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# Phenolic Antioxidants and the Protection of Low Density Lipoprotein from Peroxynitrite-Mediated Oxidations at Physiologic $CO_2$

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Dietary phenolic antioxidants have been shown to prevent LDL modifications mediated by several physiologic oxidants including peroxynitrite. However, more recent data demonstrated that $CO_2$ affected the fate of peroxynitrite in biological fluids and significantly reduced peroxynitrite scavenging by polyphenols, raising doubts concerning their antioxidant activity. We found that the oxidation of LDL lipids mediated by peroxynitrite decreased in the presence of bicarbonate, while Trp oxidation and 3-nitroTyr formation increased, suggesting a redirection of peroxynitrite reactivity toward the protein moiety. We therefore evaluated the protective activity of some phenolic antioxidants (quercetin, oleuropein, resveratrol, (+)-catechin, (−)-epicatechin, tyrosol, α- and γ-tocopherol, ascorbate) on peroxynitrite-mediated oxidation of LDL aromatic residues. Some of these phenols protected LDL Trp from oxidation better than ascorbate or α-tocopherol, although protection at 100 μM did not exceed 30−40%. However, the same phenolic antioxidants were more active in inhibiting 3-nitroTyr formation and those with a catechin structure provided significant protection ($IC_{50\%}$ 40−50 μM). Red wine, a polyphenol-rich beverage, showed a protective effect comparable to that of the most active phenolic antioxidants. Direct EPR studies showed that bicarbonate significantly increased the peroxynitrite-dependent formation of *O*-semiquinone radicals in red wine, supporting the hypothesis that polyphenols are efficient scavengers of radicals formed by peroxynitrite/$CO_2$. Ascorbate was a poor inhibitor of peroxynitrite/$CO_2$-induced LDL tyrosine nitration, but the simultaneous addition to the most active polyphenols halved their $IC_{50\%}$. In conclusion, although cooperation with other antioxidants can further decrease the $IC_{50\%}$ of polyphenolics, as demonstrated for ascorbate, their antioxidant activity appears to occur at concentrations at least 1 order of magnitude higher than their bioavailability.

**KEYWORDS: Lipoprotein; 3-nitrotyrosine; tryptophan oxidation; peroxynitrite; phenolic antioxidants; bicarbonate**

## INTRODUCTION

Notwithstanding the widespread belief that increased consumption of fruit and vegetables could significantly improve public health, the involved biological mechanisms are poorly understood. For example, it has been estimated that the incidence of cancer in The Netherlands could be reduced by as much as 19% (range 6−28%) by increasing the average intake of fruit and vegetables from the current 250 g/day to the recommended 400 g/day (*1*). Although clinical trials with antioxidant vitamins (A, C, and E) have been disappointing and raise some doubts about the role of fruit and vegetables in cancer prevention (*2*), other indications suggest that their consumption could also be associated with a lower risk of heart disease, diabetes, stroke, and obesity.

The general term "Mediterranean diet" is widely used to indicate a diet particularly rich in fruit and vegetables, and therefore, in antioxidant compounds and vitamins. Whatever the truth regarding the potential benefits of this diet, it is probable that phenolic compounds, with their powerful antioxidant properties, may play a role (*3*). Phenolic compounds are the most abundant antioxidants in our diets: a typical serving of 200 g of fruit may contain as much as 500 mg of mixed phenols. However, several studies have questioned the hypothesis that the major role of phenolic compounds is simply that of acting as antioxidants (for a review, see ref *4*) and shown that the bioavailability of phenols is limited, and their biological actions not necessarily related to the antioxidant properties (*5*−*9*). However, it has so far been impossible to identify any biological activity, other than the "antioxidant hypothesis", to explain epidemiological data suggesting the benefit of a diet rich in fruit and vegetables.

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One of the most biologically significant targets of oxidative attacks on proteins is probably the low-density lipoprotein (LDL). This lipoprotein has been unequivocally shown to be the initial target that mediates and accelerates the development of arterial lesions in atherosclerosis. Oxidized LDL stimulates its own uptake by a class of receptors named "scavenger receptors", which are expressed in macrophages as well as in "nonprofessional" phagocytes. Unlike the physiologic LDL receptor, the scavenger receptor is not down regulated when the cell cholesterol content increases and the process leads to cholesterol accumulation, cell activation, and transformation. Macrophages engulf oxidized LDL (foam cells) and initiate the atherosclerotic process.

In vitro studies have shown that LDL undergoes several oxidative modifications that may be catalyzed by reactive oxygen, chlorine, and nitrogen species (*10*). Although the exact mechanism whereby LDL is oxidized in vivo is unclear, the finding that 3-nitrotyrosine (3-nitroTyr) and 3-chlorotyrosine are present in atherosclerotic lesions (*10*, *11*) indicates that the LDL protein moiety (Apo B-100) is a target of oxidative attack and also shows that at least chlorinating and nitrating species may be physiologically relevant oxidants.

Several different nitrating pathways have been described in biological systems (*12*), but one of the most potent nitrating species so far identified is peroxynitrite, (this term refers to both the anion oxoperoxynitrate (1-), $ONOO^-$, and its conjugate acid hydrogen oxoperoxynitrate, ONOOH) the product of the fast radical−radical reaction between $^{\bullet}NO$ (nitrogen monoxide) and $O_2^{\bullet-}$ (superoxide anion) (*13*). Peroxynitrite-modified LDL acquires a negative charge (*14*), has a reduced amount of antioxidants (*15*, *16*), shows oxidation products derived from lipids (*14*), and Apo B-100 (*17*) and is scavenged by the macrophage scavenger receptor CD36 (*18*, *19*).

