Free Radical Research, 2002 VOL. 36 (6), pp. 621–631

Taylor & Francis
healthsciences

The Interaction of Resveratrol with Ferrylmyoglobin and Peroxynitrite; Protection Against LDL Oxidation

PAULA BRITO, LEONOR M. ALMEIDA and TERESA C.P. DINIS*

Laboratório de Bioquímica, Faculdade de Farmácia, Universidade de Coimbra and Centro de Neurociências, Couraça dos Apóstolos, 51, r/c, 3000-295 Coimbra, Portugal

Accepted by Professor Victor Darley-Usmar

(Received 8 August 2001; In revised form 5 November 2001)

Resveratrol (3,4′,5-trihydroxystilbene) is a natural phytoalexin synthesized in response to injury or fungal attack, found in the grape skin and wine, specially red wine. A large number of studies have demonstrated that resveratrol regulates many biological activities, namely protection against atherosclerosis by a set of pharmacological properties, including the antioxidant activity.

In this study, we explored the capacity of resveratrol in protecting low density lipoproteins (LDL) against either ferrylmyoglobin- or peroxynitrite-mediated oxidation and the underlying mechanisms of its antioxidant potential. Resveratrol efficiently decreases the accumulation of hydroperoxides in LDL promoted by ferrylmyoglobin, a potent oxidant formed by the reaction of metmyoglobin with hydrogen peroxide, in a concentration-dependent manner, promptly reducing the oxoferryl complex to metmyoglobin. Simultaneously, resveratrol is consumed as detected by the rapid decrease in the characteristic peak at 310 nm, in a similar way to that observed upon its reaction with peroxidase/H_2O_2, pointing to a mechanism of one-electron oxidation and subsequent resveratrol dimer formation.

On the other hand, resveratrol inhibits LDL apoprotein modifications induced by peroxynitrite, another potent oxidant formed by the reaction between superoxide and nitric oxide, as assessed by the decrease in apo-B net charge alterations and in carbonyl groups formation mediated by that oxidant. Resveratrol also interacts with peroxynitrite in a similar way to that observed with laccases, suggesting a mechanism of resveratrol oxidation rather than a nitration one. These mechanisms are discussed.

Considering that either ferrylmyoglobin or peroxynitrite are physiologically relevant oxidants implicated in several pathologies, including atherosclerosis, our results certainly contribute to the understanding of the antioxidant action of resveratrol and consequently provide a new approach for the cardiovascular benefits associated with moderate consumption of red wine.

Keywords: Resveratrol; Ferrylmyoglobin; Peroxynitrite; LDL; Red wine

INTRODUCTION

Resveratrol (3,4′,5-trihydroxystilbene) (Fig. 1) is a natural phytoalexin synthesized by plants, including *Vitis vinifera* (grapevine), in response to injury or fungal attack.[1]

In the grapevine, *trans*-resveratrol is produced in response to fungal infection (particularly by *Botrytis cinerea*) or to environmental stress and it is mainly present in the grapes' skin. Therefore, resveratrol is expected to occur in grape products like wine, especially red wine. Differences in the resveratrol content of wines have been reported, in dependence on several factors, namely grape cultivar, geographical origin, wine type, *Botrytis* infection and oenological practices.[1,2] Recent studies have suggested that resveratrol could be one of the wine active ingredients responsible for the reduced coronary heart disease, in moderate wine drinkers, due to a set of pharmacological properties, such as antioxidant activity,[3,4] modulation of hepatic apoliprotein and lipid synthesis,[5] and inhibition of either platelet

*Corresponding author. Fax: +351-239852569. E-mail: tcpdinis@ci.uc.pt

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290029083

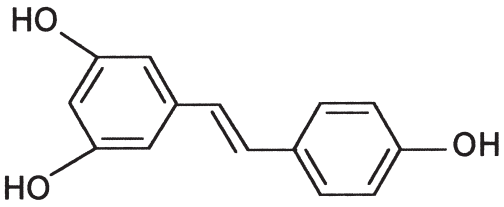

FIGURE 1 Chemical structure of *trans*-resveratrol (3, 4′, 5-trihydroxystilbene).

aggregation or proatherogenic eicosanoids production by human platelets and neutrophils.[6]

The aim of this study was mainly to explore the antioxidant activity of resveratrol in terms of its ability to protect low density lipoproteins (LDL) against oxidation promoted by either ferrylmyoglobin or peroxynitrite, two physiologically relevant oxidants involved in the pathophysiology of cardiovascular diseases.

Ferrylmyoglobin is the oxidation product of the reaction between metmyoglobin (Mb^{III}) and H_2O_2 and contains two electrophilic centers: the oxoferryl complex ($Fe^{IV} = O$), a moderately stable species and a transient protein radical.[7] This hypervalent state of myoglobin is a potent oxidant that has the ability to oxidize a variety of compounds, electron donors, and to initiate deleterious chain reactions. Such fact has biological relevance considering that ferryl haemeproteins have been found to occur *in vivo* and may, consequently, be implicated in atherogenesis and other pathologies.[8]

Peroxynitrite ($ONOO^-$) is also a highly reactive species, but not a radical, produced *in vivo* by the reaction between superoxide anion ($O_2^{\cdot -}$) and nitric oxide (NO).[9,10]

A variety of cells, including endothelial cells, neutrophils and activated macrophages, produce significant quantities of $O_2^{\cdot -}$ and NO· during inflammatory response, so, they have been considered the major sources of $ONOO^-$ *in vivo*. Several studies have reported that $ONOO^-$ is capable of oxidizing a wide variety of biomolecules, including proteins, lipids, DNA and carbohydrates.[11,12] Consequently, $ONOO^-$ has been implicated in several inflammatory disorders such as rheumatoid arthritis and atherosclerosis.[13]

On the other hand, oxidative modifications of LDL are believed to play a significant role in atherosclerosis and consequently in the development of cardiovascular diseases.[14] In fact, LDL are subject to attack by oxidant challengers that can modify both the lipid and protein moieties, making them more atherogenic than native LDL. Oxidized LDL (ox-LDL) are taken up unspecifically by scavenger receptors in macrophages, in a non-controlled process, leading to the formation of foam cells, the first event in the atherome plaque formation.[14]

Assuming that a causal relationship exists between oxidized LDL and atherosclerosis, it will be predictable that antioxidants may protect against atherosclerosis by preventing or retarding the oxidative modification of LDL. Therefore, this study intended to explore the ability of resveratrol to prevent oxidative modifications in the lipid and protein moieties of LDL, mediated either by ferrylmyoglobin or by peroxynitrite in order to contribute to the understanding of the underlying mechanisms of the antioxidant activity of resveratrol.

