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Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite

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Abstract

The protective effects of resveratrol (3, 4′, 5-trihydroxystilbene; present naturally in different plants) against the oxidative/nitrative damage of human plasma proteins induced by peroxynitrite ($ONOO^-$) were studied and compared with those of deferoxamine (DFO; a natural siderophore isolated from *Streptomyces pilosus*), which is a typical and well-known antioxidant. We also studied the effect of $ONOO^-$ on plasma lipid peroxidation and the role of tested antioxidants in this process. $ONOO^-$ at the used concentrations (0.01–1 mM) showed toxicity to human plasma components. Exposure of plasma to $ONOO^-$ (0.1 mM) resulted in an increase of the level of carbonyl groups and nitrotyrosine residues in plasma proteins (approximately 4-fold and 76-fold, respectively) and in a distinct augmentation of lipid peroxidation (approximately 2-fold). In the presence of 0.1-mM resveratrol, a distinct decrease of carbonyl group formation and tyrosine nitration in plasma proteins caused by 0.1-mM $ONOO^-$ was observed (by approximately 70% and 65%, respectively). Addition of 0.1-mM DFO to plasma also distinctly reduced the level of carbonyl groups and nitrotyrosines caused by 0.1-mM $ONOO^-$ (by approximately 50% and 60%, respectively). Moreover, these antioxidants also inhibited plasma lipid peroxidation induced by $ONOO^-$ (0.1 mM). The obtained results indicate that in vitro resveratrol, like well-known antioxidant DFO, has inhibitory effects on $ONOO^-$-mediated oxidation of proteins and lipids in human plasma.

Keywords: Plasma; Peroxynitrite; Tyrosine nitration; Carbonyl groups; Resveratrol; Deferoxamine

1. Introduction

The generation of reactive oxygen species (ROS) may occur in a large number of physiological and nonphysiological processes that include their generation as by-products of normal cellular metabolism. ROS may induce oxidative stress and damage to all types of biologic molecules. Different ROS, including superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$), lead to protein oxidation [1]. Oxidative stress causes damage to proteins by an adduction of products of glycoxidation and/or lipid peroxidation and by a direct oxidation of protein side chains [2,3]. ROS may oxidize amino acid residue side chains into ketone or aldehyde derivatives (carbonyl groups) [4]. Histidine, arginine and lysine are the most susceptible amino acids for ROS-mediated carbonyl formation [5]. It has been demonstrated that exposure of protein to $ONOO^-$ results in the oxidation of tryptophan, cysteine and methionine; nitration of tyrosine; formation of dityrosine; carbonyl group formation; and protein fragmentation [6–10]. The rate of $ONOO^-$ formation depends on the concentrations of $O_2^{\cdot-}$ and $^{\cdot}NO$, and even a relatively small increase of their concentrations may be responsible for a remarkable increase of $ONOO^-$ generation and its cytotoxic effects. It is hypothesized that, in vivo, the flux of $O_2^{\cdot-}$ is more critical to the rate of $ONOO^-$ generation than nitric oxide due to $^{\cdot}NO$ being usually in a large molar excess. Modification of proteins induced by $ONOO^-$ and its intermediates may lead to functional alterations of proteins. Our earlier studies showed that incubation of human blood platelets with $ONOO^-$ results in the changes of low-molecular-weight thiols (glutathione, cysteine and cysteinylglycine) [11] and in the depletion of free protein thiols [11] concomitant with an increase of nitrotyrosine amount in platelet proteins [12].

