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In Vitro Inhibition of Human cGMP-Specific Phosphodiesterase-5 by Polyphenols from Red Grapes

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A moderate consumption of red wine may reduce the risk of cardiovascular diseases via wine-derived phenolic compounds. A variety of biological mechanisms have been proposed for wine-derived phenolic compounds including nitric oxide-mediated vasorelaxation. This study examined whether the vasodilating effect of wine-derived phenolic compounds was associated with the inhibition of phosphodiesterases (PDEs) and, in particular, PDE5. For this purpose, human recombinant PDE5A1 isoform was prepared by expression of the full-length cDNA of PDE5A1 into COS-7 cells. Red wine and the extracts from grape skin inhibited PDE5A1 activity, whereas the seed extracts had a negligible effect. The mixture of anthocyanins inhibited the enzyme activity (IC_{50} = 11.6 μM), with malvidin-3-*O*-β-glucoside (IC_{50} = 35.4 μM) and malvidin (IC_{50} = 24.9 μM) the most potent among the monoglucosides and aglycons, respectively. *trans*-Resveratrol and *trans*-piceid exhibited negligible effects, whereas hydroxycinnamates were completely inactive. These results indicate that polyphenols-induced vasorelaxation may also be sustained by smooth muscle PDE inhibition by anthocyanins present in red wines and grapes.

KEYWORDS: Anthocyanins; red grape polyphenols; wine; phosphodiesterase-5; *Vitis vinifera*

INTRODUCTION

Wine has been an important component of human culture for over 6000 years, serving dietary and socioreligious functions (*1*). Epidemiological studies have shown a putative correlation between the moderate consumption of red wine and a lowered risk of coronary heart disease (*2*, *3*). Red wine represents a rich source of phenolics such as anthocyanins (ACs), catechins, proanthocyanidins (PAs), stilbenes, hydroxycinnamates (HCs), and other phenolics. The levels of phenolics in wines are highly variable according to grape varieties, area of cultivation, and vinification methods, and many differences exist between red and white and aged versus young wines (*4*). ACs are flavonoids widely distributed in fruits, vegetables, and red wine. They provide color to red wines and to the skin of red and black grapes (*5*). PAs, another class of plant phenol metabolites occurring in fruits, vegetables, seeds, and flowers, are usually the most abundant flavonoids in red wines. ACs and PAs are among the most important compounds in determining the quality of red wine, because they greatly influence color, bitterness, astringency, and chemical stability toward oxidation (*4*). Resveratrol, 3,4,5-trihydroxystilbene, is a naturally occurring phytoalexin produced by grapevines in response to injury. In red wines, the concentration of the trans-isomer, which is the most abundant form, ranges between 0.1 and 15 mg/L, and its corresponding 3-*O*-β-glucoside, named *trans*-piceid, is also found (2.5−14.5 mg/L) (*6*). HCs, mainly caftaric, coutaric, and fertaric acids, also occur at levels of approximately 130 and 60 mg/L in white and red wines, respectively (*7*).

Biological mechanisms proposed for red wine-derived phenolic compounds include estrogenic activity (*8*), antioxidant/antiradical activity, inhibition of platelet aggregation, modulation of lipid metabolism, inhibition of low-density lipoprotein oxidation, and proliferation of smooth muscle cells (SMC) (*2*, *9*−*12*). Last, but not least, wine polyphenols exhibit vasorelaxing effects, mainly through NO-dependent mechanisms (*13*−*15*).

The NO-dependent vasorelaxation by red wine-derived phenolic compounds has been extensively investigated: several studies reported that red wines and grapes exhibit endothelium-dependent vasorelaxation via enhanced generation and/or increased biological activity of NO, leading to elevation of cGMP levels (*16*). In vivo red wine-derived phenolic compounds were able to reduce blood pressure in normo- and hypertensive rats (*17*), and the amplitude of vasorelaxation changed as a function of the variability of wine constituents according to grape varieties, cultivar, and methods for obtaining wine (*18*). Consequently, the vasodilatory effect does not apply to all wines, and the degree of vasorelaxation is correlated to the content and the structure of the polyphenols. According to Burns et al.

