

Resveratrol, at Concentrations Attainable with Moderate Wine Consumption, Stimulates Human Platelet Nitric Oxide Production¹⁻³

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Abstract

The mechanisms through which moderate wine consumption reduces ischemic cardiovascular events are not yet fully unraveled. Grape extracts or a mixture of the polyphenols contained in wine were previously shown to increase nitric oxide (NO); however, little information is available on the effect of resveratrol, one of the main polyphenols of wine, on platelet NO production. We assessed the effects of resveratrol, at the concentrations attainable after moderate wine intake, on platelet NO production and the mechanism of this activity. Twenty healthy volunteers were studied before and after 15 d of controlled white or red wine intake (300 mL/d). After wine intake, plasma resveratrol and the release of NO by stimulated platelets increased significantly. Resveratrol, at the concentrations detected in plasma after wine intake, was incubated *in vitro* with washed platelets and several variables related to NO production and to signal transduction were measured. Resveratrol *in vitro* enhanced significantly the production of NO by stimulated platelets, the activity of platelet NO synthase (NOS), phosphorylation of protein kinase B, an activator of the endothelial NOS (eNOS), and phosphorylation of vasodilator-activated protein (VASP), an expression of the biologic activity of NO in platelets. Simultaneously, we observed decreased phosphorylation of P38 mitogen-activated protein kinase (p38MAPK), a proinflammatory pathway in human platelets, a reduction of the activity of NADPH oxidase, a major source of reactive oxygen species (ROS) and of the generation of O₂⁻ radicals, as detected by cytochrome C reduction. In conclusion, resveratrol, at concentrations attainable after moderate wine intake, activates platelet eNOS and in this way blunts the proinflammatory pathway linked to p38MAPK, thus inhibiting ROS production and ultimately platelet function. This activity may contribute to the beneficial effects of moderate wine intake on ischemic cardiovascular disease. *J. Nutr.* 138: 1602–1608, 2008.

Introduction

Epidemiological studies have shown that cardiovascular and cerebrovascular ischemic events are decreased by moderate wine consumption (1–7). Whereas the mechanism of this beneficial action is not fully established, several activities of wine, and especially of red wine, may be involved, such as the ability to raise high-density lipoprotein levels, to increase the antioxidant plasmatic potential, to improve endothelium-dependent vasodilation, and to inhibit platelet aggregation and leukocyte adhesion (2,4,8–11).

Studies in humans, as well as experiments on isolated tissues or organs, demonstrated that beneficial cardiovascular effects can be achieved also with de-alcoholized wine, suggesting that

wine has beneficial effects partly dependent on components other than alcohol (1,4,12,13). Grapes contain a variety of phenolic antioxidants, including quercetin, catechin, epicatechin, proanthocyanidins and resveratrol (5), mainly present in grape skin.

The levels of the phenolic compounds in wines are highly variable, due to differences in fruit sources as well as in processing, ranging from ~200 mg of total phenols in a glass of red wine to 40 mg in a glass of white wine (14–18). The protective effects of polyphenols against oxidative stress in biologic systems are ascribed to their capacity to chelate metal catalysts (19), activate antioxidant enzymes (20), reduce α -tocopherol radicals (21), and inhibit oxidases (22,23). Specific polyphenols interact with arachidonic acid (AA)⁷ metabolism and inhibit platelet

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³ Supplemental Figures 1–3 are available with the online posting of this paper at jn.nutrition.org.

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⁷ Abbreviations used: AA, arachidonic acid; AKT, protein kinase B; BSA, bovine serum albumin; eNOS, endothelial nitric oxide synthase; GFP, gel-filtered platelets; NOS, nitric oxide synthase; p38MAPK, p38 mitogen-activated protein kinase; PRP, platelet-rich plasma; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-DL-penicillamine; VASP, vasodilator-stimulated phosphoprotein.

thromboxane A₂ production (24). Red wine polyphenols have also been shown to increase the expression of endothelial nitric oxide (NO) synthase (eNOS) and consequently the release of NO by human umbilical vein endothelial cells *in vitro*; however, these actions were observed only with relatively high concentrations of polyphenols (25,26). It has been proposed that resveratrol, a relevant wine constituent, is involved in vascular protection. Fresh grape skin contains 50–100 µg/g of resveratrol, whereas the concentration of resveratrol in wine ranges from ~0.1 to 14 mg/L for red wine and from 0.04 to 3.5 mg/L for white wine (with the highest concentrations reported in wines from pinot noir grapes) (14,15).

In particular, concerning the role of resveratrol in potentiating NO production, most of the previous *in vitro* studies used concentrations of this flavonoid (from 1 to 1000 µmol/L) (24,27,28) much higher than those attainable in plasma after moderate wine consumption (29).

The effects of polyphenols on platelet function have been investigated by several authors and, among other activities, an increase of NO production associated with a decrease in the formation of reactive oxygen species (ROS) has been described, either when using extracts from grape components or a mixture of polyphenols mimicking the flavonoids detected in plasma after wine intake (23,29,30), but little data on the effects of resveratrol at *in vivo* attainable concentrations on platelet function are available.

In a previous study testing the hypothesis that moderate wine consumption inhibits oxidative stress in healthy subjects, we found that plasma total resveratrol (free resveratrol plus the glucuronated and sulfated metabolites, but not the hydroxylated and methylated derivatives), measured with an HPLC-electrochemical method (31), is 1.33 ± 0.3 µmol/L after white wine intake and 1.72 ± 0.1 µmol/L after red wine intake, with a mean increase of plasma resveratrol induced by wine intake of $\sim 0.7 \pm 0.4$ µmol/L (29).