The defense mechanisms against $ONOO^-$ action are very important for biologic functions of human plasma components. Arteel et al. [13] and Klotz and Sies [14] suggest that defense against $ONOO^-$ action on cell structures may be at

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the level of prevention of its formation, at the level of interception or repair of damage resulting from $ONOO^-$ reactions. Various agents were often tested for the ability to protect different molecules (lipids and proteins) from $ONOO^-$-induced changes in their structure and functions. Human diet is rich in a great variety of micronutrients with antioxidant properties. Among these, polyphenolic compounds widely distributed in fruits, vegetables and beverage such as tea, beer and wine hold an important role. However, the role of exogenous antioxidant resveratrol (3, 4′, 5-trihydroxystilbene; present naturally in grapes, fruits and a variety of medicinal plants) in the defense against $ONOO^-$ action in human plasma is still unknown. Therefore, the aim of our study was to estimate the effects of resveratrol on $ONOO^-$-induced changes in plasma proteins and lipids. Moreover, we compared the action of resveratrol with that of deferoxamine (DFO; a natural siderophore isolated from *Streptomyces pilosus*), which is a typical and well-known antioxidant. We determined lipid oxidation and protein oxidation/nitration in human plasma in the presence of antioxidants resveratrol and DFO. Our earlier studies indicate that resveratrol, like DFO, may protect proteins of blood platelet against oxidation caused by $ONOO^-$ or its intermediates [15].

2. Materials and methods

2.1. Materials

$ONOO^-$ was synthesized according to the method of Pryor and Squadrito [16]. Freeze fractionation (−70°C) of the $ONOO^-$ solution formed a yellow top layer, which was retained for further studies. The top layer typically contained 80–100 mM of $ONOO^-$ as determined spectrophotometrically at 302 nm in 0.1 M of NaOH ($\varepsilon_{302\ nm}$=1679 M/cm). Some experiments were also performed with decomposed $ONOO^-$, which was prepared by allowing $ONOO^-$ to decompose at neutral pH (7.4) in 100 mM of potassium phosphate buffer (15 min, room temperature). Resveratrol, DFO (mesylate salt), rabbit anti-dinitrophenylhydrazine (DNP) antibodies, anti-rabbit antibodies and anti-goat/sheep antibodies coupled with peroxidase were purchased from Sigma (St Louis, MO, USA). Stock solutions of resveratrol were made in 50% dimethylsulfoxide at a concentration of 25 mg/ml and kept frozen.

Sheep anti-nitrotyrosine polyclonal antibodies were from Oxis (Portland, OR, USA). Biotinylated anti-goat/mouse/rabbit antibodies and streptavidin-biotinylated horseradish peroxidase were from DAKO (Glostrup, Denmark). All other reagents were of analytical grade and were provided by commercial suppliers.

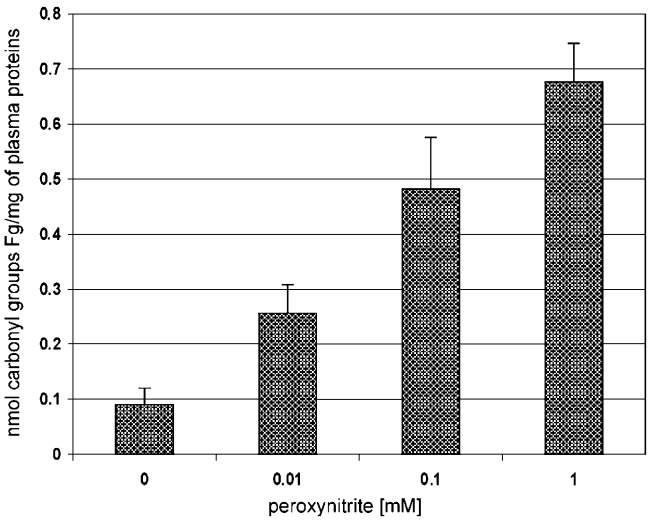

Fig. 1. DNP-reactive carbonyl formation following treatment of human plasma proteins with $ONOO^-$ (0.01–1 mM). The protein oxidation was measured by ELISA method. The results are expressed as nanomoles of carbonyl groups-Fg per milligram of plasma proteins. The results are representative of six independent experiments and are expressed as mean±S.D. The effects of three concentrations of $ONOO^-$ were statistically significant according to one-way ANOVA (P<.02). Correlation coefficient r=.835 (P<.005).

