Short Communication

Resveratrol, a Natural Phenolic Compound May Reduce Carbonylation Proteins Induced by Peroxynitrite in Blood Platelets

B. Olas, P. Nowak, M. Ponczek and B. Wachowicz

Department of General Biochemistry, Institute of Biochemistry, University of Lodz, Poland

Abstract. Resveratrol (3,4′,5-trihydroxystilbene) has a very broad range of biological properties, including antiplatelet and antioxidative activity. We investigated *in vitro* the effect of resveratrol on carbonylation of proteins (indicators of oxidative stress) in blood platelets treated with peroxynitrite ($ONOO^-$), a strong biological oxidant and inflammatory mediator. We observed that carbonylation of proteins induced by $ONOO^-$ (0.1 mmol/l), in the presence of resveratrol (0.25–0.1 mmol/l) is reduced. Resveratrol may scavenge $ONOO^-$, and may be useful in the prevention of $ONOO^-$-related diseases, such as inflammatory and cardiovascular diseases.

Key words: Resveratrol — Peroxynitrite — Carbonylation — Blood platelets

Resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin found in many plants and in numerous types of wine as a *trans*- or *cis*-isoform. Resveratrol is a secondary plant metabolite belonging to the class of stilbenes (C_6-C_2-C_6) found in relatively high concentrations in mulberries, grapes, peanuts and pine. Resveratrol shows different biological activities, including antiplatelet (Olas et al. 2002; Wang et al. 2002), anticancer and antimutagenic (Eiattar and Virji 1999), antifungal (Daniel et al. 1999), anti-inflammatory (Johnson and Maddipati 1998) and antioxidant properties (Olas et al. 2004). The antioxidative effect of resveratrol requires the presence of 4′-hydroxyl group in its ring B and the meta-hydroxyl configuration in ring A.

Reactive oxygen/nitrogen species (ROS/RNS) generated in different cells, including macrophages, neutrophils and blood platelets, have been reported to be effective materials for modulation of their functions. Nitric oxide ($^{\bullet}NO$), a unique diffusible molecular messenger in the vascular, in the fast radical-radical reaction with superoxide anion ($O_2^{-\bullet}$) generates peroxynitrite ($ONOO^-$) – a highly reactive, short lived oxidant, which may modify various types of biomolecules (DNA,

Correspondence to: Beata Olas, Department of General Biochemistry, Institute of Biochemistry, University of Lodz, Banacha 12/16, 90–237 Lodz, Poland
E-mail: olasb@biol.uni.lodz.pl

lipids and proteins) and may promote oxidative tissue and cell damage (Ischiropoulos 2003). $ONOO^-$ can exert both a nitrosative and oxidative stress. The defence mechanisms against $ONOO^-$ action are very important for biological functions of different cells, including blood platelets. Various agents were often tested for the ability to protect different components of cells from $ONOO^-$-induced changes in their structure and functions. Our earlier results showed that $ONOO^-$ initiates platelet lipid peroxidation, reacts with thiols in blood platelets (Nowak et al. 2003), induces protein modifications which include nitration of tyrosine in these cells (Olas et al. 2004). We also observed that exposure of blood platelets to $ONOO^-$ induces the inhibition of platelet activation (platelet adhesion, aggregation and secretory process). The different typical and well known antioxidants, including ebselen and deferoxamine have been shown to be a powerful scavenger against $ONOO^-$. However, the role of exogenous antioxidants in the defence against $ONOO^-$ action is not well known. Recently, we have observed that natural product may protect platelet proteins against nitration and thiol oxidation induced by $ONOO^-$ (Olas et al. 2004). The aim of our present study was to investigate *in vitro* the effect of resveratrol on the level of carbonyl protein groups (a sensitive indicator of oxidative stress (Dalle-Donne et al. 2003)) in platelets treated with $ONOO^-$. To determine the amount of carbonyl groups we used two different methods: immunologically method (ELISA) and colorimetric method. These methods determining the level of carbonyl groups with different sensitivity are based on the covalent reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH).