In the present study, we assessed the effects of resveratrol, at concentrations attainable by moderate intake of red or white wine, on platelet NO production and studied the mechanism of this activity.

Materials and Methods

Materials

Collagen from equine tendon was obtained from Mascia Brunelli. The nonenzymatic Nitric Oxide Assay kit was purchased from OXIS International. NOSdetect Assay kit, for the measurement of NO synthase (NOS) activity, was purchased from Stratagene Alexis Biochemicals. (³H)-L-Arginine was purchased from Amersham-Pharmacia Biotech, and AA, L-N^G-monomethyl-L-arginine, resveratrol, and S-nitroso-N-acetyl-DL-penicillamine (SNAP) were purchased from Sigma-Aldrich.

Anti-p38 mitogen-activated protein kinase (p38MAPK) and anti-AKT (protein kinase B) antibodies were purchased from Santa Cruz Biotechnology and anti-vasodilator-stimulated phosphoprotein (VASP) IgG1 mouse monoclonal antibody was purchased from Becton Dickinson.

Ex vivo study

Citrated (1/10 v:v trisodium citrate 3.8%) blood samples were taken at baseline and after 15 d of controlled white wine or red wine intake (300 mL/d) in the morning, under fasting conditions, from 20 healthy human volunteers with no evidence of cardiovascular disease or risk factors for atherosclerosis and who had not taken antioxidants, vitamins, or antiplatelet drugs for at least 1 mo, participating in a previously reported study (29). All gave informed consent to participate in the study, which was approved by the Ethical Committee of the University of Rome "La Sapienza."

Briefly, after a run-in period of 1 wk, during which the subjects refrained from consuming wine or alcohol and nonsteroidal antiinflam-

matory drugs, they were randomly allocated to consume a total of 300 mL/d of red (total polyphenolic concentration, 1.8 g/L) ($n = 10$, 4 males and 6 females, mean age, 45 ± 6 y) or white (total polyphenolic concentration, 0.25 g/L) ($n = 10$, 5 males and 5 females, mean age, 42 ± 5 y) wine during dinner. At baseline and after 15 d of follow-up, a blood sample was taken from each subject in the morning after a 12-h fast [for more details, see (29)].

Platelet-rich plasma (PRP) was prepared by centrifugation at $160 \times g$ for 10 min at room temperature and 250-µL aliquots (200,000 platelets/µL) were stimulated with AA 0.5 mmol/L (5 min, 37°C) in an aggregometer cuvette under continuous stirring. After stimulation, samples were centrifuged ($10,000 \times g$; 5 min, room temperature) and the cell-free supernatant was stored at -80°C for later measurement of nitrite plus nitrate. The concentration of AA used was selected on the basis of previous reports (30–32) and on the basis of the consideration that the presence of plasmatic proteins, especially albumin, acting as carriers for AA, mediate its slow release in proximity of cell membranes, preserving cell integrity (33).

Platelet-released nitrite plus nitrate. Nitrite plus nitrate was measured by the Griess reagent in the supernatant plasma of arachidonic-acid-stimulated PRP obtained by centrifugation (as described above), using a commercially available colorimetric kit (nonenzymatic nitric oxide assay kit, Oxis) after reduction of nitrate to nitrite with a redox reaction catalyzed by cadmium, as previously described (34).

Before the test, granulated cadmium was washed with water, then with 0.1 mol/L HCl and finally with 0.1 mol/L NH₄OH, pH 9.6. Samples (50 µL adjusted with water to 190 µL) were deproteinized by precipitation with ZnSO₄ [10 µL of 30% (wt:v) solution] for 15 min at room temperature, then centrifuged and the supernatants were transferred into tubes containing cadmium beads and incubated overnight at room temperature under continuous agitation. Before the assay, samples were recentrifuged to remove beads and supernatants were then added to micro-well plates; color reagent (sulphanilamide and N-[1-naphthyl] ethylenediamine dihydrochloride) was added and 540-nm absorbance was read in a microplate reader. A standard curve was built to determine nitrite concentration for each assay.

In vitro study

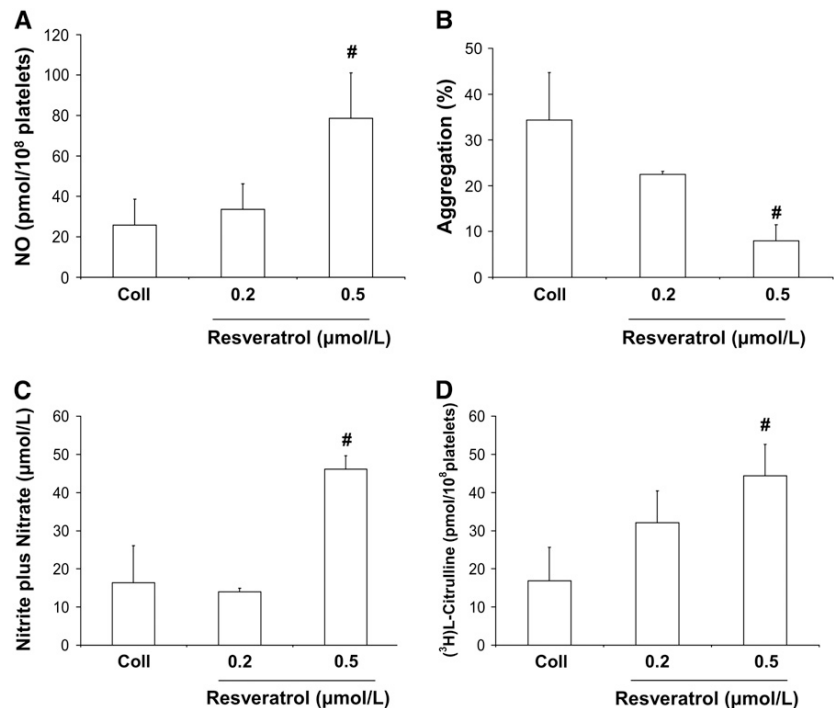
For the *in vitro* studies, preliminary experiments with increasing concentrations of resveratrol (up to 1 µmol/L) indicated that 0.5 µmol/L was the minimum concentration of resveratrol giving a potentiation of NO production (data not shown). Because this concentration is largely within the range of the increase in plasma from the *ex vivo* study, we decided to use 0.5 µmol/L as the maximum concentration for our *in vitro* studies.