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(*18*), vasodilation activity was correlated to the total content of ACs. Further investigations aiming to characterize the constituents responsible for the vasorelaxation activity showed that AC-enriched fractions and oligomeric PAs, mainly dimers, trimers, and tetramers, were the active compounds, whereas monomers (catechins) and simple phenols, such as benzoic acid, gallic acid, and HCs, were devoid of effect (*14*, *19*, *20*).

Until now, studies concerning the relaxing effects of wine polyphenols on the arterial wall focused on mechanisms mediated by NO release, whereas other possible mechanisms such as the inhibition of phosphodiesterases (PDEs) in the SMC were never considered. Indeed, rat thoracic aortic rings could be relaxed by red wine-derived phenolic compounds also when functional endothelium was removed, and the anthocyanin-enriched fractions were the most active in this regard (*19*).

When NO is released by the endothelial cells, the direct activation of guanyl cyclase leads to the accumulation of cGMP and the consequent activation of protein kinase G, which induce a decrease of smooth muscle tone via an alteration in calcium signaling (*21*). The duration and amplitude of the signal are controlled by the degradation of cGMP by cyclic nucleotide PDEs.

Depending on tissue localization and substrate specificity, PDEs are involved in the control of platelet aggregation, blood pressure, cardiac function, vascular tone, and many other functions. Physiological regulation and the role for individual PDE isoforms are reviewed by Beavo (*22*). In particular, cGMP-specific PDE5 in vascular SMC regulates cGMP levels and, consequently, the duration and extent of vasorelaxation. Accordingly, the PDE5 isoform represents the pharmacological target of the drugs for the treatment of erectile dysfunction, such as sildenafil (Viagra) (*23*).

Thus, the present work investigated whether vasorelaxation by red wine-derived phenolic compounds could also be related to the PDE inhibition; for this purpose, wine and grape polyphenols were tested on cGMP-dependent PDE5, using a recombinant form of human PDE5A1 isoenzyme (*24*).

MATERIALS AND METHODS

Materials. Culture medium Dulbecco's modified Eagle's medium, trypsin, protease inhibitors, and all of the chemical reagents for cell culture were purchased from Sigma Aldrich (Milan, Italy). Penicillin, streptomycin, and L-glutammine were from GIBCO (Grand Island, NY); fetal calf serum was provided by Mascia Brunelli S.p.A. (Milan, Italy). The COS-7 cell line was purchased from ATCC (Manassas, VA). Superfect reagent for transient transfections was obtained from Qiagen GmbH (Hilden, Germany). The expression plasmid pcDNA3 containing the full-length cDNA of PDE5A1 was a kind gift of Prof. C. S. Lin (Department of Urology, University of California, San Francisco, CA).

[^{3}H]cGMP was from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, U.K.).

DEAE-Sephadex A25 was from Pharmacia (Uppsala, Sweden). cGMP, zaprinast, *Crotalus adamanteus* snake venom, and *trans*-resveratrol were purchased from Sigma Aldrich. Sildenafil was provided by Sequoia Research Products (Oxford, U.K.).

trans-Piceid was extracted with methanol from the dried roots of *Polygonum cuspidatum*. The extract was concentrated under vacuum and taken up with ethyl acetate. The *trans*-piceid was selectively precipitated with *n*-hexane and recovered by filtration. The final purification was obtained by (i) flash chromatography on a silica column, (ii) reversed-phase HPLC chromatography (*25*), and (iii) crystallization, thus obtaining a compound of purity >98%.