It has been reported that some phenolic antioxidants can protect biological targets against peroxynitrite-mediated oxidations (*20*−*22*). In considering the possible biological role of peroxynitrite, it should be noted that in tissues, and particularly in blood plasma (*23*), the oxidation of biological targets is not performed by peroxynitrite itself, but is probably generated by radicals formed after its reaction with $CO_2$. In blood, the high concentration of $CO_2$ (1.3 mM in equilibrium with 25 mM bicarbonate) and its fast reaction with peroxynitrite leads to the prediction that a large part of the oxidant does not react directly with the majority of biological targets, but forms a short-lived adduct, $ONOOCO_2^-$, which, in the decay process to nitrate, forms about 35% of $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals (*23*, *24*). The effect of $CO_2$ is to modify peroxynitrite reactivity so that the oxidation of some targets is inhibited (mainly hydroxylation and two-electron oxidations), while nitration reactions (*25*) and some one-electron oxidations are increased (*26*, *27*). In addition, the half-life of peroxynitrite is reduced in the presence of $CO_2$ from 700 to about 12 ms (*28*), thus significantly altering and down-regulating the oxidation of internal sites of particulate targets such as bacteria (*29*), liposomes (*30*), and also LDL (*31*). Therefore, the presence of $CO_2$ is expected to inhibit the action of antioxidants in peroxynitrite-mediated oxidations (*23*), particularly that of phenolic antioxidants (*32*). As demonstrated by Ketsawatsakul et al. (*32*), at pH 7.4, the addition of 25 mM bicarbonate almost completely eliminated the ability of phenolic antioxidants to act as preventive antioxidants (i.e., to react with peroxynitrite before its reaction with other biological targets), and significantly decreased their ability to protect free tyrosine from peroxynitrite-mediated nitration. Experimental results (*32*) and considerations of kinetics (*33*) thus raise serious doubts regarding the ability of phenolic antioxidants to protect biological targets against peroxynitrite-mediated injury under physiological conditions.

These considerations prompted us to analyze the effects of bicarbonate on the peroxynitrite-mediated oxidation of LDL. Our results directed our attention toward LDL aromatic residues, which were more oxidized in the presence of bicarbonate. We therefore evaluated the protective activity of some phenolic antioxidants characteristic of a diet rich in fruits and vegetables. Our results showed that some phenolic antioxidants are able to inhibit at $\mu$M concentration peroxynitrite/$CO_2$-mediated oxidation of Trp and the formation of 3-nitroTyr in LDL. However, noticeable and unexpected differences were found between different phenolic antioxidants, and a cooperative effect was observed in the presence of ascorbate. When we extended our studies to red wine, a phenol-rich beverage, we noted protective effects comparable to those of the most active phenolic antioxidants. These results are discussed in terms of the antioxidant mechanism and possible biological relevance of dietary polyphenols.

## MATERIALS AND METHODS

**Materials.** Oleuropein ([2*S*-(2α,3*E*,4*β*)]-3-ethylidene-2-(*β*-D-glucopyranoyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetic acid 2-(3,4-dihydroxyphenyl)ethylester) was obtained from Extrasynthèse (Genay, France). All other reagents, including diethylenetriaminepentaacetic acid (DTPA); ascorbate; resveratrol (3,5,4′-trihydroxy-trans-stilbene); quercetin (3,3′,4′,5,7-pentahydroxyflavone); (−)-epicatechin ([2*R*3*R*]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol); (+)-catechin ([+]-cyanidol-3;[2*R*,3*S*]-2-[3,4-dihydroxyphenyl-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol); tyrosol (2-(4-hydroxyphenyl)-ethanol); (+)-α-tocopherol; and (+)-*γ*-tocopherol were from Sigma (St. Louis, MO).

**Phenolic Antioxidants.** Stock solutions of ascorbate, oleuropein, and tyrosol were obtained in water previously treated with chelex 100 to remove traces of transition metals. Tocopherols (α- and *γ*-), (+)-catechin, and (−)-epicatechin were dissolved in ethanol. Control experiments were performed with comparable amounts of ethanol. Resveratrol and quercetin were dissolved in diluted alkali and used immediately.

**Red Wine.** Red wine ("Teroldego Novello") was obtained from Istituto San Michele all'Adige (Trento, Italy), and its alcoholic content was 12% (v/v). Its total content of polyphenols was 1.44 g/L, as measured by reduction of the Folin−Ciocalteu. To separate the nonalcoholic fraction, the wine was evaporated at 25 °C to about half the original volume. The polyphenol content was expressed as quercetin equivs ($\mu$mol quercetin/L).

**Peroxynitrite Synthesis.** Peroxynitrite was synthesized from nitrite and $H_2O_2$, as described by Radi et al. (*34*) and treated with $MnO_2$ to eliminate excess $H_2O_2$ (6 mg/mL at 4 °C for 30 min). The mixture was then filtered three times to remove $MnO_2$. When freeze fractionated (−80 °C), peroxynitrite solution forms a yellow top layer, which was retained for further studies. The peroxynitrite concentration was determined at 302 nm ($\epsilon = 1700\ M^{-1}\ cm^{-1}$). The top layer typically contained 200−300 mM peroxynitrite. Peroxynitrite was added as a bolus to LDL samples submitted to vortexing and buffered with 150 mM phosphate, 2 $\mu$M DTPA, pH 7.4 (phosphate/DTPA). Bicarbonate was added to phosphate/DTPA and dissolved $CO_2$ (ABL 330 Gas Analyzer Radiometer, Copenhagen, Denmark) was 1.3 mM and 5 mM at 25 mM and 100 mM sodium bicarbonate, respectively. Decomposed peroxynitrite was obtained by adding peroxynitrite to phosphate/DTPA/sodium bicarbonate buffer, pH 7.4, or by neutralization with HCl before the addition of the biological target. The two methods gave comparable results.