Thus, the main purpose of the present work was to evaluate the capacity of resveratrol to inhibit both LDL lipid oxidation promoted by ferrylmyoglobin and peroxynitrite-mediated LDL protein modifications, as assessed by either the conjugated dienes formation or the increase in the relative electrophoretic mobility and carbonyl groups formation, respectively. Also, the interaction between resveratrol and oxidants was studied by spectral changes, upon reaction of resveratrol with ferrylmyoglobin and peroxynitrite.

MATERIALS AND METHODS

Chemicals

Resveratrol, diethylenetriaminepentaacetic acid (DTPA), horse heart myoglobin, peroxidase type VI A (EC.1.11.1.7), guanidine and laccase (EC.1.10.3.2) were obtained from Sigma Chemicals (St. Louis, MO). All the other reagents were of analytical grade for biochemical purposes.

Resveratrol was solubilized in ethanol containing 100 μM DTPA. The stock solution (10 mM) was stored at 4°C protected from light, for less than 2 weeks. Immediately before use, resveratrol was diluted with water purified in a Milli Q system at the required concentrations for the different assays. Horse heart myoglobin solution was dialyzed twice against phosphate buffer, before use. The first dialysis was performed against phosphate buffer (0.1 mM potassium phosphate), pH 7.4, containing 1 mM DTPA for 2 h at 4°C, and the second dialysis against a phosphate-buffered saline solution (20 mM Na_2HPO_4, 110 mM NaCl pH 7.4) (PBS) for 8 h at 4°C. The concentrations of metmyoglobin and hydrogen peroxide stock solutions were calculated on the basis of their absorbances at 632 nm ($\varepsilon = 2.1\,mM^{-1}\,cm^{-1}$) and 240 nm ($\varepsilon = 43.6\,M^{-1}\,cm^{-1}$), respectively.

Isolation of Low-density Lipoproteins

LDL particles were isolated from fresh healthy human plasma, as described by Vieira *et al.*,[15] by density gradient ultracentrifugation, at 15°C for 3 h, at 65,000 rpm in a Beckman L 80 ultracentrifuge

(Beckman Inst. Inc., Palo Alto, CA, USA) equipped with a Beckman 70.1 Ti fixed angle rotor. LDL fraction was then concentrated and dialyzed by ultrafiltration under N_2 atmosphere. LDL samples were diluted with sucrose and stored at −80°C under N_2 atmosphere.[15] Protein concentration was determined according to Lowry[16] using bovine serum albumin (BSA) as standard.

Peroxynitrite Synthesis

Peroxynitrite was synthesized in a quenched flow reactor from nitrite and H_2O_2 as described by Beckman *et al.*[17] The solution was frozen at −20°C for approximately 24 h. At this temperature, peroxynitrite concentrates into a dark yellow top layer (freeze fractionation), which was removed and stored at −80°C under N_2 atmosphere. The concentration of peroxynitrite solution was calculated from the absorbance measurement at 302 nm ($\varepsilon = 1670\,M^{-1}\,cm^{-1}$). Before use, peroxynitrite was diluted with 4 mM NaOH. To control the potential effect of residual contaminants, peroxynitrite was allowed to decompose for 1 h at 37°C in the phosphate buffer before LDL addition[18] (reverse order assay).

LDL Lipid Oxidation Induced by Ferrylmyoglobin

LDL (90 μg or 120 μg protein/ml) were incubated at 37°C in phosphate buffer medium containing 100 μM DTPA, and the oxidation reaction was initiated by addition of metmyoglobin (6 μM) followed 3 min later by hydrogen peroxide (9 μM). The extent of lipid oxidation was evaluated directly in the LDL solution, by the measurement of conjugated diene hyperoxides formation, either by the increase in the absorbance intensity at 234 nm[19] or by a more accurate and reliable method based on a second derivative spectrophotometry according to Laranjinha *et al.*[20] in a Perkin–Elmer Lambda 6 spectrophotometer, at 37°C. When present, resveratrol was added 1 min before H_2O_2.

Interaction between Resveratrol and Myoglobin

Myoglobin spectral changes in the visible region were recorded in a Perkin–Elmer Lambda 6 Spectrophotometer, at 37°C. The reaction mixtures, in 2 ml of PBS with 100 μM DTPA, contained 20 μM metmyoglobin and resveratrol at different concentrations and different addition times, as indicated in the figure legends. The oxidation reaction was started by the addition of 30 μM hydrogen peroxide. Resveratrol modification was followed by spectral analysis, between 220–360 nm. At the concentration range used, resveratrol did not show any measurable effect on the metmyoglobin spectrum.

LDL Oxidation Induced by Peroxynitrite

When the oxidation of LDL particles was induced by peroxynitrite, the experimental conditions were similar to those indicated above, but the addition of the oxidant was sequential in order to reach a steady-state concentration of 100 μM during the first 5 min (500 μM final concentration), as recommended by Sies and Arteel.[21] Apo B modifications were assessed by alterations in the net surface charge and carbonyl groups formation.

Electrophoretic Mobility

Changes in the net negative charges of apo B during peroxynitrite-mediated LDL oxidation were measured by agarose gel electrophoresis (Paragon Lipo Gel electrophoresis Kit, Beckman). LDL (360 μg protein/ml) in phosphate buffer pH 7.4 containing 100 μM DTPA, were spiked with peroxynitrite in the absence and presence of resveratrol. The electrophoretic mobility of the LDL samples was measured and expressed as Relative Electrophoretic Mobility (REM), i.e. as the ratio of the distances migrated by the treated and untreated (native) LDL samples. Lipoproteins were visualized by staining with Sudan Black B.