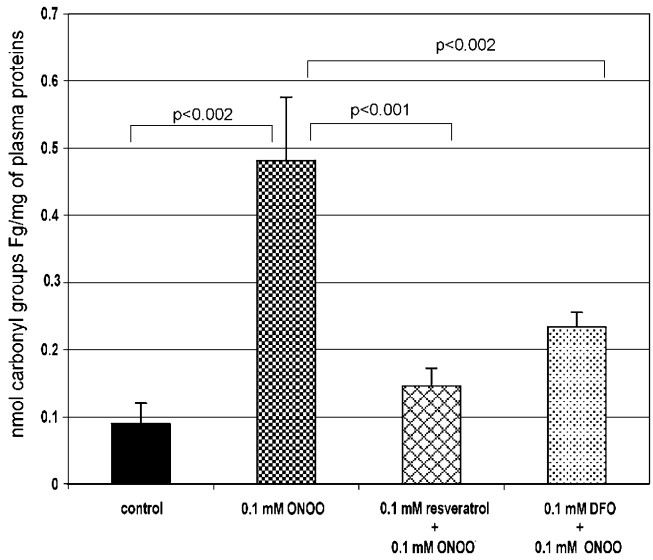

Fig. 2. The effects of resveratrol and DFO (0.1 mM) on carbonyl group formation (protein oxidation) induced by $ONOO^-$ (0.1 mM). The protein oxidation was measured immunologically using ELISA method. The results are expressed as nanomoles of carbonyl groups-Fg per milligram of plasma proteins. The results are representative of six independent experiments and are expressed as mean±S.D. The presented results are three distinct paired comparisons. The effects were statistically significant according to the paired Student's t test, ($ONOO^-$-treated plasma vs. control; resveratrol/DFO+$ONOO^-$-treated plasma vs. $ONOO^-$-treated plasma).

2.2. Incubation of plasma with antioxidants and $ONOO^-$

Human blood from healthy volunteers was collected into sodium citrate (5 mmol/L final concentration) and immediately centrifuged (3000×g, 15 min) to get plasma. $ONOO^-$ was added to plasma as a bolus to the final concentration of 0.01–1 mM and the samples were immediately vigorously mixed. Some samples of human plasma were preincubated (2 min at room temperature) with tested antioxidants

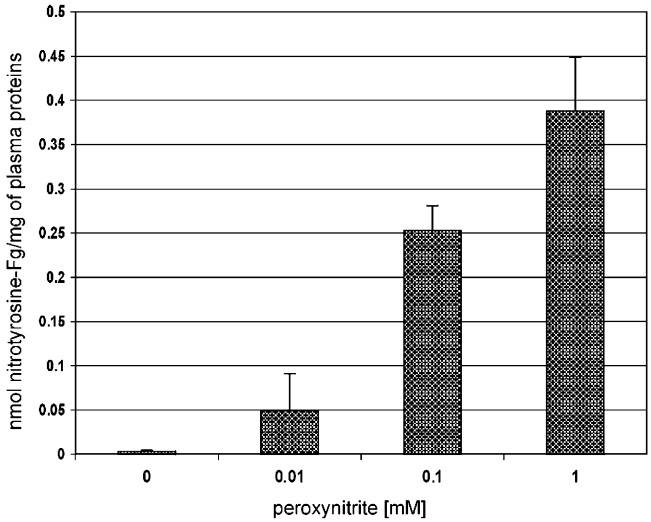

Fig. 3. The effects of $ONOO^-$ (0.01–1 mM) on nitration of tyrosine residues in plasma proteins measured by C-ELISA method. The results are expressed as nanomoles of nitrotyrosine-Fg per milligram of plasma proteins. The results are representative of eight independent experiments and are expressed as mean±S.D. The effects of three concentrations of $ONOO^-$ were statistically significant according to one-way ANOVA ($P<.0001$). Correlation coefficient $r=.822$ ($P<.01$).

(resveratrol and DFO) at final concentrations of 0.025–0.1 mM and then were treated with only one concentration of $ONOO^-$ (at a final concentration of 0.1 mM).