**Purification of LDL and Apo B-100.** Heparinized fresh human blood was obtained from normal donors following informed consent. Plasma was separated from blood by centrifugation for 5 min at 1000*g*. LDL was isolated from fresh plasma (each preparation from 8 different

donors) by sequential ultracentrifugation through a sodium bromide gradient (*35*). To remove excess sodium bromide, the sample was submitted to extensive dialysis against degassed phosphate buffered saline containing the metal chelator DTPA (2 $\mu$M). Apo B-100 was purified from LDL using the procedure previously described (*36*). Isolated LDL and Apo B-100 samples were analyzed for protein content using BCA assay (Pierce, Rockford, IL) modified (1% SDS) for lipoproteins, and LDL concentration was always expressed as protein content. Apo B-100 purity was ≥ 95% as judged by SDS gel electrophoresis in 3.5−8% polyacrylamide gradient stained with Coomassie blue. To avoid protein degradation, isolated LDL and Apo B-100 were stored under nitrogen in the presence of protease inhibitors phenylmethyl-sulfonylfluoride (0.15 mM), leupeptin (10 $\mu$g/mL), and aprotinin (10 $\mu$g/mL). The range of hydroperoxides in untreated LDL samples was 0.2−2.0 nmoles/mg protein as estimated by FOX method. Further purification of LDL by HPLC (TSK column, Bio-Rad, Richmond, CA) did not modify the basal lipid oxidation level. To compare the effects of peroxynitrite on Apo B-100 and LDL, the protein samples were diluted in 10 mM Tris-HCl and 10 mM sodium deoxycholate, pH 7.0 immediately before the addition of peroxynitrite.

**Determination of LDL Trp Fluorescence.** LDL (1.5 mg/mL in phosphate/DTPA) was diluted in water (1:300), and the oxidation of Trp residues was monitored as fluorescence loss (excitation $\lambda = 285$ nm and emission $\lambda = 327$ nm). Phenolic antioxidants up to 100 $\mu$M did not significantly change LDL tryptophan fluorescence (±4%). Higher concentrations were not tested as they interfered with the intrinsic fluorescence of LDL. The control sample received the same amount of phenolic antioxidants and decomposed peroxynitrite. The effect of the antioxidant was expressed as % of protection of LDL Trp fluorescence (i.e., % of recovered Trp fluorescence).

**Determination of Conjugated Dienes.** The formation of conjugated dienes was measured at 234 nm (*37*). The reference cuvette contained an identical amount of LDL (0.25 mg/mL) and decomposed peroxynitrite. Control experiments showed that decomposed peroxynitrite did not induce the formation of conjugated dienes in LDL. As previously reported (*38*), peroxynitrite promotes immediate oxidation of lipids with no initiation, propagation, and termination phases. We found that peroxynitrite-dependent increase in the absorption at 234 nm was almost instantaneous and did not change up to 120 min.

**Western Blot Immunoassays of 3-NitroTyr.** For Western blot analysis, proteins were separated on 3.5−8% SDS−PAGE and transferred to nitrocellulose paper at 35 V overnight. Proteins were detected by Red Ponceau staining. Blots were washed in 50 mM Tris, 0.05% Tween 20, and 150 mM NaCl buffer and blocked with 3% BSA (wt/vol) in the same buffer for 3−4 h. Washed nitrocellulose filters were incubated overnight at 4 °C with anti-3-nitroTyr monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). The specificity of the antibody was established by competition experiments using antibody preincubated with 3-nitroTyr (5 mM) for 10 min, before being added to the blots. Immunoreactive bands were detected by chemiluminescence coupled with peroxidase activity according to the manufacturer's specifications (ECL kit, Pierce). The densitometric analysis of immunoreactive bands was performed using a GS-700 densitometer (Bio-Rad). To evaluate band intensity appropriate dose-dependent curves were constructed for peroxynitrite-treated LDL. Differences < 10% in band intensity were not considered significant.

**EPR (or ESR) Measurements.** Spectra were measured on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with a variable-temperature unit (ER4111VT). Samples were drawn up into a gas-permeable Teflon tube with 0.81-mm internal diameter and 0.05-mm wall thickness (Zeuss Industrial Products, Raritan, NJ). The Teflon tube was folded four times, inserted into a quartz tube, and fixed to the EPR cavity (4108 TMH). Samples were exposed to air at 37 °C. The software supplied by Bruker (WINEPR-System) was used to correct spectra for baseline drift by a linear function and for double integration to obtain an integrated relative area. Spectrometer conditions common to all spectra were modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW; receiver gain, $1 \times 10^6$; modulation amplitude, 1 G; time constant, 164 ms; sweep time, 336 s; number of scans, 1.

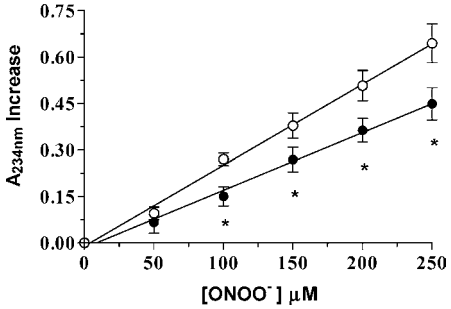



**Figure 1.** Effect of bicarbonate on the $A_{234\ nm}$ increase induced by the treatment of LDL with peroxynitrite. LDL (0.15 mg/mL) was treated with peroxynitrite in phosphate/DTPA in the absence (open symbol) or in the presence of 25 mM sodium bicarbonate (closed symbol). Data represent mean values ± SD of four different LDL preparations. *Statistically different from the appropriate control without bicarbonate ($p \leq 0.001$).

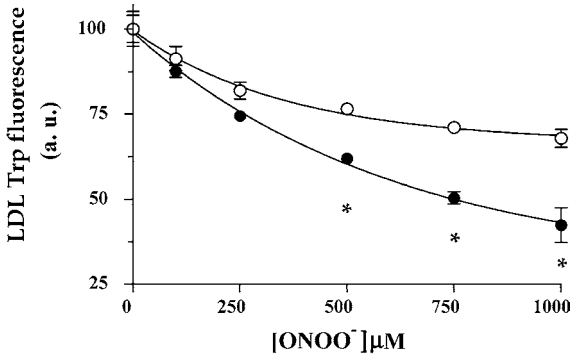



**Figure 2.** Effect of bicarbonate on peroxynitrite-dependent oxidation of LDL Trp residues. LDL (1.5 mg/mL) was treated with peroxynitrite in phosphate/DTPA in the absence (open symbols) or in the presence of 25 mM sodium bicarbonate (closed symbols). Data represent % of control value ± SD, of three different LDL preparations. *Statistically different from the appropriate control without bicarbonate ($p \leq 0.002$).