Determination of the Content of Reactive Carbonyl Groups

The apoprotein carbonyl content was determined essentially as previously described by Levine *et al.*[22] by using the 2,4-dinitrophenilhydrazine (DNPH) reagent in HCl. LDL (750 μg protein) were incubated in 1 ml PBS containing 100 μM DTPA, pH 7.4, with 500 μM peroxynitrite (final concentration), for 1 h at 37°C, in the presence of 20 μM resveratrol added 3 min before peroxynitrite. Controls without peroxynitrite or resveratrol were run in parallel. The carbonyl content was calculated from the absorbance of protein hydrazones at 370 nm, on the basis of its molar absorption coefficient, $\varepsilon_{370} = 22,000\,M^{-1}\,cm^{-1}$. As apo B in LDL is considered like a purified protein, guanidine hydrochloride was used as a blank.[23] The final reactive carbonyl groups content in apo B was expressed as nmol carbonyl/mg protein.

Interaction of Resveratrol with Peroxynitrite

Resveratrol spectral changes upon reaction of 20 μM resveratrol with 100 μM peroxynitrite or laccase (200 U), in PBS pH 7.4, containing 100 μM DTPA, were recorded in a Perkin–Elmer Lambda 6 Spectrophotometer at 37°C.

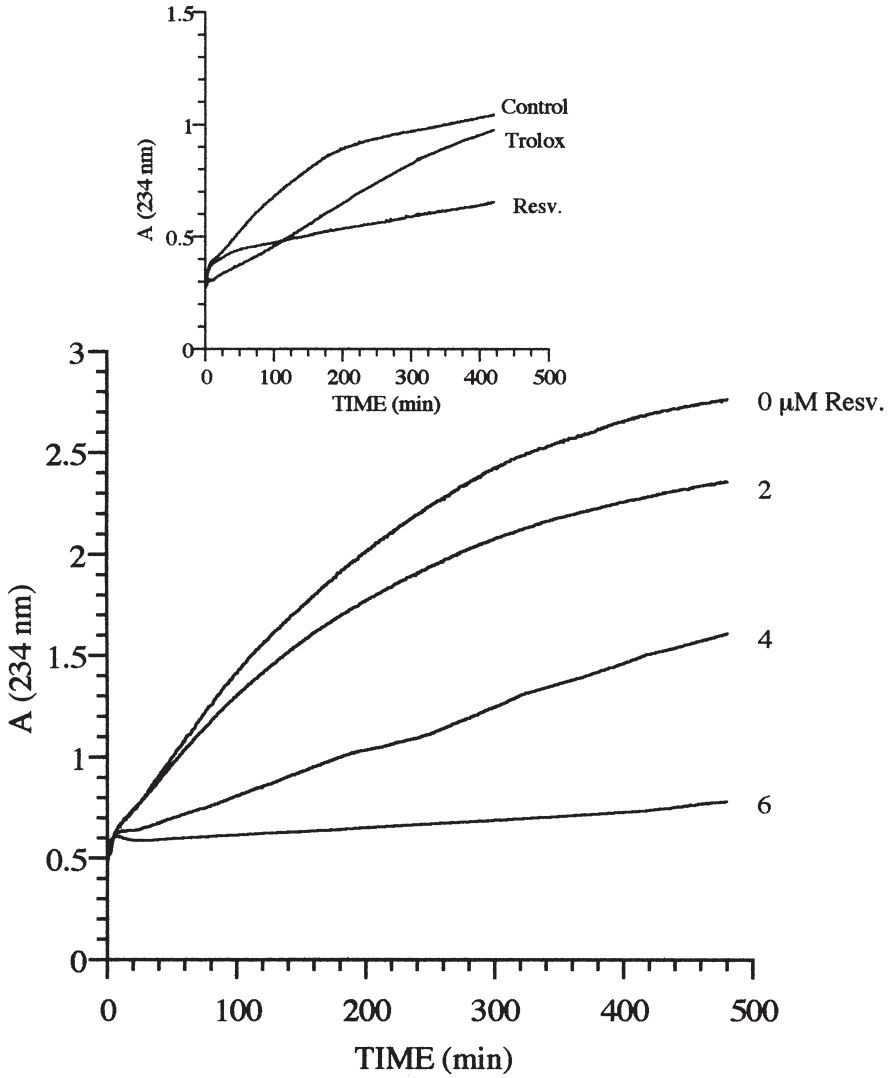

FIGURE 2 Effects of different concentrations of *trans*-resveratrol (Resv.) on conjugated dienes formation during LDL oxidation promoted by ferrylmyoglobin. LDL (90 μg protein/ml) were incubated in phosphate buffer medium containing 100 μM DTPA and 6 μM metmyoglobin at 37°C. Lipid oxidation was started by the addition of 9 μM H_2O_2 in the absence or presence of 2, 4 or 6 μM resveratrol, which was added 1 min before H_2O_2. The recordings are typical assays of three different experiments. Inset represents a typical assay where the effects of resveratrol and Trolox at the same concentration (6 μM) are compared.

RESULTS

LDL Oxidation Promoted by Ferrylmyoglobin: Effect of Resveratrol

Conjugated Diene Formation

During LDL oxidation, polyunsaturated fatty acids are converted to fatty acid hydroperoxides, the primary products of lipid oxidation, having conjugated double bonds (dienes) with a characteristic absorbance at 234 nm.[19] The time course of conjugated dienes formation, during ferrylmyoglobin-induced LDL oxidation is presented in Fig. 2. Incubation of LDL with ferrylmyoglobin, in the absence of resveratrol (control), induces a rapid increase in the absorbance at 234 nm, in contrast to that observed with other initiators, namely copper, with which a characteristic lag phase has been referred.[24] This rapid oxidation of LDL by ferrylmyoglobin has been already reported and ascribed to the ferryl radical as a direct initiator of lipid peroxidation.[25]

The increase in the absorbance intensity is significantly reduced by the pre-incubation of LDL with resveratrol (2, 4 or 6 μM) in a concentration-dependent manner, pointing out that resveratrol affords an efficient protection against the ferrylmyoglobin-mediated LDL oxidation as compared with Trolox, a hydrosoluble vitamin E analog (Fig. 2, inset). Indeed, 6 μM resveratrol practically inhibits conjugated dienes formation in our assay conditions.