$ONOO^-$ was synthesized according to the method of Pryor and Squadrito (1995). Resveratrol and rabbit anti-DNPH antibodies were purchased from Sigma (St. Louis, MO). Biotynylated anti-goat/mouse/rabbit antibody and streptavidin-biotynylated horseradish peroxidase were from DAKO (Glostrup, Denmark). All other reagents were of analytical grade and were provided by commercial suppliers. Stock solutions of resveratrol was made in 50% dimethylsulfoxide at the concentration of 25 mg/ml and kept frozen.

Human blood was collected into ACD solution (citric acid/citrate/dextrose; 5 : 1 v/v) and platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustroń (1992). The final platelet concentration was about 2.5–4 $\times$ 10^8 platelets/ml. The platelets were counted by the photometric method according to Walkowiak et al. (1989). The entire platelets washing procedure was performed in plastic tubes and carried out at room temperature. Washed human platelet suspensions in the modified Tyrode's Ca^{2+}/Mg^{2+}-free buffer (in mmol/l: 127 NaCl, 2.7 KCl, 0.5 NaH_2PO_4, 12 $NaHCO_3$, 5 HEPES, 5.6 glucose, pH 7.4) were pre-incubated (2 min, 37°C) with resveratrol at dose of 0.025–0.1 mmol/l and then were treated with $ONOO^-$ (0.1 mmol/l, 2 min, 37°C). Samples of blood platelets were dissolved in lysis buffer (2% Triton-X-100, 100 mmol/l EDTA and 100 mmol/l Tris-HCl; pH 7.4); and platelet lysates were used to study the level of carbonyl groups in proteins.

The amount of carbonyl groups present in blood platelet proteins after treatment with $ONOO^-$ and/or resveratrol was determined colorimetrically. The detec-

tion of protein carbonyls involves derivatisation of the carbonyl group with DNPH, which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product as described by Levine et al. (1990).

Detection of carbonyl groups by ELISA method (using anti-DNP antibodies) in blood platelets (control or $ONOO^-$ and/or resveratrol-treated platelets) was performed according to the procedure of Buss and Winterbourn (1997) as described previously (Olas et al. 2006).

As it was already mentioned, isolated blood platelets are an ideal system to study nitrosative and oxidative stress induced by $ONOO^-$, the physiological oxidant and inhibitor of platelet functions. Blood platelets are rich in proteins and contain potential sites for radical formation and destruction. Proteins seem to be a main target of $ONOO^-$ action in these cells. Oxidative and nitrative damage to platelet proteins mediated by $ONOO^-$ causes alterations of platelet functions (Lufrano and Balazy 2003). Our earlier studies showed that $ONOO^-$ evoked nitration of tyrosine residues in platelet proteins, and induced oxidation of protein thiols (Olas et al. 2004). Present studies may provide additional information about the effects of $ONOO^-$ on platelets, a consequence of which could be oxidation of intracellular proteins (measured by the level of carbonyl groups as a good marker of oxidative stress (Fig. 1A,B)). The formation of carbonyl groups in platelet proteins induced by $ONOO^-$ was dose-dependent ($p < 0.05$) (Fig. 1A,B). After 2 min incubation of blood platelets with $ONOO^-$ (1 mmol/l), the amount of carbonyl groups in platelet proteins distinctly increased (about 77.3% when measured by colorimetric method, $p < 0.05$, and by about 49.0% accounting using ELISA method, $p < 0.05$ (Table 1)). These alterations of platelet proteins may be responsible for inhibition in platelet function observed after their exposure to $ONOO^-$ (Nowak and Wachowicz 2001).

