (due to peroxidase-like activity encompassed by $Mb^{IV} \Leftrightarrow Mb^{III}$ transition) would be expected; however, the Soret band

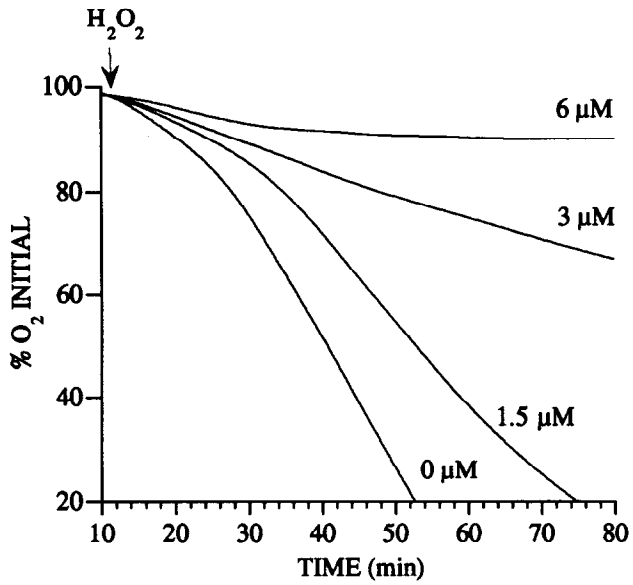

FIG. 4. Effect of chlorogenic acid on metmyoglobin/H_2O_2-induced oxidation of LDL. Time course of O_2 consumption during LDL oxidation in the absence and presence of increasing concentrations of chlorogenic acid indicated besides the traces. The reaction was initiated by H_2O_2 at the time indicated by the arrow, and chlorogenic acid was added before H_2O_2 under conditions described in Materials and Methods. Metmyoglobin or H_2O_2 alone did not exert any significant effect on the time course of O_2 consumption during the time of the experiments.

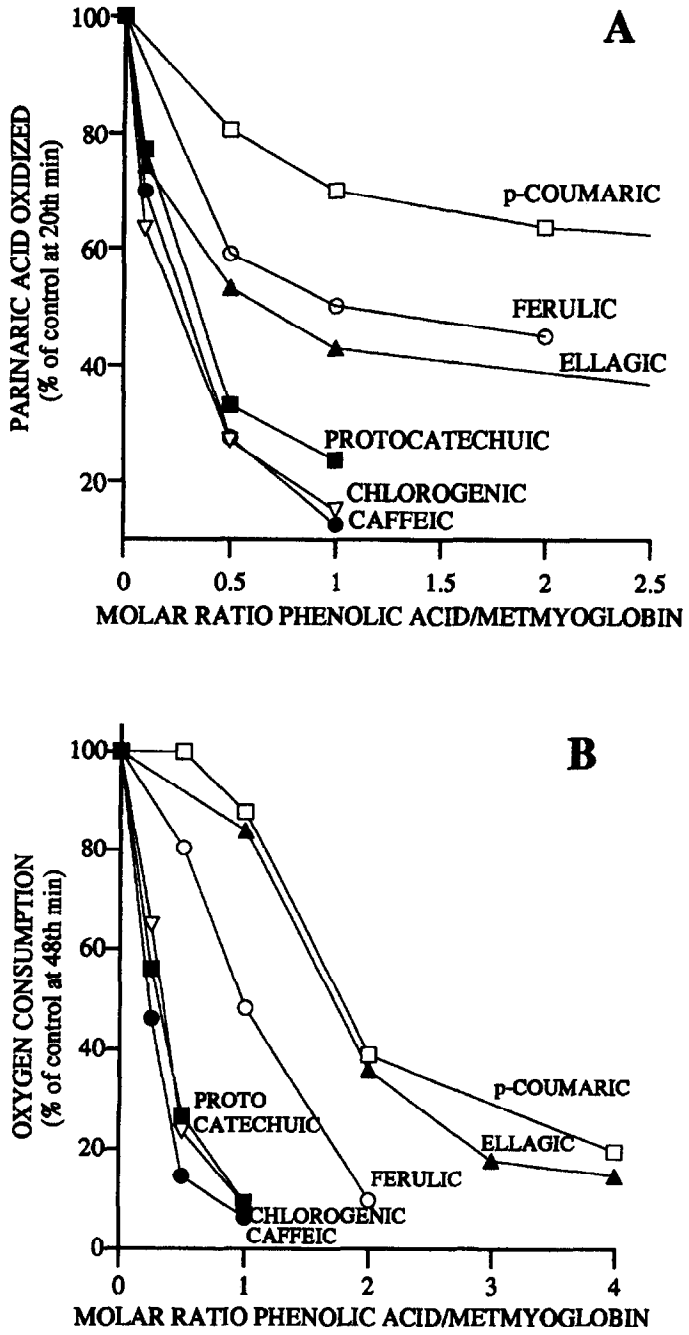

FIG. 5. Inhibition of PnA degradation in (A) LDL and of (B) LDL lipid peroxidation by chlorogenic (▽), caffeic (●), protocatechuic (■), ellagic (▲), ferulic (○), and *p*-coumaric acids (□). The amount of (A) oxidized parinaric acid and (B) O_2 consumption was taken 20 and 48 min, respectively, after starting the reaction with H_2O_2 and expressed as % of control in the absence of phenolic acid. Experimental conditions are described in Materials and Methods.