Considering that the conjugated dienes hydroperoxides have either *cis*, *trans*, or *trans*, *trans* stereochemistry with distinct absorbances at 242 and 233 nm, respectively, it is possible, by using a more accurate and reliable method based on second

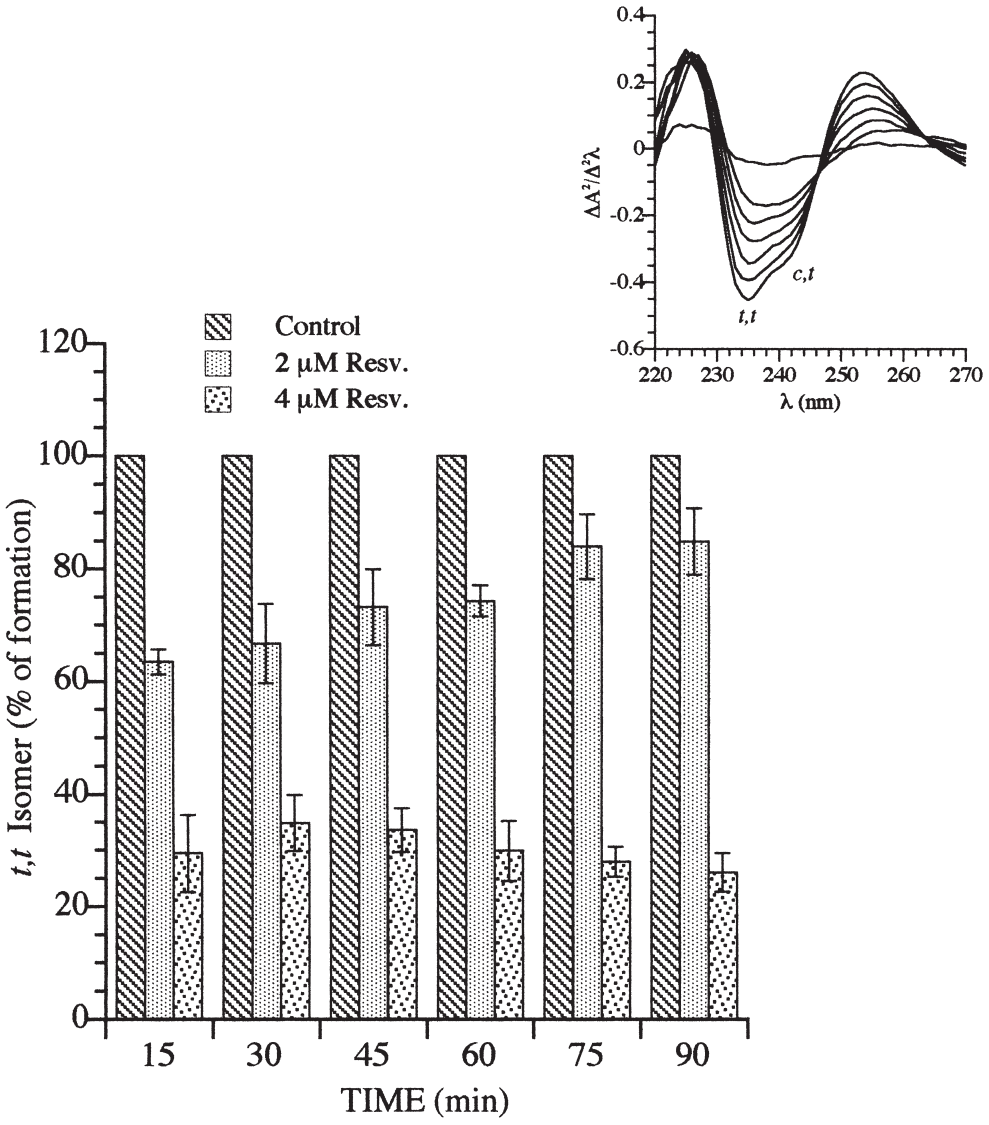

FIGURE 3 Inhibitory effects of *trans*-resveratrol on ferrylmyoglobin-induced LDL lipid oxidation, as measured by the formation of the *trans*,*trans* conjugated diene hydroperoxides, at different incubation times. The amount of this isomer was evaluated by second derivative spectrophotometry as the height of minimum peak (inset) at 233 nm, and as expressed as % of that formed in control, i.e. in absence of resveratrol. LDL (120 μg protein/ml) were incubated in the same experimental conditions as described in legend to the Fig. 2. Data shown are mean values ± SD of three different set of experiments.

derivative spectrophotometry, to confirm and simultaneously to evaluate the two isomers.[26,20] Figure 3 depicts the effect of resveratrol on the *trans*, *trans* isomer formation, in LDL under the action of ferrylmyoglobin as evaluated by the height of the minimum peak at 233 nm in the second derivative spectrum (inset), at different incubation times. In these experimental conditions, relatively low concentrations of resveratrol (2 and 4 μM) significantly decrease the conjugated diene accumulation along the reaction time as evidenced by the decrease in the *t*,*t*-isomer formation. In the presence of 6 μM resveratrol no *t*,*t*-isomer formation could be observed during the reaction time of the assay, indicating that at this concentration and for this time period resveratrol completely inhibits LDL lipid peroxidation. The inhibitory effect of resveratrol on the *cis*, *trans* isomer formation is similar to that referred to the *trans*, *trans* isomer (data not shown).

Interaction between Resveratrol and Ferrylmyoglobin

Resveratrol-mediated Redox Transitions of Ferrylmyoglobin

Figure 4 depicts spectral changes of metmyoglobin induced by H_2O_2 ($H_2O_2/Mb^{III} = 1.5$) and subsequent addition of resveratrol. After peroxide addition, the original metmyoglobin spectrum (—) with absorption peaks at 505 and 631 nm turns in the typical spectrum of ferrylmyoglobin ($Fe^{IV} = O$), with