**Statistical Analysis.** Differences between samples were analyzed statistically by the Student's *t*-test.

## RESULTS

**Bicarbonate Inhibits the Peroxynitrite-Induced Formation of Conjugated Dienes, but Promotes Trp Oxidation and 3-NitroTyr Formation.** A convenient way to investigate peroxynitrite-dependent lipid peroxidation of LDL is to measure the formation of conjugated dienes. The formation of conjugated dienes can easily be detected by the increase in absorption at 234 nm. As shown in **Figure 1**, peroxynitrite increased the formation of conjugated dienes in a dose-dependent manner, but the addition of 25 mM bicarbonate (in equilibrium at pH 7.4 with 1.3 mM $CO_2$) decreased the formation of dienes by about 30%. It is unclear if the peroxynitrite-dependent increase at 234 nm is due to the formation of conjugated dienes (*22*); however, our results agree with a previous study (*17*) on the inhibitory effects of bicarbonate on oxidation of LDL cholesterol and cholesteryl esters.

The effect of peroxynitrite on the protein moiety of LDL was investigated by measuring the loss of Trp fluorescence and the formation of 3-nitroTyr. As illustrated in **Figure 2**, peroxynitrite caused a dose-dependent decrease in LDL Trp fluorescence. The addition of bicarbonate enhanced the loss of Trp fluorescence (+ 37% at 1 mM peroxynitrite), indicating that Trp oxidation was increased.

Tyrosine residues are also preferential targets of peroxynitrite. This modification is often used as a marker of the production

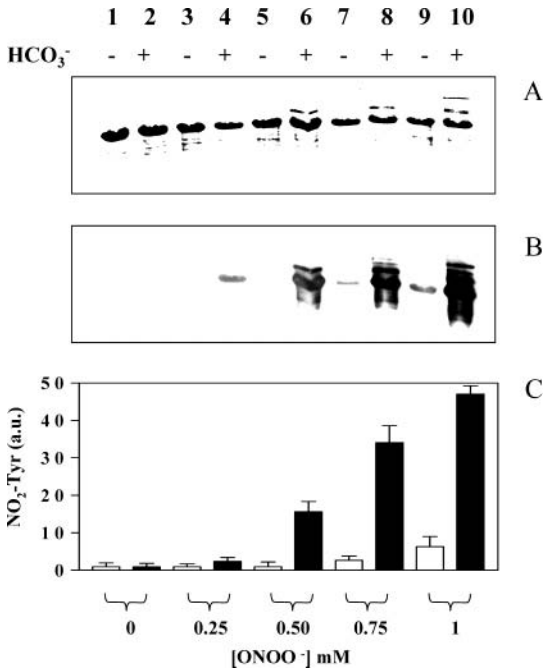



**Figure 3.** Effect of sodium bicarbonate on the formation of 3-nitroTyr in peroxynitrite-treated LDL. (**A**) Red Ponceau S staining of peroxynitrite-treated LDL. LDL (1.5 mg/mL) in phosphate/DTPA was treated with peroxynitrite in the absence (lanes 1, 3, 5, 7, 9) or in the presence (lanes 2, 4, 6, 8, 10) of 25 mM sodium bicarbonate. (**B**) Western blot analysis with anti-3-nitroTyr antibodies of gel A. (**C**) Densitometric analysis of panel B. Each lane was loaded with 7.5 $\mu$g of LDL. a. u. = arbitrary units. Data are representative of three different experiments.

of reactive nitrogen species in tissues. As shown in **Figure 3**, peroxynitrite dose-dependently induced the formation of 3-nitroTyr in LDL, and the addition of bicarbonate strongly increased the nitration yield. Moreover, **Figure 3** shows that $ONOO^-/CO_2$ induced also the formation of non SH-dependent and strongly nitrated protein aggregates. Densitometric analysis revealed that total nitration of LDL (aggregated + nonaggregated) increased about 10-fold in the presence of bicarbonate.

Bicarbonate also increased the peroxynitrite-dependent formation of 3-nitroTyr in purified Apo B-100, although the increase in nitration was smaller (2-fold increase), probably on account of the presence of the detergent (10 mM sodium deoxycholate). In fact, the addition of the detergent to LDL reduced the $CO_2$-dependent increase in 3-nitroTyr to about 1.5-fold (results not shown).

Peroxynitrite decomposed in phosphate buffer before being added to LDL did not decrease Trp fluorescence or induce 3-nitroTyr formation (results not shown).

From these results, we conclude that bicarbonate is able to redirect the reactivity of peroxynitrite and shifts LDL oxidation toward the aromatic residues of Apo B-100. The finding that peroxynitrite reacts more efficiently with proteins than with lipids of LDL has been previously reported (*22*), although these studies have been performed without $CO_2$.