Platelet preparation. Venous blood was drawn in trisodium citrate [3.8%, 1/10 (v:v)] from fasting healthy volunteers who had not ingested any drugs affecting platelet function for at least 14 d.

Platelets prepared from single volunteers were used for individual experiments. Blood was centrifuged at $160 \times g$ for 10 min at room temperature and the supernatant PRP was separated. Washed platelets were prepared by the method of Mustard, as previously described (35); we counted platelets (Genius, S.E.A.C) and adjusted then to 2×10^{11} /L using Tyrode's buffer without Ca²⁺ and Mg²⁺. For the preparation of gel-filtered platelets (GFP), PRP was passed over a Sepharose-2B column equilibrated with Tyrode's HEPES-buffered saline [140 mmol/L NaCl, 6 mmol/L HEPES, 2 mmol/L Na₂HPO₄, 2 mmol/L MgSO₄, 0.1% dextrose, pH 7.4, and 0.4% bovine serum albumin (BSA)], as described (36–38). Prior to gel filtration, PRP was incubated (1 h at room temperature) with 0.2 or 0.5 µmol/L resveratrol. After gel filtration, the cell suspension was adjusted to a final concentration of 1×10^{11} platelets/L. Platelets were activated with collagen (1.5 mg/L) for 5 min if not otherwise stated.

In preliminary experiments, the effects of resveratrol on NO production by stimulated platelets were tested using either collagen or AA as a stimulus and a similar potentiating effect of resveratrol was observed with both agonists (data not shown), as also confirmed by the strong correlation (Pearson $r = 0.833$; $P < 0.001$) of nitrite plus nitrate produced from collagen- and AA-stimulated platelets in samples treated with resveratrol (Supplemental Figs. 1 and 2).

FIGURE 1 Effect of resveratrol on collagen (Coll)-induced NO production and platelet aggregation. (A) NO release from collagen-stimulated (1.5 mg/L) gel-filtered platelets measured with a NO-sensitive electrode. (B) Maximal amplitude of aggregation of collagen-stimulated washed platelets. (C) Nitrite plus nitrate release in the supernatant of collagen-stimulated platelets. (D) NOS activity of collagen-stimulated washed platelets. Values are means \pm SEM, $n = 11$. #Different from collagen, $P < 0.05$.



Platelet NO production. A NO-selective microelectrode (Inter Medical), able to detect nanomolar amounts of gaseous NO, was adapted for use in a standard platelet aggregometer (P.I.C.A., Chronolog) to monitor platelet NO production and aggregation simultaneously, as previously described (39). The NO monitoring device consists of a headstage amplifier with a built-in power supply and 2 electrodes. The working electrode (0.2-mm diameter, made from a Pt/Ir alloy) and the counter-electrode (carbon fiber) were inserted in an aggregometer microcuvette containing a stirring bar. The aggregometer, electrode, and headstage amplifier were housed in a Faraday cage to reduce electrical interference. The electrode current was continuously recorded with a Dual Channel Chart Recorder (Chronolog) and production of NO was quantified by integrating the area under the curve. All experiments were conducted at 37°C. SNAP was used to derive a standard curve that was linear from 10 to 350 pA over the range of 10–500 nmol/L SNAP concentration; r was typically 0.97. Platelet aggregation and NO production were studied using 1.5 mg/L collagen as a stimulus. Alternatively, nitrite plus nitrate in GFP supernatants were measured by the Griess reagent, as above described.

To assess platelet NOS activity, we used a commercially available kit (NOSdetect assay kit) that measures NOS activity by monitoring the enzymatic conversion of (³H)-L-arginine to (³H)-L-citrulline. Briefly, the platelet suspension was incubated with 37 MBq/L of (³H)-L-arginine for 30 min at 37°C, then platelets were diluted with HEPES Tyrode's to a final concentration of 2×10^{11} /L; prior to trigger the reaction using 1.5 mg/L collagen as a stimulus, Ca^{2+} concentration was restored by adding $CaCl_2$ 2mmol/L. After stimulation for 5 min, samples were centrifuged, the pellet was extracted with the homogenization buffer provided with the kit, and the supernatant was added to an ion exchange resin, also provided with the kit. (³H)-L-citrulline does not bind to the resin; thus, the effluent was recovered and added to scintillation tubes and counted with a β -counter. A standard curve using increasing (³H)-L-arginine concentrations was built for each assay.

For the calculation of platelet NO production, as measured using any of the above-described methods, levels observed in the same samples after treatment with L-N^G-monomethyl-L-arginine were subtracted to give the net stimulus- and/or resveratrol-induced NO production.