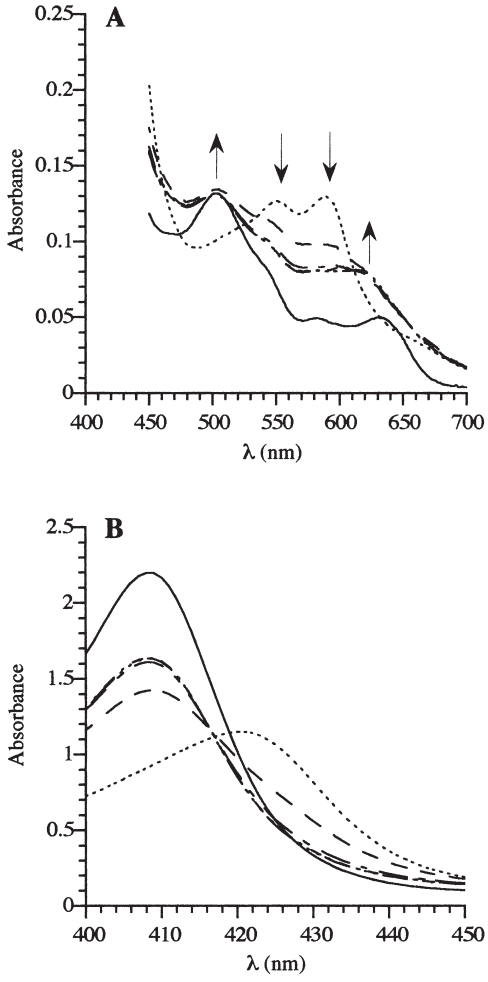

FIGURE 4 (A) Spectral changes of ferrylmyoglobin upon reaction with resveratrol. A solution of 20 μM metmyoglobin (—) in phosphate buffer was spiked with 30 μM H_2O_2 to form ferrylmyoglobin (- - -). After 10 min, 20 μM resveratrol was added and spectra were taken immediately (– – –) and further after 5 (– – –), 10 (– · – · –) and 20 min (– ··· ··· ··· –). Downward and upward arrows indicate decrease or increase, respectively, in the absorbance as the reaction proceeds. (B) Spectral changes at the Soret band, in the same experiment, with traces as in (A). Spectra are representative of at least three independent experiments.

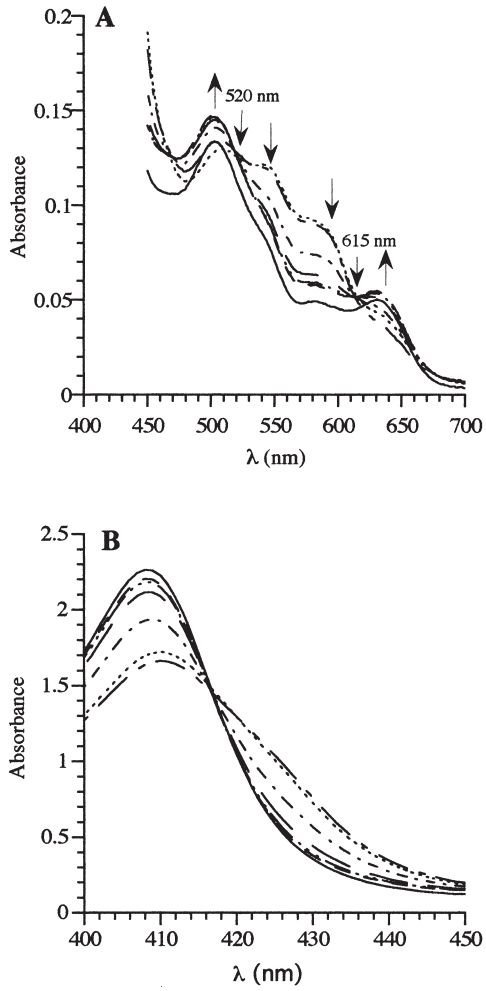

FIGURE 5 (A) Spectral changes of metmyoglobin after reaction with H_2O_2 in the presence of resveratrol. A solution of 20 μM metmyoglobin in phosphate buffer (—) containing 20 μM resveratrol was supplemented with 30 μM H_2O_2 to initiate the reaction. Spectra were taken immediately (- - -) and further, at 5 min intervals until obtaining a constant profile. (– – –) 5, (– · – · –) 10, (— —) 15, (– ··· –) 20 and (– – –) 30 min after addition of H_2O_2. Downward and upward arrows indicate decrease or increase in the absorbance as the reaction proceeds. (B) Represents the spectral changes in the Soret band in the same experiment. Traces as in (A). Spectra are representative of at least three independent experiments.

peaks at 548 and 582 nm and isosbestic points at 519 and 629 nm (- - -) (Fig. 4A). Addition of resveratrol (20 μM) to the preformed ferrylmyoglobin solution leads to rapid spectral changes (– –) in the (Fe^{IV} = O) spectrum toward a spectrum (– · –) resembling that of native metmyoglobin. However, this new spectrum is characterized by the loss of the isosbestic points and a blue shift of the characteristic absorbance peak of native Mb^{III}, at 631 nm, suggesting an irreversible modification of metmyoglobin. Also, in the Soret region (Fig. 4B), spectral changes are detected. Actually, the characteristic absorption peak at 421 nm of ferrylmyoglobin (- - -) is shifted toward that of native metmyoglobin at 408 nm (—) after resveratrol addition.

When resveratrol is present in the reaction medium, prior to the H_2O_2 addition to metmyoglobin, a rapid and transient appearance of the ferrylmyoglobin spectrum occurs without any shift of the characteristic peaks. However, this new spectrum turns back in a relatively short period of time (ca. 20 min), although the full recovery of native metmyoglobin is not achieved because a new isosbestic point appears at 615 nm (Fig. 5A). In the Soret region (Fig. 5B), only a transient decrease in the 408 nm absorption peak, characteristic of Mb^{III}, is observed and the characteristic 421 nm peak of Fe^{IV} = O is not detected.

Moreover, time courses of metmyoglobin oxidation by H_2O_2 in the presence of varying concentrations of resveratrol before or after H_2O_2 addition were followed at 556 nm, the highest