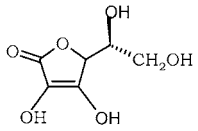
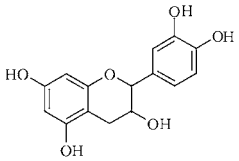
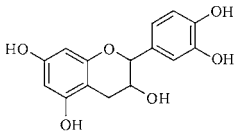
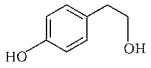
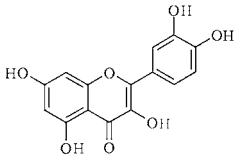
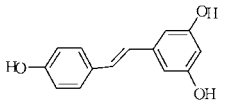
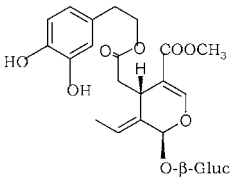
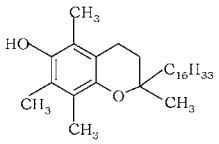
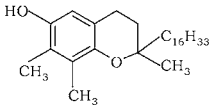
**Phenolic Antioxidants Protect LDL Trp Residues Against Peroxynitrite/$CO_2$-Mediated Oxidation.** Phenolic antioxidants were chosen for their widespread availability in vegetables (see **Table 1** for sources and chemical structures). The effects of these antioxidants on peroxynitrite/$CO_2$-mediated oxidation of LDL Trp residues were compared with those produced by ascorbate and α-tocopherol, the two most effective antioxidants of blood plasma. **Table 2** summarizes the results expressed as % of protection. It should be noted that LDL contains endogenous α-tocopherol (12.5−19.2 $\mu$M in our LDL samples), while the concentration reported in **Table 2** refers only to the exogenously added amount. Both ascorbate and α-tocopherol provided poor protection against peroxynitrite/$CO_2$-dependent Trp oxidation. Even γ-tocopherol, which differs from α-tocopherol in an unsubstituted C-5 position, making it a better trap for reactive nitrogen species (*39*), was unable to protect significantly LDL Trp residues. At 30 $\mu$M, only quercetin, the main flavonoid of our diet, and oleuropein, a major antioxidant of olive oil, provided modest but significant protection. Resveratrol was not protective, while (+)-catechin, (−)-epicatechin, and tyrosol were protective at high concentration. It should be noted, however, that protection never exceeded 30−40% at 100 $\mu$M. Control experiments showed that ethanol (used to dissolve polyphenols) did not affect Trp fluorescence loss induced by peroxynitrite.

**Phenolic Antioxidants Protect LDL Against Peroxynitrite/$CO_2$-Mediated 3-NitroTyr Formation. Table 3** shows the effects of the same phenolic antioxidants on nitration of LDL Tyr residues induced by peroxynitrite/$CO_2$. The protection afforded by phenolic antioxidants is expressed as the concentration causing a 50% reduction in 3-nitroTyr ($IC_{50\%}$). The antioxidant vitamins α- and γ-tocopherol were inefficient as inhibitors of LDL tyrosine nitration, while ascorbate, tyrosol and oleuropein were poor inhibitors ($IC_{50\%} \geq 100$ $\mu$M). Resveratrol showed intermediate activity, while phenolic antioxidants with a catechin structure (quercetin, (+)-catechin, and (−)-epicatechin) were the most active. It should be noted that, in contrast with Trp oxidation, quercetin, (+)-catechin, and (−)-epicatechin afforded efficient protection against Tyr nitration, because at 100 $\mu$M, the formation of 3-nitroTyr was completely inhibited. Control experiments showed that ethanol (used to dissolve polyphenols) did not affect 3-nitroTyr formation induced by peroxynitrite.

To verify if a polyphenol-rich beverage can protect LDL from peroxynitrite-mediated damage, we explored the effects of red wine, whose phenolic content was calculated as quercetin equiv. Previous studies showed that red wine polyphenols are potent inhibitors of peroxynitrite-driven oxidation (*40*) and the chosen wine cultivar (Teroldego Novello) has a medium-high polyphenolic content (*41*). As shown in **Figure 4**, red wine and alcohol-free red wine diluted to a polyphenol concentration of 100 $\mu$M quercetin equiv were powerful inhibitors of peroxynitrite/$CO_2$-mediated LDL Tyr nitration. Ethanol (at the concentration comparable to that of 100 $\mu$M quercetin equiv red wine sample, i.e., 0.27%) did not significantly affect peroxynitrite-induced LDL nitration. Moreover, **Figure 4** shows that peroxynitrite/$CO_2$-induced nitration and protein aggregation were largely inhibited in the presence of red wine or quercetin. The inhibitory activity of red wine and alcohol-free red wine was only slightly lower than that of antioxidants with a catechin structure (**Table 3**).

**Formation of Stable Free Radicals in Peroxynitrite-Treated Red Wine.** As discussed in the Introduction, phenolic antioxidants in the presence of a physiologic concentration of bicarbonate do not interact directly with peroxynitrite, but can scavenge $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals formed in the decay process to nitrate. This reaction is expected to form, as a first step, *O*-semiquinone radicals through hydrogen/electron donation. This plausible mechanism, however, has not been proven by EPR partly on account of the difficulties involved in detecting *O*-semiquinone radicals directly or after spin trapping with nitrone or nitroso compounds. From this point of view, red wine presents a clear advantage, because upon oxidative challenge,

**Table 1.** Source of Antioxidants in Foods

| Antioxidant | Chemical formula | Source |
|---|---|---|
| Ascorbate |  | Tomatoes, peppers, citrus fruits |
| (+)-Catechin |  | Grapes, white wine |
| (-)-Epicatechin |  | Red wine, apples |
| Tyrosol |  | Olive oil |
| Quercetin |  | Onion, lettuce, apple red wine, grapes, broccoli, kale |
| *trans*-Resveratrol |  | Red wine |
| Oleuropein |  | Olive oil |
| α-Tocopherol |  | Seed oils, olive oil |
| γ-Tocopherol |  | Seed oils, spices |

it can show long-lived radicals, hypothesized to be associated with its polyphenolic content (*42*). We therefore performed direct EPR studies of peroxynitrite-treated red wine. As shown in **Figure 5**, spectrum A, peroxynitrite induced the formation in red wine of a single resonance centered at $g = 2.004$. The $g$ value and its unusual high stability identify this signal as stable *O*-semiquinone radicals involved in redox equilibrium (*42*). Relevantly, the signal intensity increased significantly (2-fold) in the presence of bicarbonate (**Figure 5**, spectrum B). The $g = 2.004$ signal was not detectable if peroxynitrite was allowed to decompose before the addition of red wine (**Figure 5**, spectrum C), ruling out oxidative processes mediated by peroxynitrite decomposition or contaminating products. Alcohol-free red wine treated with peroxynitrite/$CO_2$ led to the formation