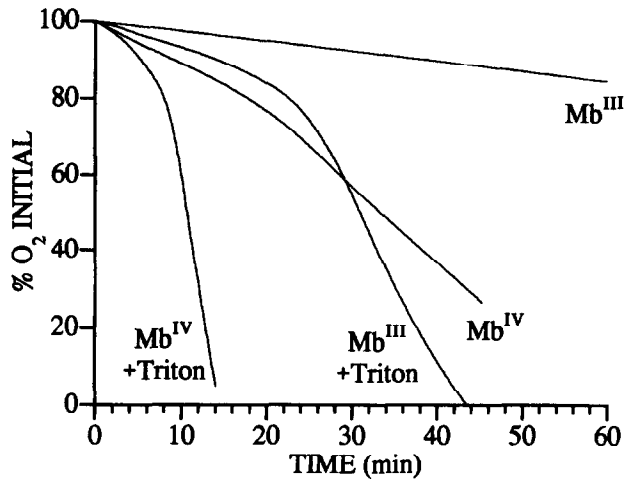

FIG. 6. Effect of Triton X-100 on O_2 consumption resulting from interaction of LDL with ferryl (Mb^{IV}) and metmyoglobin (Mb^{III}). The reaction was carried out under the standard conditions described in the Methods section. The curves of O_2 consumption in the presence of 6 μM metmyoglobin (Mb^{III}) and 6 μM metmyoglobin/9 μM H_2O_2 (Mb^{IV}) and 0.8 mM Triton X-100 (Mb^{III} + Triton and Mb^{IV} + Triton) are identified in the figure. Triton X-100 alone did not exert any effect on the rate of O_2 consumption.

at 421 nm as well as the characteristic peaks of Mb^{IV} at 548 and 582 nm remained unshifted, although decreasing in intensity. However, the reduction of the oxoferryl moiety in the presence of LDL can occur to a limited extent not detectable by absorption spectroscopy.

A pre-incubated mixture of Mb^{III} and H_2O_2 for 5 min (i.e. after decay of protein radical) was less efficient in catalysing LDL oxidation, suggesting a role for the protein radical in LDL damage. Nevertheless, the phenolic acids—particularly the catechol derivatives of cinnamic acid, chlorogenic, and caffeic acids—were previously shown to be efficient quenchers of aqueous peroxyl radicals inhibiting LDL oxidation [37], suggesting that potential myoglobin peroxyl radical-derived damage can be inhibited by these compounds. Recently, the occurrence of alkoxyl and peroxyl radicals from the decomposition of *tert*-butyl hydroperoxide by ruptured erythrocytes was also implicated in the oxidation of LDL [38].

Parinaric acid oxidation inside LDL shortly after addition of H_2O_2 to Mb^{III} suggests that the accessibility of the oxidant to lipid substrates in LDL is not kinetically hampered; diffusible oxidizing species (singlet oxygen and hydroxyl radical) are apparently not involved in myoglobin/H_2O_2-catalysed fatty acid oxidation [34]. Moreover, the propagation rate of LDL lipid peroxidation measured by O_2 consumption was preceded by a lag phase. In the presence of Triton X-100 (Fig. 6), the rate increases but the lag is not completely suppressed, suggesting a major role for intrinsic antioxidants in LDL resistance to oxidation rather than a restricted access of the oxidant to LDL lipids. It is noteworthy that in the presence of Triton X-100, metmyoglobin (without any added peroxide) was able to induce oxidation of LDL (Fig. 6). This observation is in line with the suggestion that the oxidation of LDL by metmyoglobin depends on lipid hydroperoxides within the particle [31], since the interaction of Mb^{III} with endogenous peroxides (as well as other LDL components) would be greatly enhanced in the presence of Triton X-100. This observation further supports the finding that reduction of the ferryl to the met form in myoglobin by the phenolic acids inhibits (or to a very large extent delays) the oxidation of LDL.

In conclusion, LDL peroxidation initiated by metmyoglobin/hydrogen peroxide is an efficient process, and is inhibited by dietary phenolic acids. The chemical structure of phenols plays a major role in this process, and the catechol derivatives of cinnamic and benzoic acids were the most effective inhibitors. The protection of LDL against oxidation is assigned to oxoferryl reduction to ferric form, but a broader chemical activity (e.g. peroxyl radical quenching) may be part of the mechanisms of LDL protection from oxidation by metmyoglobin/hydrogen peroxide. The absorption, metabolism, and toxicity of phenolic acids remain to be clearly elucidated; however, the permanent ingestion in diet and the presence *in vivo* [49] argue for evaluating them as physiologic antioxidants, particularly when they can regenerate α-tocopherol at the LDL surface [51].

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