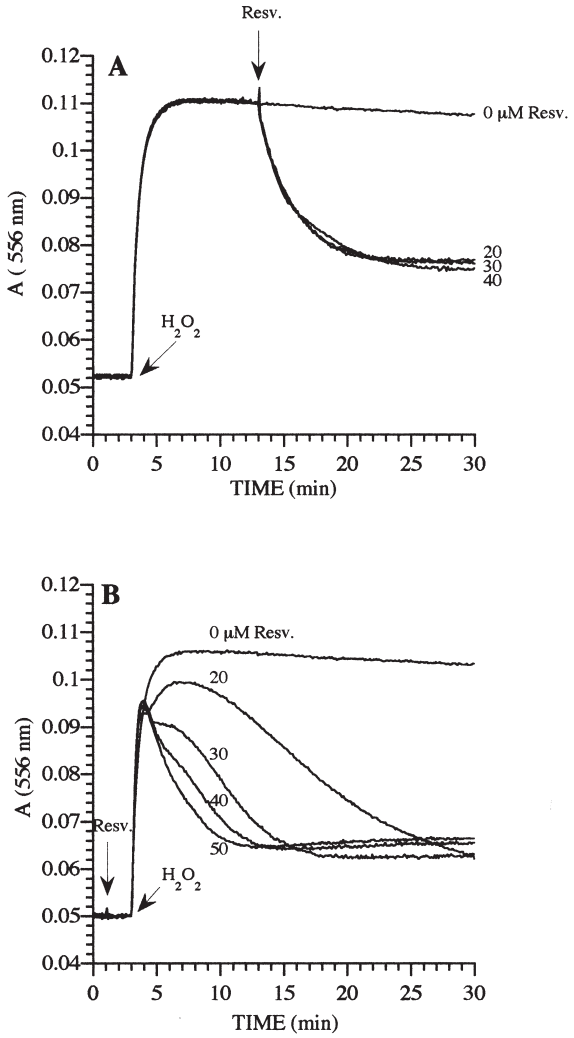

FIGURE 6 Time courses on metmyoglobin oxidation by H_2O_2 in the presence of varying concentrations of resveratrol, added after (A) or before (B) H_2O_2. The absorbance intensity 556 nm and 20 μM metmyoglobin in phosphate buffer was recorded during 3 min and afterwards, the oxidation reaction was started by the addition of 30 μM H_2O_2. In (A) resveratrol was added 10 min latter and in (B) resveratrol was previously added (2 min before) as indicated by the arrows. Recordings are representative assays of at least three independent experiments.

sensitivity wavelength for the metmyoglobin ↔ ferrylmyoglobin redox transitions (Fig. 6). When resveratrol is added after oxidation of metmyoglobin by H_2O_2, the kinetic pattern is reverted, although not completely, and the effect is concentration independent (Fig. 6A). However, when different concentrations of resveratrol are added before starting ferrylmyoglobin formation (Fig. 6B), the kinetic profile of metmyoglobin oxidation is resveratrol concentration-dependent in the first minutes (about 30 min for the lower concentration and 15 min for the others). Also, in this case, in the concentration range used, we do not observe the full recovery of metmyoglobin.

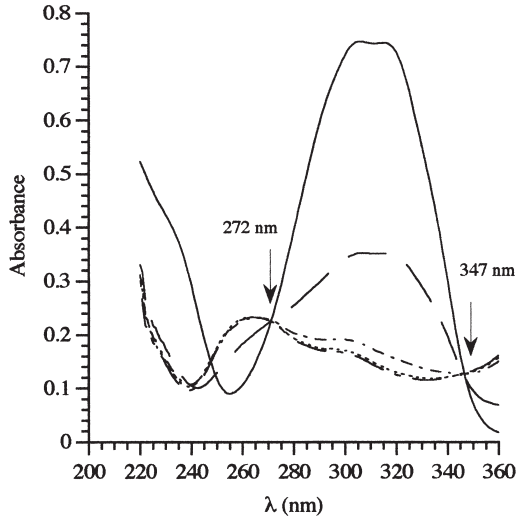

FIGURE 7 Ultraviolet spectral changes of resveratrol after interaction with ferrylmyoglobin. Absorption spectrum of 20 μM resveratrol (—) in phosphate buffer and spectral changes after reaction with 6 μM Mb^{III}/9 μM H_2O_2 at different incubation times. The reaction was started by addition of H_2O_2 and spectra were taken immediately (– –) and after 1 (– · – · –), 3 (- - -) and 5 min (– – –). Arrows point to the isosbestic points. Spectra are representative of at least three independent experiments.

Ultraviolet Spectral Changes of Resveratrol Promoted by Ferrylmyoglobin

The consumption of resveratrol as a result of its reaction with ferrylmyoglobin was accompanied by UV spectrophotometry. Figure 7 displays deep spectral changes of resveratrol (—) during its reaction with ferrylmyoglobin. Two phases may be observed in the spectral profile. An initial very fast phase presenting a substantial and rapid decrease in the absorption maximum peak at 310 nm followed by another one characterized by the appearance of a new peak at 260 nm and two isosbestic points at 272 and 347 nm.

To search the chemical nature of the products formed after interaction of resveratrol with ferrylmyoglobin, resveratrol was used as substrate of horseradish peroxidase (HRP). Moreover, we studied the effect of alkalization of the reaction mixture on the resveratrol spectrum upon interaction with ferrylmyoglobin.

Spectral changes of resveratrol, promoted by HRP catalysis, resembled those described for Mb^{III}/H_2O_2 (data not shown). Considering that the oxoferryl complex exhibits a peroxidase like activity similar to the compound II of HRP,[7] those results point to an one-electron oxidation of resveratrol mediated by ferrylmyoglobin.

On the other hand, it has been reported that alkalization of the resveratrol solution results in a shift of the peak at 310 nm to higher wavelength (base shift), due to the presence of the free *p*-hydroxy group (Fig. 1) in the *trans*-stilbene unit.[27] However, the alkalization of the reaction mixture (resveratrol

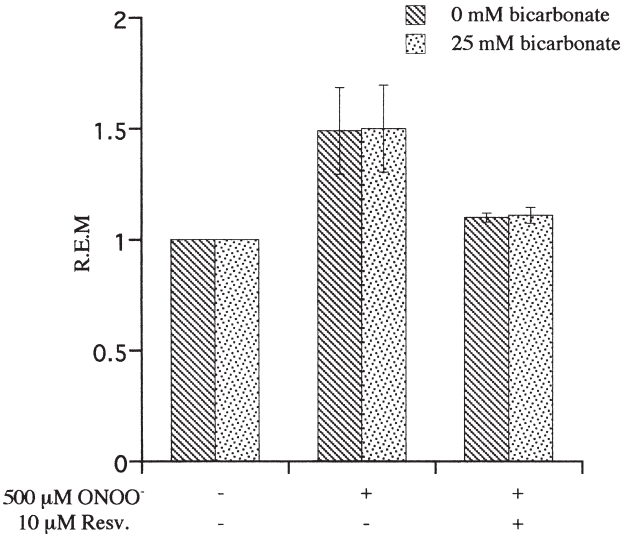

FIGURE 8 Effect of resveratrol on the peroxynitrite-induced increase in the relative electrophoretic mobility (REM) of LDL. LDL (360 μg protein/ml) were challenged by peroxynitrite (500 μM) in the absence (−) or presence (+) of 10 μM resveratrol in phosphate buffer with or without 25 mM bicarbonate. Results represent three different experiments and are expressed as mean values ± SD.