Preparation of Grape Extracts. The skins and seeds of fresh berries of five different varieties of red grape, namely, Barbera, Cabernet Sauvignon, Nebbiolo, Sagrantino, and Teroldego, collected at harvest, were extracted by means of a selective extraction method with a winelike solution (*4*), to obtain quantitative information about the localization of the active fraction of the polyphenols in the berry. A wine produced with Cabernet Sauvignon grape, vintage 2000, aged in barrique, was obtained from the Iasma winery. Spectrophotometric analyses of total polyphenols reactive to Folin−Ciocalteu, expressed as (+)-catechin (mg/L), and of PAs by conversion to anthocyanidins by acid-catalyzed cleavage of the interflavonoid bonds, expressed as cyanidin (mg/L), were carried out under the conditions described in the literature (*26*). The spectrophotometric assay chosen for the analysis of PAs was considered to be adequate because it has already proven to be in agreement with the normal-phase HPLC method for the quantification of this class of wine phenolics (*27*).

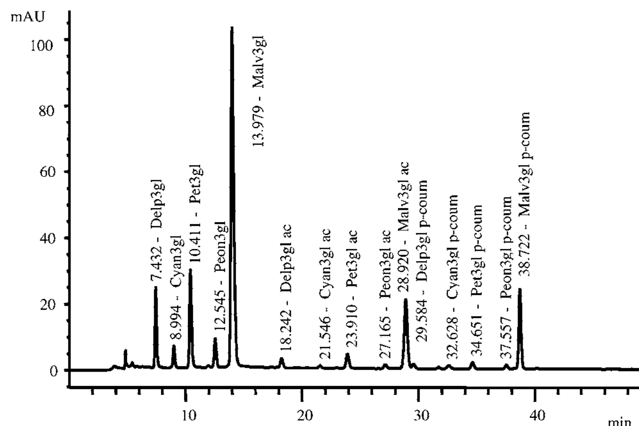


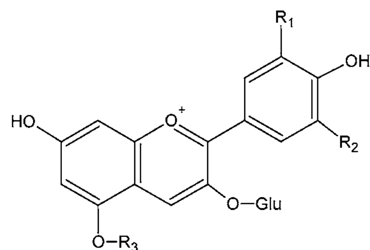
Figure 1. Representative profile of a grape extract analyzed by reversed phase HPLC at 520 nm, as described under Materials and Methods: delphinidin 3-glucoside (Delp3gl), cyanidin 3-glucoside (Cyan3gl), petunidin 3-glucoside (Pet3gl), peonidin 3-glucoside (Peon3gl), malvidin 3-glucoside (Malv3gl), and their corresponding acetic acid (ac) and *p*-coumaric acid esters (p-coum).

The analysis of ACs was performed by a reversed-phase HPLC method as previously described (*28*). Delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, and their relevant acetic acid and *p*-coumaric acid esters were quantified at 520 nm with a calibration curve with malvidin-3-glucoside chloride and expressed as milligrams per liter. The sum of these 15 compounds gives the total amount of ACs. **Figure 1** reports a representative analysis of the standardized mixture of grape ACs.

Preparation of the Standardized Mixture of Grape ACs, Single AC, and the Corresponding Aglycons. The standardized mixture of ACs employed for this experiment was isolated from the skin of grape (*Vitis vinifera* cv. Cabernet Sauvignon) according to the procedure already reported (*29*). It had the following composition, measured as percentage HPLC area at 520 nm: delphinidin 3-glucoside, 11.89%; cyanidin 3-glucoside, 1.94%; petunidin 3-glucoside, 13.83%; peonidin 3-glucoside, 9.26%; malvidin 3-glucoside, 47.18%; delphinidin 3-(6-*O*-acetyl)glucoside, 2.15%; cyanidin 3-(6-*O*-acetyl)glucoside, 0.24%; petunidin 3-(6-*O*-acetyl)glucoside, 2.21%; peonidin 3-(6-*O*-acetyl)-glucoside, 1.24%; malvidin 3-(6-*O*-acetyl)glucoside, 7.49%; delphinidin 3-(6-*O*-*p*-coumaroyl)glucoside, 0.12%; cyanidin 3-(6-*O*-*p*-coumaroyl)-glucoside, 0.03%; petunidin 3-(6-*O*-*p*-coumaroyl)glucoside, 0.40%; peonidin 3-(6-*O*-*p*-coumaroyl)glucoside, 0.17%; malvidin 3-(6-*O*-*p*-coumaroyl)glucoside, 1.86%. Delphinidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside were extracted from *V. vinifera* (cv. Cabernet Sauvignon) grape and purified by means of sequential chromatography on three different absorbents (Polyclar SB100, Sephadex LH 20, and Lichrospher 100 RP-18), according to the procedure described earlier and crystallized as chloride salts with purity >98% (*29*).