In the present experiments, we studied the defence properties of resveratrol which has antiplatelet activity (Wang et al. 2002) and antioxidant properties in blood platelets (Olas et al. 2004). The present study provides more information about antioxidant activity of resveratrol. We show for the first time, that resveratrol may protect platelet proteins against oxidation (measured by level of carbonyl groups) caused by $ONOO^-$ or its intermediates (Fig. 2A,B). In the presence of resveratrol at the highest used concentration (0.1 mmol/l), a distinct inhibition of carbonyl groups in platelet proteins occurred (about 34.6% (Fig. 2A) or about 22.6% (Fig. 2B), respectively). However, in reverse orders of addition resveratrol at the same concentration had not protective action on protein oxidation induced by $ONOO^-$ ($p > 0.05$) (Fig. 2A,B). The range of resveratrol concentrations (0.025–0.1 mmol/l) is similar to that used in studies of other authors (Brito et al. 2002). Tested concentrations of resveratrol correspond to physiological level in human plasma. Scalbert and Williamson (2000) have presented that plasma level of different phenols is estimated in the low micromolar range. Brito et al. (2002) have reported that resveratrol may prevent the effects of $ONOO^-$ and minimize the LDL modification, by the inhibition (about 70%) of carbonyl groups formation.

In conclusion, the present study suggested that modification of platelet pro-

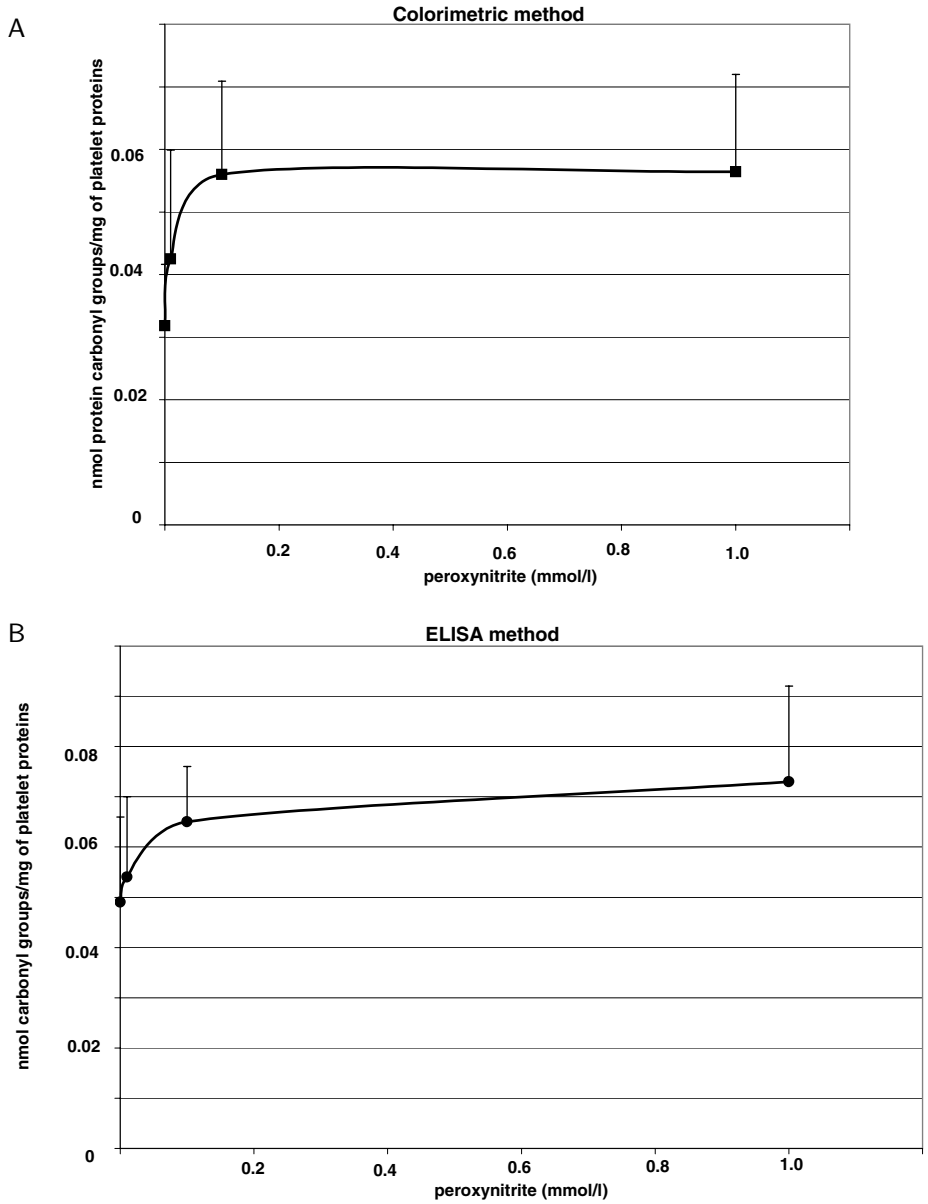

Figure 1. Effect of $ONOO^-$ on protein oxidation in human platelets. Human blood platelets were exposed to $ONOO^-$ (0.01–1 mmol/l) for 2 min at 37℃. The protein oxidation was measured colorimetrically (A) or immunologically (using ELISA method) (B). The results are expressed as nmol protein carbonyl groups/mg of platelet proteins. The results are representative of three independent experiments, and are expressed as mean ± SEM. The effects were statistically significant according to Student's t-test, $p < 0.05$ ($ONOO^-$-treated platelets versus control platelets).