**Table 2.** Effect of Antioxidants on Trp Oxidation Mediated by 1 mM Peroxynitrite/$CO_2$

| antioxidant | ($\mu$M) | Trp fluorescence (% of protection ± SD) | |
|---|---|---|---|
| ascorbate | 30 | 4.6 ± 8.2 | ($n = 4$) |
| | 60 | 8.4 ± 8.3 | ($n = 4$) |
| | 100 | 14.1 ± 9.4[b] | ($n = 5$) |
| α-tocopherol [a] | 30 | 1.0 ± 7 | ($n = 3$) |
| | 60 | 6.5 ± 5.5 | ($n = 3$) |
| | 100 | 7.3 ± 5 | ($n = 3$) |
| γ-tocopherol [a] | 30 | 1.3 ± 4.3 | ($n = 3$) |
| | 60 | 6.8 ± 10.1 | ($n = 3$) |
| | 100 | 3.5 ± 6.0 | ($n = 3$) |
| quercetin | 30 | 16.4 ± 6.0[b] | ($n = 4$) |
| | 60 | 25.4 ± 9.6[c] | ($n = 4$) |
| | 100 | 41.2 ± 10.9[c] | ($n = 4$) |
| oleuropein | 30 | 17.0 ± 12.1[b] | ($n = 5$) |
| | 60 | 20.2 ± 9.0[c] | ($n = 5$) |
| | 100 | 35.4 ± 10.0[c] | ($n = 5$) |
| resveratrol | 30 | 6.0 ± 6.6 | ($n = 5$) |
| | 60 | 5.6 ± 11.5 | ($n = 5$) |
| | 100 | 14.2 ± 19.2 | ($n = 5$) |
| (+)-catechin | 30 | 7.7 ± 11.8 | ($n = 3$) |
| | 60 | 21.7 ± 8.1[b] | ($n = 3$) |
| | 100 | 37.0 ± 3.7[c] | ($n = 3$) |
| (−)-epicatechin | 30 | 5.7 ± 3.4 | ($n = 3$) |
| | 60 | 21.4 ± 12.0[b] | ($n = 3$) |
| | 100 | 34.7 ± 7.6[c] | ($n = 3$) |
| tyrosol | 30 | 1.1 ± 9.7 | ($n = 4$) |
| | 60 | 4.9 ± 12.7 | ($n = 4$) |
| | 100 | 26.0 ± 4.4[c] | ($n = 4$) |

[a] Added concentrations. [b] $p \leq 0.05$. [c] $p \leq 0.005$.

**Table 3.** Effect of Antioxidants on 3-NitroTyr Formation Induced by 1 mM Peroxynitrite/$CO_2$

| antioxidant | $IC_{50\%}$[a] ($\mu$M) |
|---|---|
| α-tocopherol | n.i.[b] |
| γ-tocopherol | n.i.[b] |
| ascorbate | 180 ± 20 |
| tyrosol | 156 ± 32 |
| oleuropein | 100 ± 10 |
| resveratrol | 78 ± 12 |
| (+)-catechin | 44 ± 9 |
| (−)-epicatechin | 50 ± 7 |
| quercetin | 40 ± 12 |
| red wine[c] | 64 ± 10 |
| alcohol-free red wine[c] | 67 ± 11 |
| quercetin + 100 $\mu$m ascorbate | 18 ± 7 |
| (+)-catechin + 100 $\mu$m ascorbate | 27 ± 8 |
| (−)-epicatechin + 100 $\mu$m ascorbate | 16 ± 5 |

[a] Expressed as the concentration causing a 50% reduction in 3-nitroTyr. [b] Not inhibitory up to 100 $\mu$M. [c] Expressed as quercetin equiv.

of a signal of comparable intensity (**Figure 5**, spectrum D) and the addition of LDL did not modify its intensity (**Figure 5**, spectrum E). In the absence of red wine, no radical signal was detected after treatment of LDL with peroxynitrite/$CO_2$ (**Figure 5**, spectrum F).

**Effect of the Simultaneous Addition of Ascorbate and Phenolic Compounds.** Ascorbate is an important antioxidant defense of blood plasma (*43*) and protects LDL against oxidative damage mediated by reactive oxygen (*44*) and nitrogen (*45*) species. However, our data showed that this vitamin has a poor protective effect against peroxynitrite/$CO_2$-mediated oxidation of LDL aromatic residues (**Tables 2** and **3**). To investigate whether ascorbate can exhibit synergistic interaction with phenolic antioxidants, we chose the most active phenols ((+)-catechin, oleuropein, and quercetin for Trp oxidation, and (+)-catechin, (−)-epicatechin, and quercetin for Tyr nitration) and tested their effects on peroxynitrite/$CO_2$-mediated oxidation of LDL aromatic residues in the presence of 100 $\mu$M ascorbate.