All other standards of ACs were obtained in the chloride form from Polyphenols Laboratories (Sandnes, Norway) and were certified from the producer for a purity >97% (HPLC, 280 and 520 nm). The structures of the ACs used for the experiments are reported in **Figure 2**.

Molar Absorptivity of the Anthocyanins. The pure standards of ACs used for the study were dissolved in the solution ethanol/water/



COMPOUND	R_1	R_2	R_3
Malvidin 3-glucoside	OCH_3	OCH_3	H
Peonidin 3-glucoside	OCH_3	H	H
Delphinidin 3-glucoside	OH	OH	H
Petunidin 3-glucoside	OH	OCH_3	H
Cyanidin 3-glucoside	OH	H	H
Pelargonidin 3-glucoside	H	H	H
Malvidin 3,5-diglucoside	OCH_3	OCH_3	Glu
Cyanidin 3,5-diglucoside	OH	H	Glu

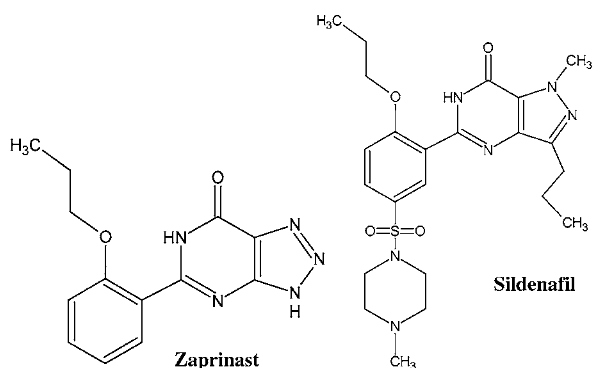


Figure 2. Structures of the ACs and reference compounds used for the experiments.

HCl (70:30:1), at a pH value of 1.26. Two solutions were prepared containing, respectively, 5 and 10 mg/L of each compound, thus obtaining final concentrations in the range (0.7−3.9) $\times$ 10^{-5} mol/L. The spectral characteristics of the known solutions were recorded on a Hitachi U-2000 UV−vis spectrophotometer using quartz cells with a 10 mm optical path. The molecular weight used for molar absorptivity calculations included the weight of a chloride counterion. The average experimental values of the molar absorptivities of each standard anthocyanin, measured both at λ_{max} and at 520 nm, are reported in **Table 1**.

Preparation of the Standardized Mixture of HCs. The HCs were extracted by pressing grape berries (*V. vinifera* cv. Rhine Riesling) in the presence of ascorbic acid (1 g/kg) and sodium metabisulfite (1 g/kg). The juice was treated with bentonite (1 g/L) for 12 h at 4 °C to remove proteins. The HCs were then absorbed by stirring the juice with 20 g/L of activated carbon and diatomaceous earth (1:1). The absorbent was washed with water, and the HCs were eluted with methanol/acetic acid (99:1) and brought to dryness in rotary evaporator. The final purification of the HCs was obtained by (i) flash chromatography on a column packed with TSK gel Toyopearl HW-40S (Toso Haas) and elution of the HCs fraction with the mixture (1:4) methanol and acid water (0.5% acetic acid) and (ii) crystallization of HCs dissolved in diethyl ether, stabilized with copper gauze (Carlo Erba, Milan, Italy) with *n*-hexane. The final composition of the standardized mixture of grape HCs (expressed as equivalent area of *trans*-caffeic acid, HPLC, 320 nm) was *trans*-caftaric acid (88.07%), *trans-p*-cutaric acid (7.45%), and *trans*-fertaric acid (4.48%).