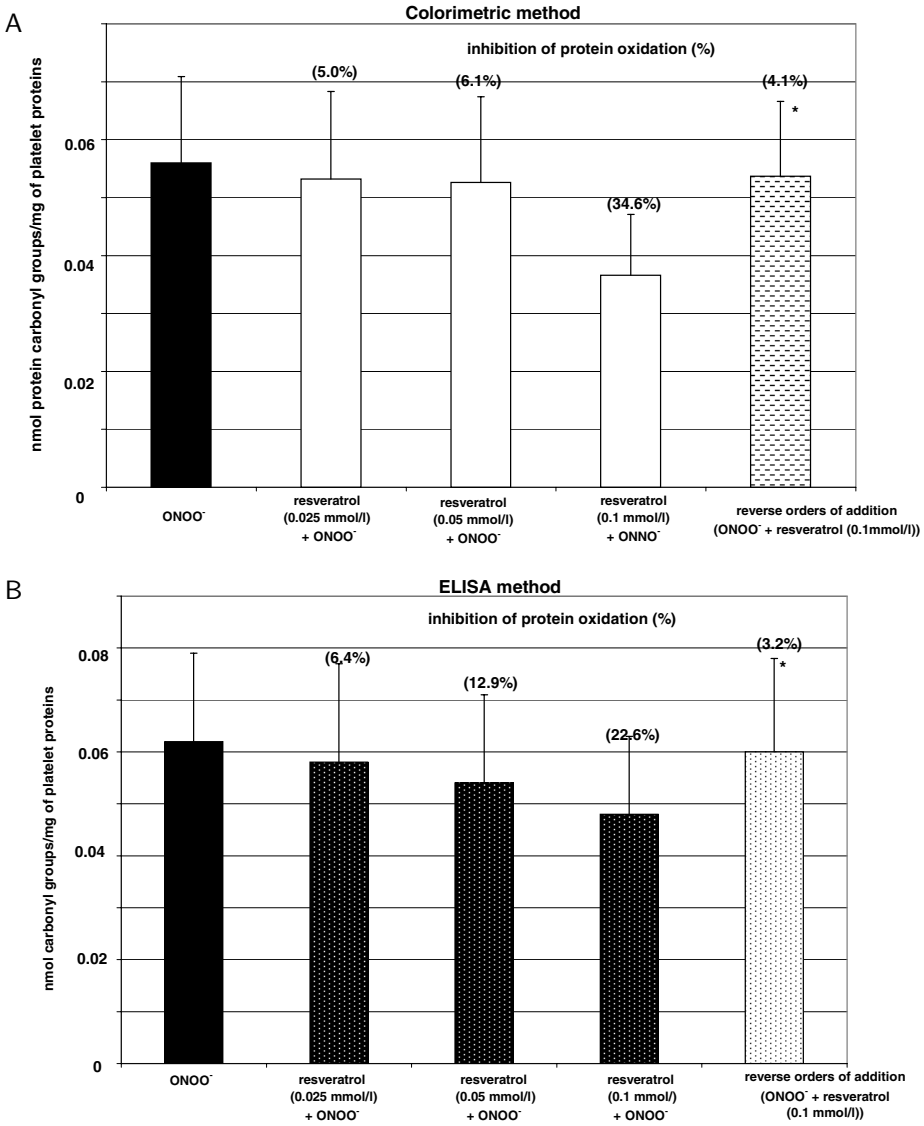

Figure 2. Effects of incubation of blood platelets with resveratrol (0.025–0.1 mmol/l, 2 min, 37°C) on protein oxidation in platelets treated with $ONOO^-$ (0.1 mmol/l, 2 min, 37°C). The protein oxidation was measured colorimetrically (A) or immunologically (using ELISA method) (B). The results are expressed as nmol protein carbonyl groups/mg of platelet proteins. The results are representative of six independent experiments, and are expressed as mean ± SEM. The effects were statistically significant according to Student's *t*-test, $p < 0.05$ (resveratrol + $ONOO^-$ (0.1 mmol/l)-treated platelets *versus* $ONOO^-$ (0.1 mmol/l)-treated platelets), * $p > 0.05$ (reverse orders of addition $ONOO^-$ (0.1 mmol/l) + resveratrol (0.1 mmol/l)-treated platelets *versus* resveratrol (0.1 mmol/l) + $ONOO^-$ (0.1 mmol/l)-treated platelets).

Table 1. Effects of $ONOO^-$ (2 min, 37°C) on the level of carbonyl groups (marker of protein oxidation) in platelet proteins. Stimulation of the protein oxidation was expressed as percentage of that recorded for control blood platelets. The results are representative of three independent experiments, and are expressed as mean ± SEM. The effects were statistically significant according to Student's *t*-test, $p < 0.05$ ($ONOO^-$-treated platelets *versus* control platelets)