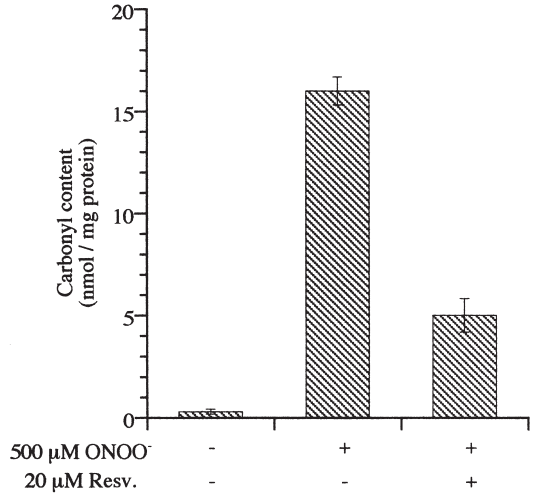

FIGURE 9 Protective effect of resveratrol on peroxynitrite-induced LDL apoprotein modifications as evaluated by carbonyl groups content: Reactive carbonyl groups were determined by the DNPH method as described in "Material and Methods", in the absence (−) and presence (+) of 20 μM resveratrol. Results represent three independent experiments and are expressed as mean values ± SD.

plus ferrylmyoglobin) did not induce any base shift of the long wavelength chromophore, denoting the absence of the free *p*-hydroxy group in the *trans*-stilbene unit (not shown).

LDL Oxidation Promoted by Peroxynitrite: Effect of Resveratrol

Increase in the Net Negative Charge

Incubation of LDL with synthesized peroxynitrite induces an increase in the electrophoretic mobility of lipoprotein particles relative to native LDL, indicating an increase in the net negative charge of LDL (Fig. 8). Such modifications could be ascribed to oxidative or non-oxidative changes in the apolipoprotein associated with potential modifications of amino acid residues. When LDL were pre-incubated with 10 μM resveratrol before treatment with peroxynitrite, the electrophoretic mobility of the lipoprotein decreases in about 80% relatively to that observed in the absence of resveratrol, suggesting that this compound prevents the changes of apo B responsible to its surface charge. Moreover, the results were similar when the assay was carried out in the presence of bicarbonate.

Carbonyl Groups Formation

An early marker for protein oxidation is the carbonyl groups formation. So, we quantified the apoprotein carbonyls formed after incubation of LDL with peroxynitrite in the absence and presence of resveratrol. Figure 9 shows the results obtained. Peroxynitrite induces the carbonyl groups formation, but pre-incubation of LDL with 20 μM resveratrol strongly inhibits their formation. These results point

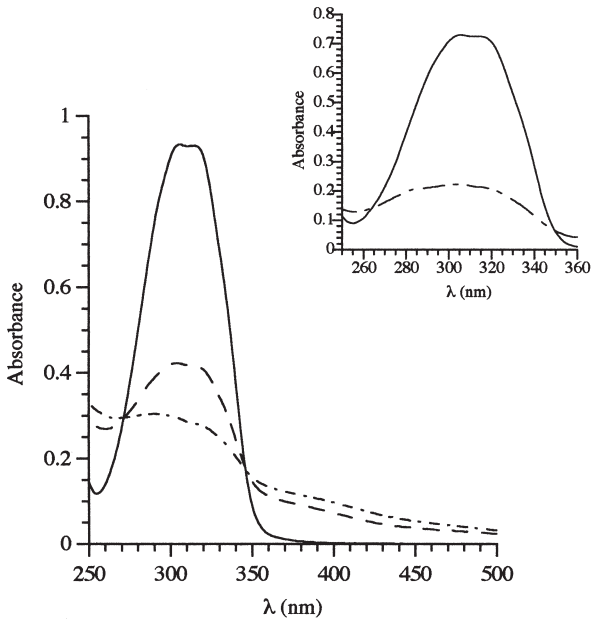

FIGURE 10 Spectral changes of resveratrol upon interaction with peroxynitrite. Absorption spectra of 20 μM resveratrol were taken in a medium containing 20 mM Na_2HPO_4 12 H_2O, 110 mM NaCl, 100 mM DTPA, pH = 7.4 in the absence (—) and presence of 100 (– –) and 250 μM (–·–·–) peroxynitrite. The spectral changes promoted by this oxidant are identical to those catalyzed by laccase (inset). Here, spectra of 20 μM resveratrol, in the referred medium, were taken in the absence (—) and presence of 200 U laccase (– – –). Worthy of notice that the isosbestic points are very similar. Spectra are representative of at least three independent experiments.

to resveratrol as an efficient protector of LDL oxidative damage induced by peroxynitrite.

Spectrophotometric Assays

In order to clarify the product formed upon the reaction of resveratrol with peroxynitrite, the changes in the UV spectrum of resveratrol were recorded after the interaction with this oxidant (Fig. 10). Increasing concentrations of peroxynitrite abolish the 310 nm absorbance peak of resveratrol, but do not lead to the appearance of the characteristic peak of nitrophenol at 430 nm, pointing that nitration does not occur in these experimental conditions.[28]

To support this conclusion, these UV spectra were also compared with those obtained in the presence of laccase (Fig. 10, inset). Laccases are polyphenol oxidases recognized by their abilities to catalyse the oxidation and oxidative polymerization reactions of phenolic substrates.[29,30] So, considering that laccase promotes spectral changes in resveratrol similar to those observed in the presence of peroxynitrite, this result provides evidence about the oxidation reaction between resveratrol and peroxynitrite.

DISCUSSION

In view of that resveratrol could be an active ingredient of wine that may explain the French paradox,[1] it is pertinent to investigate its antioxidant capacity. Frankel *et al.*[3] were the first to report that resveratrol could inhibit copper-induced low density lipoprotein peroxidation. This observation was confirmed by Belguendouz *et al.*,[31] which also showed that resveratrol protects LDL from oxidation promoted by peroxyl radicals generated by thermal degradation of an azocompound. Therefore, resveratrol appears to protect LDL lipids against oxidation both copper chelation and free radical scavenging mechanisms, respectively.[31,32] More recently, the ability of resveratrol to inhibit LDL oxidation induced by UV radiation and NADPH- or ADP-Fe^{3+} has been reported.[33] In this last case, the mechanisms involved has been suggested to be the scavenging of free radicals and not iron chelation as previously proposed by Belguendouz *et al.*[31] Besides the ability to protect lipids against peroxidative degradation, resveratrol has also been reported to protect the protein moiety of LDL challenged by copper or AAPH.[32]

The present study provides more information about the antioxidant activity of resveratrol, by using two relevant physiological oxidants—ferrylmyoglobin and peroxynitrite—supporting the statement that resveratrol protects LDL from both lipid oxidation and protein modifications.

