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) 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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5 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-cysteine; 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 ~0.7 ± 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. NODetect Assay kit, for the measurement of NO synthase (NOS) activity, was purchased from Stratagene Alexis Biochemicals. ³¹H]-Arginine was purchased from Amersham-Pharmacia Biotech, and AA, l-N¹⁵-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 antiinflamm-

**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 × 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 (33); we counted platelets (Genius, S.E.A.C) and adjusted them to 2 × 10⁹/µL 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 mM NaCl, 6 mM HEPES, 2 mM Na₂HPO₄, 2 mM 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 × 10¹² platelets/L. Platelets were activated with collagen (1.5 µg/mL) 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).
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.L.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 (NO detect assay kit) that measures NOS activity by monitoring the enzymatic conversion of \(^{(14)}\)-arginine to \(^{(14)}\)-citrulline. Briefly, the platelet suspension was incubated with 37 MBq/L of \(^{(14)}\)-arginine for 30 min at 37°C, then platelets were diluted with HEPES Tyrode’s to a final concentration of 2 x 10^11/L before 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, the supernatants were transferred into spectrophotometer cuvettes, and the reduction of cytochrome c was measured at 550 nm. To calculate the O\(_2^-\) generation evaluated by cytochrome c reduction. Generation of superoxide anion radicals (O\(_2^-\)) in resting platelets and in platelets stimulated with collagen (6 mg/L), after preincubation with resveratrol (0.1 and 0.5 \(\mu\)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 x 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 O\(_2^-\), an extinction coefficient for cytochrome c of 18,700 (mol/L)\(^{-1}\) cm\(^{-1}\) was used (42,43).

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 \(\mu\)L of platelet suspension (2 x 10^11/L), previously fixed with (2%) paraformaldehyde (0.1% BSA) for 60 min at room temperature and then permeabilized with digitonin (100 \(\mu\)mol/L) for 30 min at room temperature (40). Unbound antibody was removed by adding 0.1% BSA PBS and subsequent centrifugation at 3000 x g for 3 min (twice). Fluorescence intensity was analyzed on an Epics XL-MCL cytometer (Coulter Electronics) equipped with an argon laser at 488 nmol/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.
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 ± 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 ± 0.3 μmol/L for white wine and from 0.71 ± 0.02 to 1.72 ± 0.1 μmol/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 ± 0.3 μmol/L after red wine and 0.6 ± 0.3 μmol/L after white wine, with a mean increase of 0.7 ± 0.4 μmol/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 ± 3.4 μmol/L to 24.1 ± 3.6 μmol/L (P = 0.037)] or white wine [from 10.6 ± 2.4 μmol/L to 19.8 ± 4.8 μmol/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 ± 12.0 pmol/10^8 platelets of NO (Fig. 1A). Preincubation with resveratrol (0.2–0.5 μmol/L) produced an increase of NO production that was significant at the concentration of 0.5 μmol/L (Fig. 1A). At the same time, platelet aggregation decreased and this was significant at 0.5 μmol/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 ± 9.8 μmol/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 μmol/L resveratrol (Fig. 1C).

Platelet NOS activity upon stimulation with collagen was 16.9 ± 8.7 pmol ([H]l-citrulline/10^8 platelets). In resveratrol-treated platelets, ([H]l-citrulline production increased significantly at 0.5 μmol/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 μmol/L) dose-dependently enhanced AKT phosphorylation induced by collagen, with a significant increase when the concentration was at least 0.1 μmol/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 μmol/L) dose-dependently inhibited p38 MAP-kinase phosphorylation, with a significant reduction when the concentration was at least 0.1 μmol/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 μmol/L), VASP phosphorylation increased further, with a significant rise when the concentration was at least 0.1 μmol/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 ± SEM, n = 5. *Different from collagen alone, P < 0.001.
Platelet NADPH oxidase activity and cytochrome c reduc-
tion. 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 μmol/L) compared with AA alone (Fig. 3A). Preincubation with resveratrol (0.1–0.5 μmol/L) decreased AA-mediated \( \text{O}_2^- \) formation, with a significant reduction when the concentration was at least 0.1 μmol/L (Fig. 3A). Confirmatory experiments were conducted with a higher concentration of NADPH (25 mmol/L), giving comparable results (data not shown). Generation of \( \text{O}_2^- \) in control platelets was enhanced by stimulation with collagen (Fig. 3B) and preincubation with resveratrol (0.1–0.5 μmol/L) decreased collagen-mediated \( \text{O}_2^- \) production, with a significant reduction already at 0.1 μmol/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 superna-
tant 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 incor-
poration of \(^3\text{H}\)l-citrulline in platelets preincubated with \(^3\text{H}\)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 \(^3\text{H}\)l-citrulline in platelets preincubated with \(^3\text{H}\)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 PKB 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 PKB-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, p38 MAPK phosphorylation induces the activation of platelet integrin \(\alpha_{\text{IIb}}\beta_3\).
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 activation.

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 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**