Blood platelets treated with $ONOO^-$ (mmol/l)	Stimulation of protein oxidation (%) (measured by colorimetric method)	Stimulation of protein oxidation (%) (measured by ELISA method)
0.01	33.6 ± 5.7	10.2 ± 3.2
0.1	76.1 ± 7.9	32.6 ± 4.8
1	77.3 ± 8.1	49.0 ± 6.6

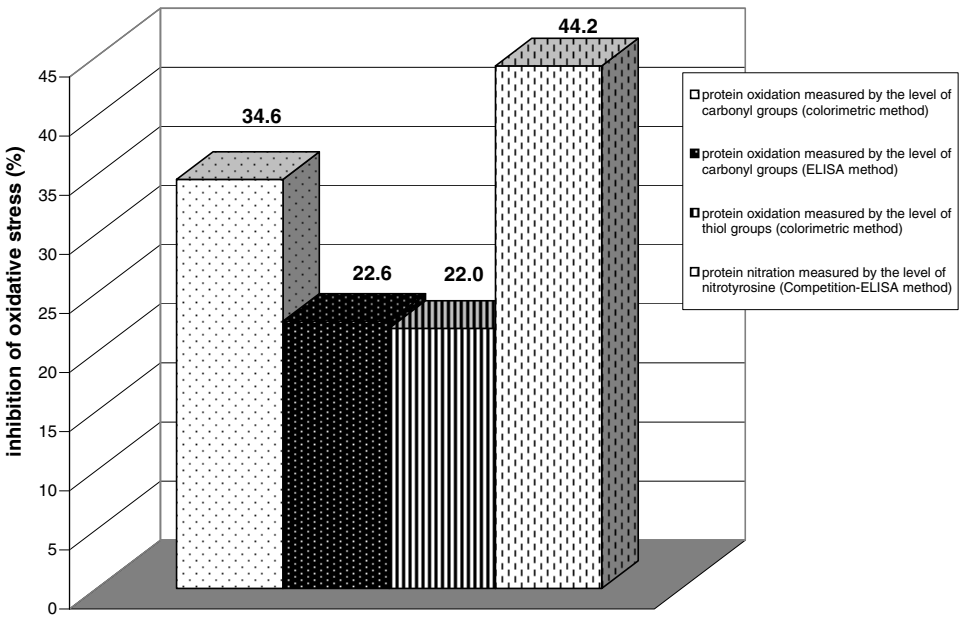

Figure 3. Effect of resveratrol (0.1 mmol/l) on oxidative stress in blood platelets treated with $ONOO^-$ (0.1 mmol/l). Inhibition of protein oxidation and nitration by resveratrol were expressed as percentage of that recorded for blood platelets treated with $ONOO^-$ alone.

teins (not only nitration, oxidation of thiol groups, but also change in the level of carbonyl groups) induced by $ONOO^-$ may lead to functional alterations of proteins and enzyme activities, but the active biophenolic component – resveratrol

(presents in grapes and in numerous types of wine, especially in red wine) may scavenge $ONOO^-$ (Fig. 3), and therefore may be potentially useful in the prevention of the $ONOO^-$-related diseases, such as inflammatory and cardiovascular diseases.