2.3. Determination of nitrotyrosine in the proteins of human plasma by a competition ELISA method

Detection of nitrotyrosine-containing proteins by a competition ELISA (C-ELISA) method in plasma (control or antioxidants and $ONOO^-$-treated plasma) was performed according to the procedure of Khan et al. [17] as described previously [15]. The nitro-fibrinogen (Fg; at concentrations of 0.5 μg/ml and 3–6 mol nitrotyrosine/mol protein) was prepared for use in the standard curve. The linearity of the C-ELISA method was confirmed by the construction of a standard curve ranging from 10 to 500 nM nitrotyrosine-Fg equivalent. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitro-Fg equivalents. The amount of nitrotyrosine present in Fg after treatment with $ONOO^-$ (at a final concentration of 1 mM) was determined spectrophotometrically (at pH 11.5, $\varepsilon_{430\ nm}=4400$ M/cm) [6].

2.4. Detection of carbonyl groups in human plasma proteins by ELISA method

Detection of carbonyl groups by ELISA method (using anti-DNP antibodies) in plasma (control or antioxidants and $ONOO^-$-treated plasma) was carried out according to a method described by Buss et al. [18]. Human plasma proteins reacted with DNP and then proteins were nonspecifically adsorbed to an ELISA plate. The $ONOO^-$-treated Fg (10 nmol of carbonyl groups/mg of Fg) was prepared for use in the standard curve. The linearity of the ELISA method was confirmed by the construction of a standard curve ranging from 0.1 to 10 nmol carbonyl groups/mg of Fg. The amount of carbonyl groups present in Fg after treatment with $ONOO^-$ (at a final concentration of 1 mM) was determined spectrophotometrically as described by Nowak and Wachowicz [19].

2.5. Production of thiobarbituric acid-reactive substances in human plasma

Incubation of plasma (control and treated with $ONOO^-$ and/or antioxidants) was ceased by cooling the samples in an ice bath. Samples of plasma were transferred to an equal volume of 20% (vol/vol) cold trichloroacetic acid in 0.6 M of HCl and centrifuged at 1200×*g* for 15 min. A total of 1 vol of clear supernatant was mixed with 0.2 vol of 0.12-M thiobarbituric acid in 0.26-M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nanomoles of thiobarbituric acid-reactive substances (TBARS) [20].

2.6. Data analysis

The statistical analysis was done by several tests. To eliminate uncertain data, the Q-Dixon test was performed. All the values in this study are expressed as mean±S.D. The statistical analysis was performed with one-way ANOVA for repeated measurements. The significance of the correlation coefficient *r* was analyzed by *t* test. The statistically

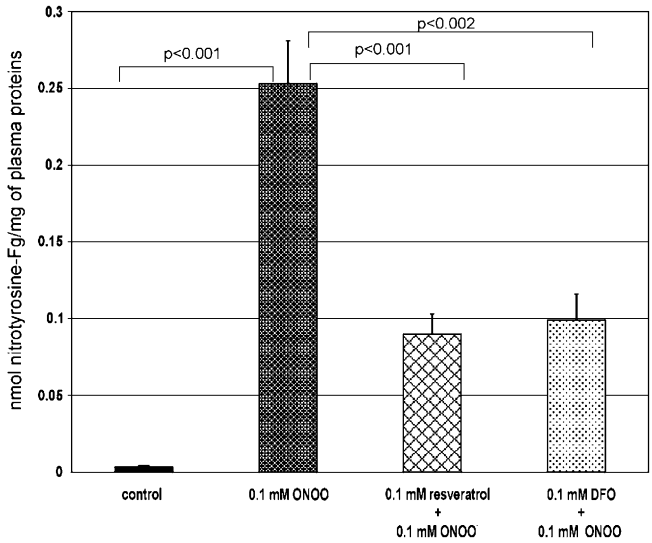

Fig. 4. The effects of resveratrol and DFO (0.1 mM) on nitration of tyrosine residues in plasma proteins induced by $ONOO^-$ (0.1 mM). The tyrosine nitration was measured by C-ELISA method and results are expressed as nanomoles of nitrotyrosine-Fg per milligram of plasma proteins. The results are representative of three independent experiments and are expressed as mean±S.D. The presented results are three distinct paired comparisons. The effects were statistically significant according to the paired Student's *t* test ($ONOO^-$-treated plasma vs. control; resveratrol/DFO+$ONOO^-$-treated plasma vs. $ONOO^-$-treated plasma).