Platelet p38MAP kinase, AKT, and VASP activities. p38MAP kinase, AKT, and VASP phosphorylation in resting or collagen- (6 mg/L) stimulated washed platelets was analyzed by flow cytometry using specific antibodies (p38MAPK IgG goat polyclonal, anti AKT IgG goat polyclonal, and anti

VASP IgG1 mouse monoclonal). In all assays, an irrelevant isotype-matched antibody was used as a negative control.

Antibodies (1 mg/L) were added to 200 μ L of platelet suspension (2×10^{11} /L), previously fixed with (2%) paraformaldehyde (0.1% BSA) for 60 min at room temperature and then permeabilized with digitonin (100 μ mol/L) for 30 min at room temperature (40). Unbound antibody was removed by adding 0.1% BSA PBS and subsequent centrifugation at $3000 \times g$ for 3 min (twice). Fluorescence intensity was analyzed on an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 488 nm/L. For every histogram, 50,000 platelets were counted to determine the proportion of positive platelets. We reported antibody reactivity in arbitrary units obtained by multiplying the number of positive events resulting from platelet stimulation by the mean values of the fluorescence observed when the specific antibody was used and by correcting for the values obtained in unstimulated samples treated with the same antibody.

Platelet NADPH oxidase activity. Measurement of platelet NADPH oxidase activity was carried out in platelet homogenates according to Seno et al. (41). Washed platelets were suspended in homogenate buffer containing: 50 mmol/L Tris/HCl (pH 7.4), 1.0 mmol/L EDTA, 2.0 mmol/L leupeptin, and 2.0 mmol/L pepstatin A and then homogenized. Platelet homogenates were incubated for 10 min at 37°C with 100 μ mol/L NADPH in the presence of resveratrol (0.1 and 0.5 μ mol/L) or its vehicle. The assay solution contained 400 μ L Tyrode's buffer and 0.25 mmol/L lucigenin. After preincubation at 37°C for 3 min, we started the reaction by adding 100 μ L of platelet homogenates together with AA 0.5 mmol/L or its vehicle. The chemiluminescent signal was expressed as relative chemiluminescence units for a mean of 10 min and corrected for protein concentration (relative chemiluminescence units/mg) (34,41). Protein concentrations were determined by the method of Lowry.

O₂⁻ generation evaluated by cytochrome c reduction. Generation of superoxide anion radicals (O₂⁻) in resting platelets and in platelets stimulated with collagen (6 mg/L), after preincubation with resveratrol (0.1 and 0.5 μ mol/L) or its vehicle, was measured by cytochrome c reduction, as described earlier (42,43). After a 10-min activation, platelets were sedimented by centrifugation at $2000 \times g$ for 5 min, the supernatants were transferred into spectrophotometer cuvettes, and the reduction of cytochrome c was measured at 550 nm. To calculate the molar concentration of O₂⁻, an extinction coefficient for cytochrome c of $18,700 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ was used (42,43).

Statistical analysis

For the *ex vivo* studies, the comparisons among groups before and after red or white wine intake were conducted using 1-way and repeated-measures ANOVA or, in case of nonhomogeneous variances (assessed with the Levene's test), with nonparametric tests (Wilcoxon's and Kolmogorov-Smirnov (z) tests; StatViewII software, Abacus Concepts) [for more details, see ref. (29)].

For the *in vitro* studies, statistical analyses were carried by the ANOVA followed by the Bonferroni post hoc test for selected pairs (GraphPad Prism 4.01 for Windows, GraphPad Software), as indicated. A P -value of < 0.05 was considered significant. Data are expressed as means \pm SEM.

Results

Ex vivo study

Platelet nitrite plus nitrate production. After 15 d of controlled wine intake by healthy volunteers, plasma resveratrol concentrations increased from 0.72 ± 0.3 to $1.33 \pm 0.3 \mu\text{mol/L}$ for white wine and from 0.71 ± 0.02 to $1.72 \pm 0.1 \mu\text{mol/L}$ for red wine. The increased plasma resveratrol concentration, assessed by HPLC after the hydrolysis of the samples with β -glucuronidase and sulfatase, was $1.0 \pm 0.3 \mu\text{mol/L}$ after red wine and $0.6 \pm 0.3 \mu\text{mol/L}$ after white wine, with a mean increase of $0.7 \pm 0.4 \mu\text{mol/L}$ (29).

Nitrite plus nitrate concentrations in the supernatant of AA (0.5 mmol/L)-stimulated PRP increased significantly after 15 d of controlled red [from $13.9 \pm 3.4 \mu\text{mol/L}$ to $24.1 \pm 3.6 \mu\text{mol/L}$ ($P = 0.037$)] or white wine [from $10.6 \pm 2.4 \mu\text{mol/L}$ to $19.8 \pm 4.8 \mu\text{mol/L}$ ($P = 0.01$)] intake. The changes in the nitrite plus nitrate released by activated platelets after red or white wine intake did not differ.

In vitro studies

Platelet NO production. GFP stimulated with collagen (1.5 mg/L) released $25.8 \pm 12.0 \text{ pmol}/10^8$ platelets of NO (Fig. 1A). Preincubation with resveratrol (0.2 – $0.5 \mu\text{mol/L}$) produced an

increase of NO production that was significant at the concentration of $0.5 \mu\text{mol/L}$ (Fig. 1A). At the same time, platelet aggregation decreased and this was significant at $0.5 \mu\text{mol/L}$ resveratrol ($P = 0.046$) (Fig. 1B).

Platelet-derived NO was measured also as the nitrite plus nitrate concentration in the supernatant of stimulated platelets. Under control conditions, GFP stimulated with collagen released $16.3 \pm 9.8 \mu\text{mol/L}$ nitrite plus nitrate in the supernatant. When GFP were preincubated with various concentrations of resveratrol, an increase in the release of nitrite plus nitrate occurred at $0.5 \mu\text{mol/L}$ resveratrol (Fig. 1C).