Expression of Human Recombinant PDE5A1. Human recombinant PDE5A1 was prepared by expression of the full-length cDNA of PDE5A1 into COS-7 cells as previously described (*24*). Briefly, COS-7

Table 1. Average Experimental Values of Molar Absorptivity of Available Standard Compounds

pigment	source[a]	molar absorptivity, max	wavelength, max	molar absorptivity, 520 nm
pelargonidin chloride	1	26154	529	25357
cyanidin chloride	1	17559	544	14421
peonidin chloride	1	24584	543	20335
delphinidin chloride	1	18492	553	12602
petunidin chloride	1	17359	552	12006
malvidin chloride	1	28987	553	20101
pelargonidin 3-glucoside chloride	1	22447	515	22066
cyanidin 3-glucoside chloride	1	26758	534	24823
peonidin 3-glucoside chloride	1	25803	531	24476
delphinidin 3-glucoside chloride	2	21600	544	17302
petunidin 3-glucoside chloride	2	33518	543	27207
malvidin 3-glucoside chloride	2	30433	542	25454
cyanidin 3,5-diglucoside chloride	1	33358	529	31967
malvidin 3,5-diglucoside chloride	1	33760	538	28469

[a] 1, pure standard from Polyphenols Laboratories; 2, pure standard isolated from Cabernet Sauvignon grape.

cells were cultured in 100 mm dishes (10^6 cells/dish) and were transfected with expression plasmid pcDNA3 containing the full-length cDNA of PDE5A1 (10 μg), using Superfect reagent. Forty-eight hours post-transfection, cells were collected in buffer containing 40 mM Tris-HCl, pH 7.5, 15 mM benzamidine, 15 mM 2-mercaptoethanol, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 5 mM EDTA. Cells were homogenized in a Dounce homogenizer on ice with 25 strokes. Insoluble materials were removed by centrifugation (Centrifuge 5810 R, Eppendorf s.r.l., Milan, Italy) of the cell lysate at 14000*g* for 20 min at 4 °C. The supernatant was diluted with an equal volume of glycerol, and the total protein concentration was determined according to the method of Bradford (*30*). Cell lysates were stored at −80 °C.

Enzyme Assay. PDE5A1 activity was determined according to the method of Kincaid and Manganiello (*31*). Cell lysate (70 μL at 0.2 mg of protein/mL) was incubated with 0.5 μM cGMP and 60000 cpm [^{3}H]-cGMP in 40 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$ and 2 mg/mL bovine albumin; final reaction volume was 100 μL. After 30 min of incubation at 37 °C, the reaction was stopped with 0.1 N HCl; the pH was then adjusted to 7 with 0.1 N NaOH, and samples were incubated for a further 30 min at 37 °C with 100 μL (1 mg/mL) of nucleotidase (*Crotalus adamanteus* snake venom) to cleave GMP to the corresponding nucleoside. Guanyl nucleoside was then separated from the unreacted products by anion exchange chromatography, using DEAE-Sephadex A25. The eluted radioactive guanosine was counted in a β-scintillation counter. PDE5A1 activity was expressed as picomoles of product formed per minute per milligram of protein.

Cabernet Sauvignon wine (containing 2108 mg/L of total phenols) and the polyphenols extracted separately from skins and seeds of five different grape cultivars growing in Italy were assayed at the concentration of 25 μg/mL (calculated as total phenols). Control samples were added with the vehicle used to resuspend the extracts. Successively, *trans*-resveratrol, *trans*-piceid, the standardized mixture of ACs from Cabernet Sauvignon grapes, single 3-*O*-β-glucosides of malvidin, delphinidin, cyanidin, petunidin, peonidin, pelargonidin, and their corresponding aglycons (1−100 μM), and the standardized mixture of HCs (1−100 μM) were assayed. Sildenafil (0.5−500 nM) and zaprinast (0.1−100 μM), two selective inhibitors of PDE5, were used as reference compounds (**Figure 2**). In our experimental conditions, IC_{50} values of sildenafil and zaprinast were 75.2 nM and 9.8 μM, respectively, close to the values reported in the literature (*24*).