significant differences were also assessed by applying the paired Student's *t* test.

3. Results

Our studies showed that incubation of plasma with $ONOO^-$ resulted in a dose-dependent increase of carbonyl groups in plasma proteins (Fig. 1). Protein carbonyl formation caused by $ONOO^-$ (0.01–1 mM) was detected by ELISA method (Fig. 1) and by Western blot analysis (data are not presented). We have shown that

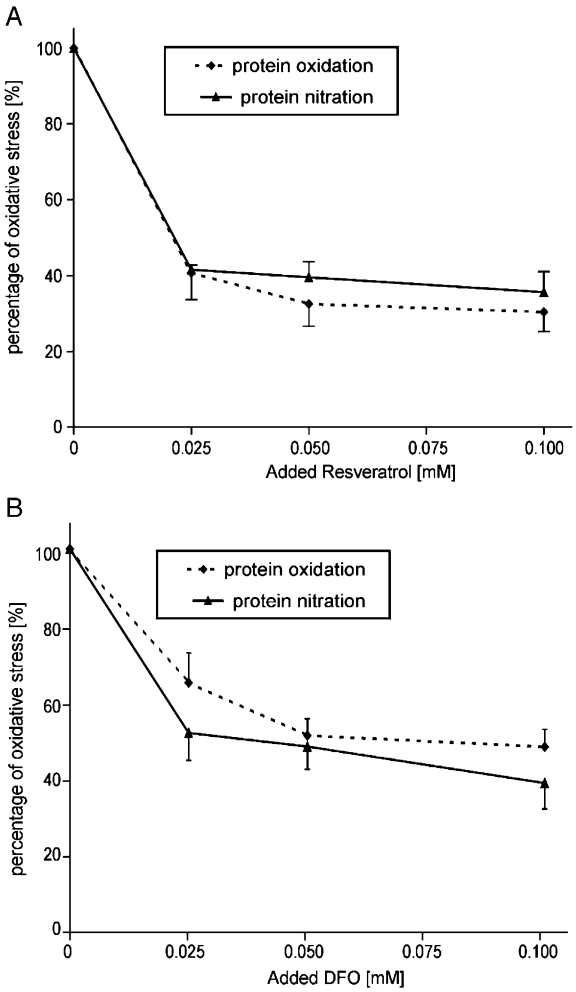

Fig. 5. Inhibition of $ONOO^-$-dependent oxidative stress (protein oxidation and nitration) in the presence of resveratrol (A) and DFO (B) at concentrations of 0.025–0.1 mM. The concentration of $ONOO^-$ used in the experiments was 0.1 mM. The results are representative of three independent experiments and are expressed as mean±S.D. The effects of three concentrations of antioxidants were analyzed by one-way ANOVA. The effects of resveratrol on protein oxidation were statistically significant ($P<.05$) (Fig. 5A). The effects of resveratrol on protein nitration were not statistically significant ($P>.05$) (Fig. 5A). The effects of DFO on both processes (protein oxidation and nitration) were statistically significant ($P<.05$) (Fig. 5B). Statistical analysis of correlation: protein oxidation, $r=-.703$ ($P<.05$) for resveratrol and $r=-.680$ ($P<.05$) for DFO; protein nitration, $r=-.390$ ($P>.05$) for resveratrol and $r=-.699$ ($P<.05$) for DFO.