Platelet NOS activity upon stimulation with collagen was $16.9 \pm 8.7 \text{ pmol } (^3\text{H})\text{L-citrulline}/10^8$ platelets. In resveratrol-treated platelets, $(^3\text{H})\text{L-citrulline}$ production increased significantly at $0.5 \mu\text{mol/L}$ (Fig. 1D).

Collagen-induced AKT phosphorylation. Stimulation with collagen induced the phosphorylation of platelet AKT compared with unstimulated platelets ($P < 0.005$) (Fig. 2A); preincubation with resveratrol (0.1 – $0.5 \mu\text{mol/L}$) dose-dependently enhanced AKT phosphorylation induced by collagen, with a significant increase when the concentration was at least $0.1 \mu\text{mol/L}$ (Fig. 2A).

p38 MAP-kinase phosphorylation. In collagen-stimulated platelets, p38 MAP-kinase was markedly phosphorylated compared with resting platelets ($P < 0.005$) (Fig. 2B). Preincubation with resveratrol (0.1 – $0.5 \mu\text{mol/L}$) dose-dependently inhibited p38 MAP-kinase phosphorylation, with a significant reduction when the concentration was at least $0.1 \mu\text{mol/L}$ (Fig. 2B).

VASP phosphorylation. VASP phosphorylation tended to increase upon collagen stimulation (Fig. 2C). When platelets were preincubated with resveratrol (0.1 – $0.5 \mu\text{mol/L}$), VASP phosphorylation increased further, with a significant rise when the concentration was at least $0.1 \mu\text{mol/L}$ (Fig. 2C and Supplemental Fig. 3).

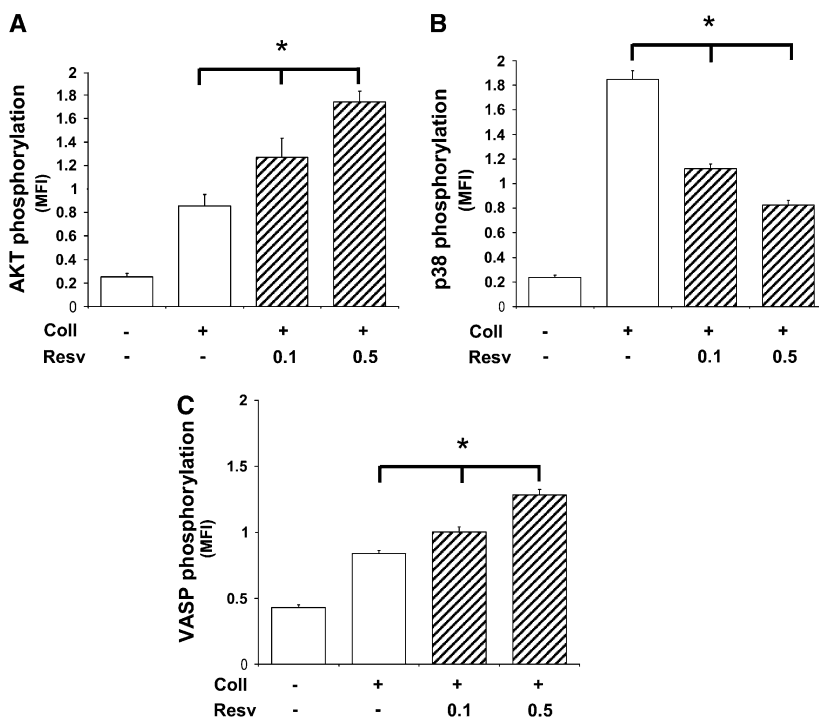


FIGURE 2 Effect of resveratrol (Resv) on AKT (A), p38 MAPK (B), and VASP phosphorylation (C) in collagen (Coll)-stimulated platelets. Data are expressed as means \pm SEM, $n = 5$. *Different from collagen alone, $P < 0.001$.

Platelet NADPH oxidase activity and cytochrome c reduction. Platelet NADPH oxidase activity was enhanced by stimulation with AA (0.5 mmol/L) and an especially strong enhancement was observed when AA was added together with NADPH (100 μ mol/L) compared with AA alone (Fig. 3A). Preincubation with resveratrol (0.1–0.5 μ mol/L) decreased AA-mediated O_2^- formation, with a significant reduction when the concentration was at least 0.1 μ mol/L (Fig. 3A). Confirmatory experiments were conducted with a higher concentration of NADPH (25 mmol/L), giving comparable results (data not shown). Generation of O_2^- in control platelets was enhanced by stimulation with collagen (Fig. 3B) and preincubation with resveratrol (0.1–0.5 μ mol/L) decreased collagen-mediated O_2^- production, with a significant reduction already at 0.1 μ mol/L.

Discussion

In a previous *in vivo* study, in which we analyzed the relationship between the plasma concentrations of polyphenols and the antioxidant activity of red and white wine intake in healthy human volunteers, we found that several aspects of *ex vivo*

platelet function were inhibited (29). Based on this observation and on previous reports suggesting that some wine components may favor platelet NO production (23,39,43), we decided to evaluate the release of NO degradation products in the supernatant of platelets stimulated with AA prepared from healthy volunteers consuming red or white wine in controlled conditions.

Our results showed a marked increase of platelet-derived nitrite plus nitrate after wine consumption, which was similar for both red and white wine. In these subjects, the increase of platelet-released nitrite plus nitrate was paralleled by an increase in plasma resveratrol (29). We thus decided to test the hypothesis that resveratrol directly induces NO production from human blood platelets at concentrations attainable during moderate daily wine consumption.