Acknowledgements. This work is supported by the grant No. 506/810 from University of Lodz.

References

Brito P., Almeida L. M., Dinis T. C. (2002): The interaction of resveratrol with ferrylmyoglobin and peroxynitrite; protection against LDL oxidation. Free Radic. Res. **36**, 621—631

Buss H., Chan T. P., Sluis K. B., Domigan N. M., Winterbourn C. C. (1997): Protein carbonyl measurement by a sensitive ELISA method. Free Radic. Biol. Med. **23**, 361—366

Dalle-Donne I., Rossi R., Giustarini D., Milzani A., Colombo R. (2003): Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta **329**, 23—38

Daniel O., Meier M. S., Schlatter J., Frischhnecht P. (1999): Selected phenolic compounds in cultivated plants: ecologic functions, health implications, and modulation by pesticides. Environ Health Perspect. **107**, 109—114

Eiattar T. M. A., Virji A. S. (1999): Modulating effect of resveratrol and quercetin on oral cancer cell growth and proliferation. Anti-Cancer Drugs **10**, 187—193

Ischiropoulos H. (2003): Biological selectivity and functional aspects of protein tyrosine nitration. Biochem. Biophys. Res. Commun. **305**, 776—783

Johnson J. L., Maddipati K. R. (1998): Paradoxical effects of resveratrol on the two prostaglandin H synthases. Prostaglan. Lipid Med. **56**, 131—143

Levine R. L., Garland D., Oliver C. N., Amici A., Climent I., Lenz A. G., Ahn B. W., Shaltiel S., Stadtman E. R. (1990): Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. **186**, 464—478

Lufrano M., Balazy M. (2003): Interactions of peroxynitrite and other nitrating substances with human platelets: the role of glutathione and peroxynitrite permeability. Biochem. Pharmacol. **65**, 515—523

Nowak P., Wachowicz B. (2001): Studies on pig blood platelet responses to peroxynitrite action. Platelets **12**, 376—381

Nowak P., Olas B., Bald E., Glowacki R., Wachowicz B. (2003): Peroxynitrite-induced changes of thiol groups in human blood platelets. Platelets **14**, 375—379

Olas B., Wachowicz B., Saluk-Juszczak J., Zielinski T. (2002): Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin. Thromb. Res. **107**, 141—145

Olas B., Nowak P., Kołodziejczyk J., Wachowicz B. (2004): The effects of antioxidants on peroxynitrite-induced changes in platelet proteins. Thromb. Res. **113**, 399—406

Olas B., Nowak P., Kolodziejczyk J., Ponczek M., Wachowicz B. (2006): Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. J. Nutr. Biochem. **17**, 96—102

Pryor W. A., Squadrito G. L. (1995): The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. **268**, L699—722

Scalbert A., Williamson G. (2000): Dietary intake and bioavailability of polyphenols. J. Nutr. **130**, S2073—2085

Wachowicz B., Kustroń J. (1992): Effect of cisplatin on lipid peroxidation in pig blood platelets. Cytobios **70**, 41—47

Walkowiak B., Michalak E., Koziołkiewicz W., Cierniewski C. S. (1989): Rapid photometric method for estimation of platelet count in blood plasma or platelet suspension. Thromb. Res. **56**, 763—766

Wang Z., Huang Y., Zou J., Cao K., Xu Y., Wu J. M. (2002): Effects of red wine and wine polyphenol resveratrol on platelet aggregation *in vivo* and *in vitro*. Inter. J. Mol. Med. **9**, 77—79

Final version accepted: April 18, 2006