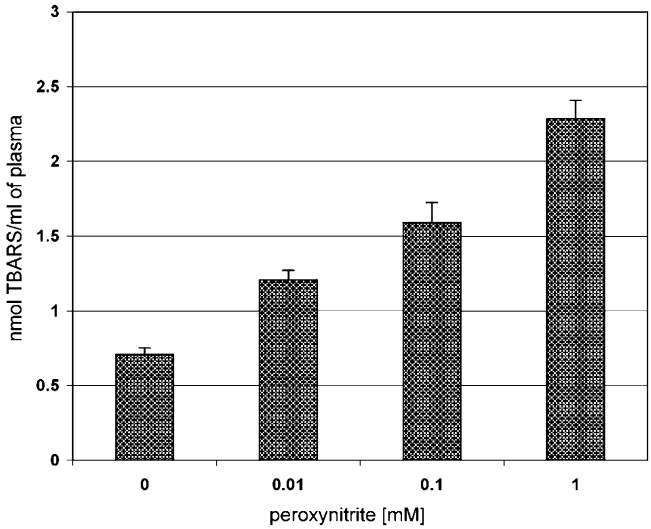

Fig. 6. Effect of $ONOO^-$ (0.01–1 mM) on plasma lipid peroxidation expressed as TBARS (marker of lipid peroxidation). The results are representative of six independent experiments and are expressed as mean±S.D. The effects of three concentrations of $ONOO^-$ were statistically significant according to one-way ANOVA ($P<.001$). Correlation coefficient $r=.805$ ($P<.001$).

two tested antioxidants (0.1 mM) distinctly diminished formation of carbonyl groups in plasma proteins treated with 0.1 mM of $ONOO^-$. Resveratrol and DFO reduced carbonyl group formation by approximately 70% and 50%, respectively (Fig. 2).

Exposure of plasma to $ONOO^-$ resulted also in an increase of nitrotyrosine amount in plasma proteins, as determined by a C-ELISA method (Fig. 3) and by Western blot analysis (data are not presented). Tyrosine nitration of plasma proteins caused by $ONOO^-$ (0.01–1 mM) was dose dependent. We have shown that resveratrol and DFO (0.1 mM) diminished (by approximately 65% and 60%, respectively) tyrosine nitration in plasma proteins induced by 0.1 mM of $ONOO^-$ (Fig. 4). In the presence of tested antioxidants at different concentrations, the reduction of plasma protein oxidation and nitration induced by $ONOO^-$ (0.1 mM) was observed (Fig. 5A and B). Contrary to the dose-dependent effects of DFO (Fig. 5B), resveratrol in the range of the used concentrations (0.025, 0.05 and 0.1 mM) caused only a dose-dependent reduction of protein oxidation (carbonyl groups; Fig. 5A). Resveratrol at the lowest concentration (0.025 mM) reduced the alterations in plasma proteins caused by $ONOO^-$, but the increase of its concentration has no statistical significant effects ($P>.05$) on plasma protein nitration (Fig. 5A).

Incubation of human plasma with $ONOO^-$ at the concentrations of 0.01–1 mM resulted in a distinct dose-dependent increase of TBARS (Fig. 6). After 2-min incubation of plasma with $ONOO^-$ (0.1 mM), the amount of TBARS increased approximately two-fold (Fig. 6); however, in the presence of tested antioxidants (0.1 mM),