Statistical Analysis. A two-way analysis of variance followed by post hoc analysis using the Scheffe procedure was used to test for statistical differences. Differences with $p < 0.05$ were considered to be significant. Concentration−inhibition curves were analyzed as reported elsewhere (*32*), and each point represents the mean of six replications ± the standard deviation (SD).

Table 2. Effect of Cabernet Savignon Wine and the Polyphenols Extracted from Grape Skin and Seeds of Different Cultivars of Italian Grape on PDE5A1 Activity[a]

	PAs, mg/L	ACs, mg/L	PDE5A1,[b] % vs control
Cabernet Sauvignon wine	2434	31.4	78.8 ± 2.0**
Cabernet Sauvignon skin	908	38.1	74.7 ± 3.6**
Cabernet Sauvignon seeds	1104	0	91.6 ± 5.7
Teroldego skin	1111	87.4	73.6 ± 5.9**
Teroldego seeds	930	0	99.7 ± 0.7
Barbera skin	1017	350.7	78.3 ± 2.7**
Barbera seeds	233	0	98.5 ± 2.5
Nebbiolo skin	2371	64.6	85.1 ± 4.6**
Nebbiolo seeds	785	0	93.3 ± 2.2*
Sagrantino skin	3545	352.1	85.7 ± 3.5**
Sagrantino seeds	1232	0	96.7 ± 4.5

[a] PAs and ACs were quantified as described under Materials and Methods. The amounts of PAs and ACs are expressed as cyanidin and malvidin-3-monoglucoside chloride, respectively. [b] Data on PDE5A1 activity are expressed as percent activity vs control (mean ± SD, $n = 9$), testing all of the mixtures at 25 μg/mL of total polyphenols. *, $p < 0.001$; **, $p < 0.0001$.

RESULTS AND DISCUSSION

Control PDE5A1 activity was 51.9 ± 9.1 pmol min^{-1} (mg of protein)$^{-1}$ (mean ± SD, $n = 14$). In the first set of experiments, the effect of Cabernet Sauvignon wine and of the polyphenols extracted from grape skin and seeds on in vitro activity of PDE5A1 was tested. The ACs as well as the HCs, the PAs, and the resveratrols are ubiquitous components in the skin of red grapes and therefore present in the skin extracts, whereas the seed polyphenols are almost exclusively constituted of PAs with a minor presence of the flavanol monomers (*33*). The seed flavanols are galloylated and nongalloylated PAs of DP 2−19, mDP ~3, constituted mainly of the three subunits (−)-epicatechin, (+)-catechin, and (−)-epicatechin 3-gallate in the ratio 3:1:1 and linked by 4 → 8 and 4 → 6 interflavanic bonds (*33*). As shown in **Table 2**, red wine and the extracts from grape skin inhibited the activity of PDE5A1, whereas all of the seed extracts were inactive or had a negligible effect. The composition of the flavanols in the grape skin has been suggested to differ from those in the seeds for having a lower content of monomers and a higher DP of the PAs (2−83, mDP ~33) and for containing in their structure, in addition to the three subunits present in grape seeds PAs, also a considerable amount of (−)-epigallocatechin (*34*). Their structure is not completely clarified because inconsistencies between the terminal subunits identified with different methods have been observed (*35*).

The similar chemical nature of seeds and skin PAs and the lack of effect of the seeds extracts led us to hypothesize that PAs, among the polyphenols present in the skin extracts, do not contribute significantly to the inhibitory effect against PDE5A1.

To evaluate whether the effect on PDE5A1 activity could be linked to the presence of ACs, we tested a mixture of ACs, isolated from Cabernet Sauvignon. As shown in **Figure 3A**, the mixture inhibited PDE5A1 in a concentration-dependent fashion, statistically significant inhibition starting at 1 μM. At 100 μM, the inhibition was almost complete. The IC_{50}, calculated by considering an average MW of 527, was 11.6 μM, which is very close to that of zaprinast (9.8 μM). Our results are in agreement with a previous study by Ferretti et al. (*36*), who found that ACs from *Vaccinium myrtillus* inhibited PDEs from different tissues. Other investigations were not consistent with our data (*37*, *38*). The controversial results could be ascribed to the experimental conditions for PDE testing, the type of PDE isoform used, and the source of ACs mixture. Our results confirmed the hypothesis that the effect of grape skin extracts on PDE was due to the presence of ACs.