Polyphenols, and in particular resveratrol, inhibit ROS production from stimulated platelets (27,29,34). Platelets, like endothelial cells, contain not only the L-arginine-NOS pathway but also a phagocyte-type NADPH oxidase that is a major source of ROS (41,42). Indeed, Pignatelli et al. (44) have recently demonstrated that human platelets express gp91^{phox}, the catalytic unit of NADPH oxidase, and that this enzyme is crucial for the production of ROS by platelets, because patients with hereditary deficiency of gp91^{phox} have an almost complete suppression of platelet superoxide anion production.

In our *in vitro* experiments, the stimulation of platelets with collagen induced the production of NO, both when NO was measured directly by an ultrasensitive electrochemical device and by the assay of the NO degradation products nitrite plus nitrate in the supernatant of stimulated platelets.

The activity of the platelet eNOS enzyme was also increased, upon collagen stimulation, as documented by an increased incorporation of (³H)L-citrulline in platelets preincubated with (³H)L-arginine.

Preincubation of blood platelets with low concentrations of resveratrol, i.e. concentrations corresponding to the increased plasma concentrations produced by moderate wine intake, significantly increased platelet NO production, both as detected directly by a NO-sensitive electrode and by the measurement of NO-degradation products in the supernatant of stimulated platelets. This increase was the consequence of the stimulation of platelet NOS activity, as documented by the increased formation of (³H)L-citrulline in platelets preincubated with (³H)L-arginine.

Incubation with resveratrol also produced an increase of AKT phosphorylation in platelets, one of the effectors of NOS activation; in fact, agonist-dependent stimulation of PI3K and/or activation of calmodulin-dependent protein kinase kinase can activate AKT to phosphorylate eNOS, concomitantly to the increase in cytoplasmic calcium. The calcium-dependent activation of calmodulin will stimulate calmodulin-dependent protein kinase kinase and the recruitment of calmodulin and perhaps Hsp90 to eNOS, facilitating rapid eNOS activation and the burst-like release of NO (42–50). How phosphorylation of eNOS by AKT enhances NO release is not completely known, but it is likely to be related to changes in the sensitivity of the enzyme to calcium-activated calmodulin (46).

The increase of NOS activity and the consequent enhanced release of NO produced biologically relevant effects in platelets as shown by the enhanced PKG-dependent VASP phosphorylation induced by preincubation with resveratrol. p38 MAPK phosphorylation also decreased in resveratrol-preincubated platelets, which is likely to be an effect of the enhanced NO formation (43,49). When activated, p38 MAPK is able to induce a strong production of ROS (50). Moreover, p38MAPK phosphorylation induces the activation of platelet integrin $\alpha_{IIb}\beta_3$;

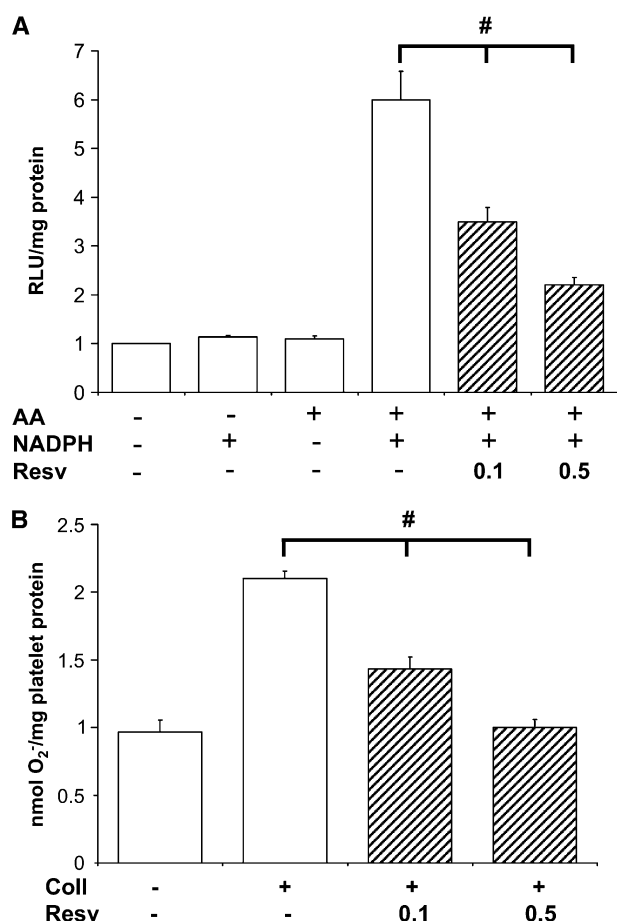


FIGURE 3 Effect of resveratrol (resv) on NADPH oxidase activity (A) and ROS production (B) by stimulated, washed human platelets. (A) NADPH oxidase activity of platelets stimulated with 0.5 mmol/L AA. (B) Cytochrome C-dependent ROS production by platelets stimulated with 6 mg/L collagen (Coll). Data are expressed as means \pm SEM, $n = 5$. #Different from stimulus alone, $P < 0.001$.

thus, the inhibition of p38MAPK phosphorylation is relevant for the suppression of platelet aggregation.

Together, these data suggest that resveratrol increases platelet NO production via PI3K-dependent AKT phosphorylation and the subsequent phosphorylation and activation of eNOS. The increased NO production in turn induces an increase of cGMP-dependent effects, such as VASP phosphorylation and inhibition of p38MAPK phosphorylation, with the consequent inhibition of platelet activation and a relevant reduction of ROS production (29,35,51). Recently, data showing resveratrol-dependent inhibition of p38MAPK in platelets have been reported (50).