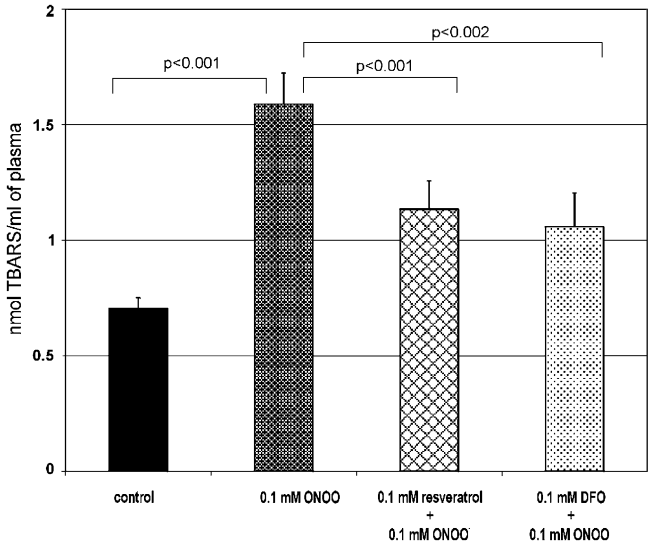

Fig. 7. The effects of resveratrol or DFO (0.1 mM) on the level of TBARS in plasma treated with $ONOO^-$ (0.1 mM). The results are representative of six independent experiments and are expressed as mean±S.D. The presented results are three distinct paired comparisons. The effects were statistically significant according to the Student's *t* test, ($ONOO^-$-treated plasma vs. control; resveratrol/DFO+$ONOO^-$-treated plasma vs. $ONOO^-$-treated plasma).

the TBARS level in plasma was reduced (Fig. 7). Resveratrol, like DFO, at a concentration of 0.1 mM had a very similar inhibitory effect (inhibition by approximately 25%) (Fig. 7).

In control experiments, we have observed that the decomposed $ONOO^-$ did not change the level of TBARS. It also did not cause the tyrosine nitration in plasma protein, nor did protein oxidation (data are not presented).

4. Discussion

$ONOO^-$ may evoke oxidative/nitrative stress in various cells or tissues. It can induce undesirable effects on biologic systems and cause damage to different molecules, including lipids or proteins. Human plasma is rich in proteins and lipids and contains potential sites for radical formation and destruction; therefore, oxidative damage to plasma proteins mediated by $ONOO^-$ causes alterations of plasma protein functions [21,22]. We have shown that in vitro exogenous $ONOO^-$ induces oxidation of plasma proteins expressed as an increase of carbonyl groups. The obtained increase of carbonyl groups in plasma proteins is consistent with that in the literature [22]. The concentrations of $ONOO^-$ used in our experiments were relatively high, but the lifetime of $ONOO^-$ at a physiological pH is very short, its halftime being of the order of 1 s, and exposure to a bolus of 250 μM of $ONOO^-$ is equivalent to 7 min of exposure to a steady-state $ONOO^-$ concentration of 1 μM [23]. These concentrations could be readily formed at sites of inflammation, where production of rates of $NO^{\cdot}$ and $O_2^{-\cdot}$ radicals considerably increases [23,24].

The present studies indicate that $ONOO^-$ may not only induce plasma protein oxidation by forming carbonyl groups but also distinctly evoke nitration of protein tyrosine residues (measured by C-ELISA method and Western blot analysis). Reactions mediated by $ONOO^-$ — oxidation and nitration — are competitive processes, but oxidation takes place in the absence of carbon dioxide whereas nitration is enhanced by the presence of CO_2. A large tyrosine nitration of serum albumin and Fg in vitro was observed when $ONOO^-$ was used at the concentrations of 1–1.5 mM. Our experiments showed that nitration of different plasma proteins occurred not only at a concentration of 1 mM $ONOO^-$ but also in plasma treated with $ONOO^-$ at lower concentrations (0.01 and 0.1 mM). Moreover, $ONOO^-$ is a powerful oxidant that induces lipid peroxidation in plasma. Our results of tyrosine nitration in plasma proteins and lipid peroxidation stimulated by $ONOO^-$ are in agreement with those in the literature [22,25–27]. The presence of antioxidants (vitamin C, uric acid, glutathione and other thiols) in plasma inhibits $ONOO^-$-mediated oxidation [28]. Bilirubin in plasma is also an effective antioxidant of $ONOO^-$ [29].