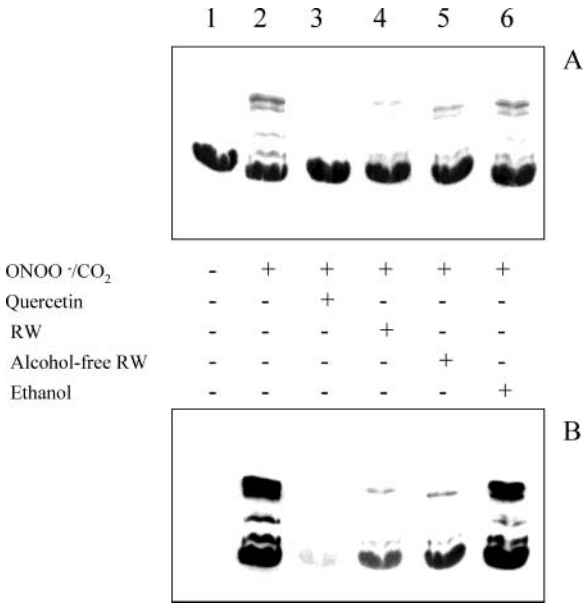



**Figure 4.** Effects of quercetin, red wine (RW), alcohol-free RW or ethanol on the formation of 3-nitroTyr in peroxynitrite/$CO_2$-treated LDL. (**A**) Red Ponceau S staining of peroxynitrite/$CO_2$-treated LDL. Lane 1: untreated LDL (1.5 mg/mL) in phosphate/DTPA. Lane 2: sample as in lane 1, but treated with 1 mM peroxynitrite/$CO_2$. Lanes 3–5: samples as in lane 2, but in the presence of quercetin (100 $\mu$M), RW (100 $\mu$M, quercetin equiv), alcohol-free RW (100 $\mu$M, quercetin equiv), respectively. Lane 6: sample as in lane 2, but in the presence of 0.27% ethanol. (**B**) Western blot analysis with anti-3-nitroTyr antibodies of gel A. Each lane was loaded with 7.5 $\mu$g of LDL. Data are representative of three different experiments.

Ascorbate did not significantly increase the protection afforded by 30−60 $\mu$M (+)-catechin, oleuropein, or quercetin against peroxynitrite/$CO_2$-mediated Trp oxidation (results not shown). A small increase in Trp protection (+17.3 ± 7.5%), attributable to the addictive effect of ascorbate, was observed when the three phenolic compounds were tested at 100 $\mu$M.

Interestingly, the inhibition of 3-nitroTyr formation by quercetin, (+)-catechin, and (−)-epicatechin was significantly increased in the presence of 100 $\mu$M ascorbate. The $IC_{50\%}$ of these phenolic antioxidants was halved in the presence of ascorbate (**Table 3**). Therefore, these results indicate that ascorbate was more efficient as a co-inhibitor of Tyr nitration than of Trp oxidation.

## DISCUSSION

In this report, we show that some common phenolic antioxidants can protect LDL aromatic residues against peroxynitrite-mediated oxidation, even in the presence of physiological concentrations of $CO_2$. These phenolics were chosen on the basis of their presence in a diet rich in fruit and vegetables. The issue of the protective action by phenolic antioxidants of peroxynitrite-mediated oxidation has been previously addressed using different biological targets (*20*−*22*), including LDL (*21*, *22*), but to our knowledge, this is the first study performed at physiologic concentrations of $CO_2$ and focused on LDL Trp and Tyr residues. In the presence of $CO_2$, the pathway of peroxynitrite decay and its lifetime are significantly modified, and the likelihood that this class of antioxidants has some protective ability has been previously questioned (*32*, *33*). We found that

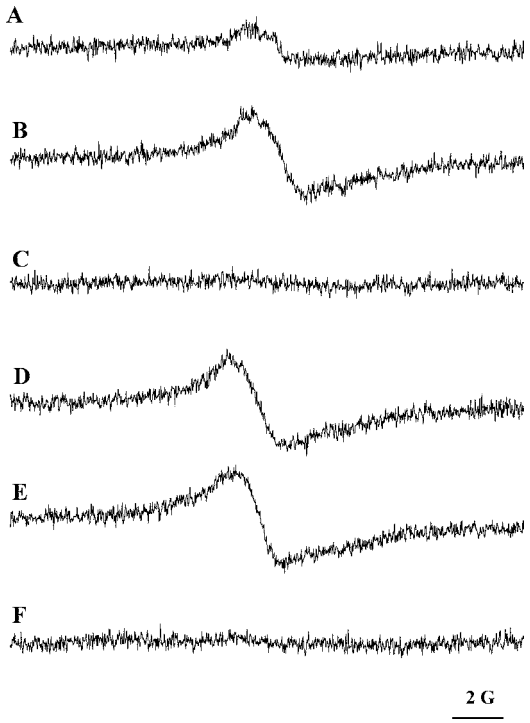



**Figure 5.** EPR spectra at 37 °C of peroxynitrite-treated red wine. (**A**) Red wine (3.4 mM, quercetin equiv) 1 min after treatment with 5 mM peroxynitrite. (**B**) Sample as in (**A**), but in the presence of 100 mM sodium bicarbonate. (**C**) Sample as in (**B**), but with decomposed peroxynitrite. (**D**) Alcohol-free red wine (3.4 mM, quercetin equiv) 1 min after treatment with 5 mM peroxynitrite/$CO_2$. (**E**) Sample as in (**D**), but in the presence of LDL (1.5 mg/mL). (**F**) LDL (1.5 mg/mL) 1 min after treatment with 5 mM peroxynitrite/$CO_2$. All samples were in phosphate buffer (0.15 M), DTPA (0.1 mM), pH 7.4. Spectrometer conditions were as described in Materials and Methods.

the oxidation of LDL aromatic residues by peroxynitrite was increased in the presence of $CO_2$, but in the $\mu$M concentration range, the protective action of some phenolic antioxidants was still observed. Notably, the active concentration ($IC_{50\%}$) of these polyphenols is not significantly different from that observed in the absence of $CO_2$, as previously reported using free tyrosine as nitration target (*32*). Although the structure−activity relationship was not a focus of this study, we observed a clearly higher activity in the phenolic antioxidants with a catechin structure.

**Antioxidant Mechanism.** Direct EPR studies show that peroxynitrite-treated red wine forms *O*-semiquinone radicals whose intensity is significantly increased in the presence of bicarbonate. This suggests that red wine polyphenols are better scavengers of radicals derived from $ONOOCO_2^-$ than from ONOOH. The decay pathway of $ONOOCO_2^-$ differs from that of ONOOH in that $CO_3^{\bullet-}$ is produced instead of the hydroxyl radical ($OH^\bullet$), while both pathways involve the formation of the $^\bullet NO_2$ (*23*, *24*). The two major differences in the presence of $CO_2$ are, therefore, $CO_3^{\bullet-}$ formation and a shorter lifetime of peroxynitrite (*28*). Compared with the broad reactivity of $OH^\bullet$, the reactivity of $CO_3^{\bullet-}$ is more selective for aromatic residues (*46*−*48*), and this may explain the increase in oxidation of Trp and Tyr residues by peroxynitrite in the presence of $CO_2$ (*23*, *24*, *26*, *27*).