Previous studies demonstrated that polyphenols inhibit NADPH oxidase activity by modulating the PKC pathway (25–29). We studied AA-induced NADPH oxidase activity and collagen-induced cytochrome c-dependent ROS production in platelets and found that resveratrol inhibits NADPH oxidase activity and ROS production at the same concentrations that induce NO production. Superoxide generated by NADPH-oxidase is one of the most effective agents to scavenge NO via the formation of peroxynitrite. Therefore, the enhanced release of NO from platelets upon treatment with resveratrol is probably the result of both an enhanced NO-synthase activity and a diminished NO inactivation.

These findings are in agreement with some of the inhibitory effects previously observed in platelets using polyphenols mixtures (13,24–26,39). Thus, the present study suggests that the wine polyphenol resveratrol alone, at the concentrations produced in plasma by moderate intake of wine, either red or white, exerts on human platelets a NO production-stimulating effect. This activity may be complemented by other activities of the polyphenols contained in wine, such as inhibition of LDL oxidation, increased HDL, the general improvement of the lipoprotein profile (51) and, at the cellular level, the down-regulation of PKC-mediated NADPH oxidase activation, to generate the well-known protective effects of moderate wine consumption against cardiovascular ischemic events (6,18).

Literature Cited

1. Gronbaek M, Sorensen TI. Alcohol consumption and risk of coronary heart disease. Studies suggest that wine has additional effect to that of ethanol. *BMJ*. 1996;313:365.
2. Gronbaek M, Dies A, Sorensen TI, Becker U, Schnohr P, Jensen G. Mortality associated with moderate intakes of wine, beer, or spirits. *BMJ*. 1995;310:1165–9.
3. St Leger AS, Cochrane AL, Moore F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet*. 1979;1:1017–20.
4. German JB, Walzem RL. The health benefits of wine. *Annu Rev Nutr*. 2000;20:561–93.
5. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr*. 2001;74:418–25.
6. Di Castelnuovo A, Rotondo S, Iacoviello L, Donati MB, De Gaetano G. Meta-analysis of wine and beer consumption in relation to vascular risk. *Circulation*. 2002;105:2836–44.
7. de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation*. 1999;99:779–85.
8. Halpern MJ, Dahlgren AL, Laakso I, Seppanen-Laakso T, Dahlgren J, McAnulty PA. Red-wine polyphenols and inhibition of platelet aggregation: possible mechanisms, and potential use in health promotion and disease prevention. *J Int Med Res*. 1998;26:171–80.
9. de Lorimier AA. Alcohol, wine, and health. *Am J Surg*. 2000;180:357–61.
10. Murase T, Kume N, Hase T, Shibuya Y, Nishizawa Y, Tokimitsu I, Kita T. Gallates inhibit cytokine-induced nuclear translocation of NF- κ B and expression of leukocyte adhesion molecules in vascular endothelial cells. *Arterioscler Thromb Vasc Biol*. 1999;19:1412–20.
11. Ferrero ME, Bertelli AE, Fulgenzi A, Pellegatta F, Corsi MM, Bonfrate M, Ferrara F, De Caterina R, Giovannini L, et al. Activity in vitro of resveratrol on granulocyte and monocyte adhesion to endothelium. *Am J Clin Nutr*. 1998;68:1208–14.
12. Das DK, Sato M, Ray PS, Maulik G, Engelman RM, Bertelli AA, Bertelli A. Cardioprotection of red wine: role of polyphenolic antioxidants. *Drugs Exp Clin Res*. 1999;25:115–20.
13. Vinson JA, Teufel K, Wu N. Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in hamster model. *Atherosclerosis*. 2001;156:67–72.
14. Mark L, Nikfardjam MS, Avar P, Ohmacht R. A validated HPLC method for the quantitative analysis of trans-resveratrol and trans-piceid in Hungarian wines. *J Chromatogr Sci*. 2005;43:445–9.
15. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J*. 2003;17:1975–85.
16. Kopp P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the “French paradox”? *Eur J Endocrinol*. 1998;138:619–20.
17. Zern TL, Fernandez ML. Cardioprotective effects of dietary polyphenols. *J Nutr*. 2005;135:2291–4.
18. de Gaetano G, Cerletti C. Wine and cardiovascular disease. *Nutr Metab Cardiovasc Dis*. 2001;11:47–50.
19. Ferrali M, Signorini C, Caciotti B, Sugherini L, Ciccoli L, Giachetti D, Comporti M. Protection against oxidative damage of erythrocyte membranes by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett*. 1997;416:123–9.
20. Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann N Y Acad Sci*. 2002;957:146–61.
21. Hirano R, Sasamoto W, Matsumoto A, Itakura H, Igarashi O, Kondo K. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. *J Nutr Sci Vitaminol (Tokyo)*. 2001;47:357–62.
22. Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, Pieters L, Vlietinck AJ, Vanden Berghe D. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod*. 1998;61:71–6.
23. Vitseva O, Varghese S, Chakrabarti S, Folts JD, Freedman JE. Grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. *J Cardiovasc Pharmacol*. 2005;46:445–51.
24. Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin Chim Acta*. 1995;235:207–19.
25. Leikert JF, Rathel TR, Wohlfart P, Cheyner V, Vollmar AM, Dirsch VM. Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. *Circulation*. 2002;106:1614–7.
26. Wallerath T, Poleo D, Li H, Förstermann U. Red wine increases the expression of human endothelial nitric oxide synthase. *J Am Coll Cardiol*. 2003;41:471–8.
27. Olas B, Zbikowska HM, Wachowicz B, Krajewski T, Buczyński A, Magnuszewska A. Inhibitory effect of resveratrol on free radical generation in blood platelets. *Acta Biochim Pol*. 1999;46:961–6.
28. Hsieh TC, Juan G, Darzynkiewicz Z, Wu JM. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1) and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G(2). *Cancer Res*. 1999;59:2596–601.
29. Pignatelli P, Ghiselli A, Buchetti B, Carnevale R, Natella F, Germanò G, Fimognari F, Di Santo S, Lenti L, et al. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. *Atherosclerosis*. 2006;188:77–83.
30. Hong Y, Gengo FM, Rainka MM, Bates VE, Mager DE. Population pharmacodynamic modelling of aspirin- and ibuprofen-induced inhibition of platelet aggregation in healthy subjects. *Clin Pharmacokinet*. 2008;47:129–37.
31. Cesari F, Marucci R, Caporale R, Paniccia R, Romano E, Gensini GF, Abbate R, Gori AM. Relationship between high platelet turnover and platelet function in high-risk patients with coronary artery disease on dual antiplatelet therapy. *Thromb Haemost*. 2008;99:930–5.