Our data show that the LDL lipid oxidation promoted by ferrylmyoglobin, as evaluated by conjugated diene hydroperoxides production, is efficiently inhibited by pre-incubation of lipoproteins with relatively low concentrations of resveratrol (Figs. 2 and 3). This antioxidant activity of resveratrol may be explained by the reduction of ferrylmyoglobin to metmyoglobin, which is consistent with the optical changes of Mb^{III} observed after the sequential addition of H_2O_2 and resveratrol. In fact, resveratrol shows capacity to revert the spectrum of ferrylmyoglobin to that of metmyoglobin (Figs. 4 and 5), although, more efficiently when resveratrol is present in the medium before H_2O_2 addition (Fig. 5). This observation is consistent with others reported in literature, namely the reduction of ferrylmyoglobin by Trolox,[34] ascorbate[35] and dietary phenolic acids.[36] Like dietary phenolic acids,[36] resveratrol does not fully reverse the structural changes, induced by H_2O_2 in the heme moiety, as reflected by the blue shift of the 631 nm peak (Fig. 4A) and does not retrieve the absorbance value at 408 nm of Mb^{III} (Fig. 4B). Moreover, similarly to those phenolics when resveratrol is present in the medium before H_2O_2 addition, no shift of the characteristic peak of Mb^{III} is observed (Fig. 5A) and the absorbance in the Soret region is practically recovered (Fig. 5B). On the other hand, resveratrol is also oxidized upon reaction with ferrylmyoglobin, to a product chemically related to itself, as suggested by the two isosbestic points at 272 and 347 nm (Fig. 7). Similar spectral changes are observed by HRP catalysis.

Langcake and Price[27] have shown that oxidative dimerization *trans*-resveratrol occurs after treatment with HRP and H_2O_2, yielding a *trans*-dehydrodimer as the major product. The product of reaction between resveratrol and Mb^{III}/H_2O_2 seems to be a similar compound, given that, after alkalization of the reaction mixture no base shift to the longer wavelength of the chromophore is observed (data not shown), denoting the absence of the free *p*-hydroxy group in the *t*-stilbene unit. This dehydrodimer of *trans*-resveratrol probably derives from the association of two presumed radical intermediates centered in the *p*-hydroxy substituted aromatic rings, providing evidence for oxidation of resveratrol within the context of one-electron transfer steps.

Peroxynitrite is also a powerful oxidant that has been reported to induce lipid peroxidation and protein modifications.[28,37] However, recent work in our laboratory have shown that the primary consequence of the interaction of peroxynitrite with LDL is the apoprotein damage, and that such damaging effects are inhibited by dietary phenolic acids.[38]

Resveratrol, in our assay conditions, is able to prevent the effects of peroxynitrite, minimizing the

LDL modifications, as assessed by the decrease in the electrophoretic mobility and by the inhibition in about 70% of carbonyl groups formation (Figs. 8 and 9).

In order to get a more complete evaluation of the peroxynitrite scavenging activity of resveratrol, the assays were also performed in the presence of 25 mM bicarbonate. Actually, physiological concentrations of bicarbonate can modify the ability of dietary phenolics to prevent the peroxynitrite-mediated reactions, as recently demonstrated.[39] However, in our assay conditions, bicarbonate does not affect the resveratrol capacity in protecting LDL against peroxynitrite damage.

It has been reported that the protection afforded by antioxidants, namely hidroxynnamates and flavonoids, to peroxynitrite-induced modifications is mediated through nitration and/or oxidation reactions.[28,40] In our experimental conditions, no nitration reaction is observed, as indicated by the absence of the characteristic absorbance peak of the nitrophenol at 430 nm (Fig. 10). In fact, at the resorcinol ring, such reaction will be difficult to occur, since the potential positions (2-, 4- and 6-) activated by the presence of the hydroxyl groups are, also, sterically hindered (Fig. 1). In ring B, a nitration reaction could occur at 3′- and 5′-positions due to partial activation by the *p*-hydroxy group. Nevertheless, the yield of nitrophenol formation, by direct reaction of phenols with peroxynitrite, is low in the absence of added carbonate to the reaction medium.[41] Therefore, we do not exclude the nitration of resveratrol by peroxynitrite but in a too low extent to be detected spectrophotometrically.

On the other hand, spectral changes of resveratrol upon interactions with peroxynitrite are similar to those observed with laccases (Fig. 10, inset), suggesting that resveratrol is preferentially oxidized by peroxynitrite. From the reaction of resveratrol with laccases, five different dimers have been identified until now.[30] Therefore, our results suggest that resveratrol in the presence of peroxynitrite is able to donate hydrogen leading to the formation of phenoxyl radicals that can react together giving a mixture of resveratrol dimers.

In conclusion, resveratrol putatively protects LDL apoprotein modifications mediated by peroxynitrite by an oxidative reaction, rather than a nitration one, leading to dimers formation. Moreover, our data point out that resveratrol, in relatively low concentrations, efficiently inhibits oxidation of LDL lipid and protein moieties promoted by ferrylmyoglobin and peroxynitrite, respectively, providing new insights into the biochemical mechanisms underlying its antioxidant activity. Additionally, the results of this study, the first one realized with two biologically relevant oxidants, show that resveratrol can efficiently outcompete the multiple reactions that peroxynitrite can undergo.

By protecting lipids and in particular apoB-100 domain from oxidative damage, it is likely that *in vivo*, resveratrol will reduce the non-specific uptake of ox-LDL by macrophages, and may therefore, be relevant in the context of protection against atherosclerosis. Thus, our results provide a new approach for cardiovascular benefits associated with moderate consumption of wine.

Acknowledgements

We are greatly indebted to Prof. João Laranjinha and to Prof. Vítor Madeira for their advice and helpful suggestions. This work was supported by PRAXIS XXI (Proj. C/AGR/10239/98). Paula Brito was a recipient of a grant from PRAXIS XXI (BIC).

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