We examined the defense properties of resveratrol, which is a phenolic antioxidant present in grapes and in numerous types of wine, especially in red wine. Resveratrol shows various biologic activities including antiplatelet, anticancer, antimutagenic, antifungal, anti-inflammatory and antioxidant properties [12,15,30,31]. The antioxidative effect of resveratrol requires the presence of the 4′-OH group in Ring B and the meta-OH configuration in Ring A [32]. The present study provides more information about the antioxidant activity of resveratrol when physiological oxidant $ONOO^-$ is used. We also compared the antioxidative action of resveratrol with the effect of DFO, which is a known antioxidant and may also react with peroxyl radicals and $ONOO^-$ [33,34]. Our earlier results showed that resveratrol may partly reduce the formation of nitrotyrosine in platelet proteins and thiol oxidation [12,15]. The obtained results indicate that resveratrol in vitro may also protect plasma proteins against oxidation and nitration caused by $ONOO^-$ or its intermediates. Moreover, we observed that resveratrol suppresses the $ONOO^-$ toxicity measured as the level of TBARS — marker of lipid peroxidation. The range of resveratrol concentrations (0.025–0.1 mM) is similar to that used in studies of other authors [35], and the tested concentrations of resveratrol correspond to physiological levels in human plasma. The plasma level of different phenols is estimated in the low micromolar range [36].

Mechanisms of resveratrol action on plasma components are not clear. Brito et al. [35] have reported that resveratrol may prevent the effects of $ONOO^-$ and minimize LDL modifications by the inhibition (approximately 70%) of carbonyl group formation and by the reduction of LDL lipid peroxidation. Our results demonstrated that the amount of total carbonyl groups in protein plasma (measured by ELISA method) in the presence of resveratrol is reduced by approximately 65%. Epicatechin, a

polyphenolic antioxidant from tea, is also active against nitration [26,37–39] but not oxidation of thiols [37]. It suggests that the reaction between epicatechin and $ONOO^-$ does not exist but that the reaction of an intermediate, the tyrosyl radical, with epicatechin may occur. The beneficial effect of resveratrol can be attributed to scavenging of $ONOO^-$ [40]; however, the interaction of $ONOO^-$ with the phenolic seems to be slow to play a role under physiological conditions. In biologic systems, in the presence of CO_2, the decomposition of $ONOO^-$ yields 30–35% of carbonate radical ($CO_3^{\bullet -}$) and nitrogen dioxide ($NO_2^{\bullet}$), which are also strong oxidants; natural phenols are the efficient scavengers of $CO_3^{\bullet -}$ and $NO_2^{\bullet}$ [41]. The presented results indicate that consuming dietary antioxidant supplements with resveratrol seems to have protective effects against impairment of plasma proteins caused by a strong biologic oxidant and inflammatory mediator.

DFO both in vitro and in vivo is frequently used in biochemical and biologic studies to define the contribution of iron-mediated processes during oxidative stress. The results of our present study suggest that antioxidant DFO, which is a transition metal chelator, protects not only against protein oxidation induced by $ONOO^-$ but also against nitration of tyrosine in plasma proteins. The mechanism of DFO protection is independent of iron chelation [42]. DFO protects plasma protein plasminogen from $ONOO^-$-induced inactivation and reduces the extent of tyrosine nitration [43]. DFO does not react with $ONOO^-$ but inhibits $ONOO^-$-dependent free radical-mediated processes. The inhibitory effect of DFO on tyrosine oxidation/nitration is well explained by its reactions with $ONOO^-$-derived $^{\bullet}OH$, $^{\bullet}NO_2$ and/or $CO_3^{\bullet -}$. DFO also reacts with tyrosyl radicals. Moreover, DFO is the inhibitor at the presence and absence of CO_2 [42]. The concentration of DFO (0.025 mM) used in the present experiments is similar to that used in studies of other authors [44].

In conclusion, we suggest that resveratrol, like typical antioxidant DFO, may interact with $ONOO^-$ or its intermediates and that its defense against $ONOO^-$ seems to be at the level of interception. Our results may provide a molecular basis for the well-documented protective cardiovascular effects and anti-inflammatory action of red wine, which contains high concentrations of resveratrol.

Acknowledgment

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