32. Rao GH, Johnson GG, Reddy KR, White JG. Ibuprofen protects platelet cyclooxygenase from irreversible inhibition by aspirin. *Arterioscler Thromb Vasc Biol.* 1983;3:383–8.
33. Porcellati S, Gresele P, Stasi M, Buratta S, Horrocks LA, De Franceschi S, Nenci GG, Goracci G. Albumin prevents TxB₂ formation from thrombin-stimulated human platelets by sequestering the liberated arachidonic acid in the extracellular space. *Platelets.* 1995;6:381–7.
34. Pignatelli P, Di Santo S, Buchetti B, Sanguigni V, Brunelli A, Violi F. Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. *FASEB J.* 2006;20:1082–9.
35. Nardini M, Ghiselli A. Determination of free and bound phenolic acids in beer. *Food Chem.* 2004;84:137–43.
36. Gresele P, Migliacci R, Procacci A, De Monte P, Bonizzoni E. Prevention by NCX 4016, a nitric oxide-donating aspirin, but not by aspirin, of the acute endothelial dysfunction induced by exercise in patients with intermittent claudication. *Thromb Haemost.* 2007;97:444–50.
37. Falcinelli E, Guglielmini G, Torti M, Gresele P. Intraplatelet signaling mechanisms of the priming effect of matrix metalloproteinase-2 on platelet aggregation. *J Thromb Haemost.* 2005;3:2526–35.
38. Vezza R, Roberti R, Nenci GG, Gresele P. Prostaglandin E2 potentiates platelet aggregation by priming protein kinase C. *Blood.* 1993;82:2704–13.
39. Freedman JE, Parker C III, Li L, Perlman JA, Frei B, Ivanov V, Deak LR, Iafrafi MD, Fols JD. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. *Circulation.* 2001;103:2792–8.
40. Schwarz UR, Geiger J, Walter U, Eigenthaler M. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets: definition and detection of ticlopidine/clopidogrel effects. *Thromb Haemost.* 1999;82:1145–52.
41. Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata KI, Kawashima S, et al. Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb Res.* 2001;103:399–409.
42. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal.* 2006;8:691–728.
43. Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, Violi F. gp91phox-dependent expression of platelet CD40 ligand. *Circulation.* 2004;110:1326–9.
44. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by AKT-dependent phosphorylation. *Nature.* 1999;399:601–5.
45. Stojanovic A, Marjanovic JA, Brovkovich VM, Peng X, Hay N, Skidgel RA, Du X. A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. *J Biol Chem.* 2006;281:16333–9.
46. Schäfer A, Flierl U, Kobsar A, Eigenthaler M, Ertl G, Bauersachs J. Soluble guanylyl cyclase activation with HMR1766 attenuates platelet activation in diabetic rats. *Arterioscler Thromb Vasc Biol.* 2006;26:2813–8.
47. Wollny T, Aiello L, Di Tommaso D, Bellavia V, Rotilio D, Donati MB, de Gaetano G, Iacoviello L. Modulation of haemostatic function and prevention of experimental thrombosis by red wine in rats: a role for increased nitric oxide production. *Br J Pharmacol.* 1999;127:747–55.
48. Schwarz UR, Kobsar AL, Koksche M, Walter U, Eigenthaler M. Inhibition of agonist-induced p42 and p38 mitogen-activated protein kinase phosphorylation and CD40 ligand/P-selectin expression by cyclic nucleotide-regulated pathways in human platelets. *Biochem Pharmacol.* 2000;60:1399–407.
49. Anter E, Thomas SR, Schulz E, Shapira OM, Vita JA, Keaney JF Jr. Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols. *J Biol Chem.* 2004;279:46637–43.
50. Shen MY, Hsiao G, Liu CL, Fong TH, Lin KH, Chou DS, Sheu JR. Inhibitory mechanisms of resveratrol in platelet activation: pivotal roles of p38 MAPK and NO/cyclic GMP. *Br J Haematol.* 2007;139:475–85.
51. Castilla P, Echarri R, Dávalos A, Cerrato F, Ortega H, Teruel JL, Lucas MF, Gómez-Coronado D, Ortuño J, et al. Concentrated red grape juice exerts antioxidant, hypolipidemic, and antiinflammatory effects in both hemodialysis patients and healthy subjects. *Am J Clin Nutr.* 2006;84:252–62.