It is interesting to note that at 100 $\mu$M, the compounds with a catechin structure can almost completely inhibit LDL Tyr nitration, while the inhibition of Trp oxidation did not exceed 40%. This result can be rationalized if one considers that the most widely accepted mechanism of peroxynitrite/$CO_2$-mediated modifications of Tyr residues is its oxidation to the Tyr radical followed by a reaction with $^\bullet NO_2$ to form 3-nitroTyr or with another Tyr radical to form Tyr-Tyr (this last reaction is unfavorable in proteins for steric hindrance). Also, Trp is oxidized by peroxynitrite/$CO_2$ to Trp radical, but in oxygenated solutions, the Tyr radical lives longer than the Trp radical, and thus, could be more easily scavenged by phenolic antioxidants. In fact, the lifetime of the Trp radical is shorter due to its fast reaction with molecular oxygen with formation of another short-lived species, a Trp peroxyl radical (*49*). One consequence of this last reaction is that phenolic antioxidants have to compete with molecular oxygen for the scavenging of Trp radicals. On the contrary, Tyr radicals are unusually stable in proteins, so that they are often detectable by direct EPR for long times at physiological temperature (*27*).

The poor ability of vitamins C and E to protect LDL aromatic residues against peroxynitrite/$CO_2$ mediated oxidation was surprising. Indeed, we found that ascorbate has a clear cooperative effect with phenolic antioxidants for the protection of LDL Tyr nitration, a synergistic effect likely linked to the ability of ascorbate to regenerate phenolic antioxidants in the aqueous phase. This suggests that, in addition to the above considerations on the lifetime of Tyr and Trp radicals, the antioxidant mechanism depends also on the partition coefficient of the antioxidant as well as on the diffusibility of peroxynitrite/$CO_2$-derived radicals through the LDL protein (in proteins Tyr is often in a more hydrophilic environment than Trp and should be more accessible to the charged $CO_3^{\bullet-}$ radical, while the $^\bullet NO_2$ radical freely permeates hydrophobic environments). Because the protection afforded by ascorbate and tocopherols was weak and their distribution is more selective (aqueous and lipid phases, respectively), it is tempting to speculate that polyphenols are more active because of their amphipathic properties and that diffusion between the hydrophilic and hydrophobic phases may help to protect (repair) LDL aromatic residues from peroxynitrite/$CO_2$-mediated oxidation. Taking all the above considerations together, it may be concluded that the antioxidant mechanism of phenolics depends not only on their reducing power and partition coefficient but also on the characteristics (diffusibility, reactivity, lifetime, etc.) of both oxidant and target.

**Biological Relevance.** Despite the consistent finding that phenolic antioxidants possess a strong antioxidant potential, recent studies demonstrate that their bioavailability is limited and depends on variables such as metabolic transformation and the interaction with other nutrients and with gut microflora (*50*). After consumption of 10−500 mg of total polyphenols, plasma concentration of any individual rarely exceed 1 $\mu$M (*50*), while this as well as other studies (see the references reported in (*9*)) showed that the antioxidant activity of dietary polyphenols in vitro can be demonstrated at higher concentrations.

On the other hand, at least for peroxynitrite, the relatively high concentration of antioxidants needed to protect LDL aromatic residues was somewhat expected, based on the following considerations. First, phenolic antioxidants have not only antioxidant but also prooxidant effects (*51*). Although we observed no phenolic-dependent increase in the oxidation of Trp and Tyr residues, it cannot be completely ruled out that *O*-semiquinone radicals may restrict the antioxidant efficiency through secondary oxidations. Second, LDL is a large protein rich in aromatic residues (151 Tyr and 37 Trp) and 1.5 mg LDL/mL corresponds to a Tyr and Trp concentration of 453 and 111 $\mu$M, respectively. Although only a fraction of these residues is expected to be oxidizable by peroxynitrite/$CO_2$, it is not unreasonable to suppose that LDL Trp and Tyr residues

represent favorable targets, unless the antioxidant is present at comparable concentrations. In line with these considerations, the $IC_{50\%}$ of active phenolic antioxidants against peroxynitrite/$CO_2$-mediated LDL Tyr nitration was 40−100 $\mu$M, a value that is slightly better than or similar to that of urate ($IC_{50\%}$ = 100 $\mu$M) a biologically relevant peroxynitrite scavenger (*48*). In conclusion, although cooperation with other antioxidants can further decrease the $IC_{50\%}$ of polyphenolics, as demonstrated for ascorbate in this and other studies (*52*), their antioxidant activity appears to occur at concentrations at least 1 order of magnitude higher than their bioavailability (*50*).

Despite these considerations, it cannot be concluded that a polyphenol-rich diet does not provide significant protection against peroxynitrite-mediated oxidation or that the relevance of phenolic antioxidants for human health is restricted to mainly pharmacological uses. In fact, the concentration of polyphenols in the gut is significantly higher than that in plasma (mM versus $\mu$M), so that at the level of the gastro-intestinal tract, a diet rich in fruit and vegetables would be expected to perform a relevant protective and preventive action (*9*). Moreover, atherosclerosis may result, at least in part, from oxidative processes promoted by oxidant species contained in foods or generated during digestion (*53*), against which a diet rich in phenolic antioxidants is probably an important first defense. Finally, uncertainties in the polyphenol intake remain (*50*), and more than 4000 flavonoids have been found in plants, fruits, and vegetables. The cooperative and synergistic effects generated between endogenous antioxidants, polyphenols, and their metabolites as well as their relative bioavailability are largely unknown and need to be studied in greater detail.

## ABBREVIATIONS USED

Apo B-100, apolipoprotein B-100; DTPA, diethylenetriaminepenta-acetic acid; LDL, low-density lipoprotein; 3-Nitro-Tyr, 3-Nitrotyrosine.

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