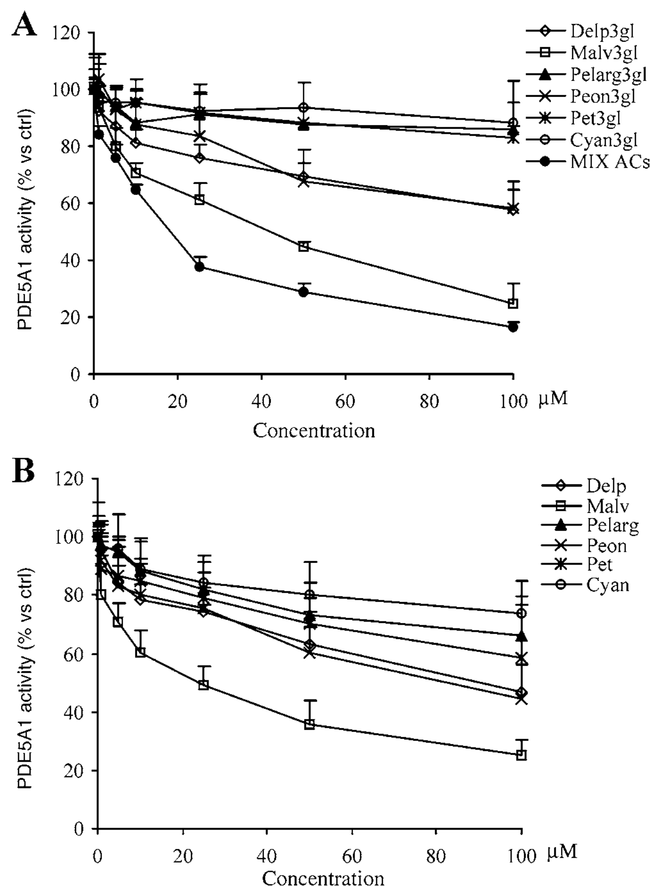


Figure 3. Effect of standardized mixture of ACs, the single AC (**A**), and the corresponding aglycons (**B**) on PDE5A1 activity. Results are expressed as percent of control activity ± SD. Control enzyme activity was 51.9 ± 9.1 pmol min^{-1} (mg of protein)$^{-1}$ (mean ± SD, $n = 14$).

To investigate how the single glucosides contributed to the overall effect, we tested six among the most representative ACs. Even if not present in red grape, pelargonidin 3-*O*-β-glucoside was also tested, to obtain structural information required for the efficacy on PDE5A1. Malvidin 3-*O*-β-glucoside was found to be the most potent, with an IC_{50} of 35.4 μM. None among the tested glucosides accounted for the total effect of the mixture (**Figure 3A**). It is worth recalling that the ACs mixture, like in the wine or in the skin extracts (**Figure 1**), contains also a non-negligible amount of ACs esterified with *p*-coumaric or acetic acid, which could possibly explain the higher activity of the mixture. The unavailability of these compounds in a pure isolated form is an obstacle to the verification of this hypothesis.

Because it is not defined yet if glucosides reach the site of effect as such or after deconjugation to give the corresponding aglycons, the latter were also tested for PDE activity. The concentration−inhibition curves for the aglycons are reported in **Figure 3B**. Malvidin was still the most potent, with an IC_{50} of 24.9 μM, but glucosidation diminished the inhibitory effect on PDE5A1 activity. This observation was further confirmed because the corresponding 3,5-diglucosides of malvidin and cyanidin tested on PDE5A1 activity at 50 μM were found to be inactive [57.01 ± 2.5 and 57.4 ± 4.1, respectively, vs control PDE activity of 62.9 ± 0.7 pmol min^{-1} (mg of protein)$^{-1}$]. The degree of inhibition for both glucosides and aglycons followed

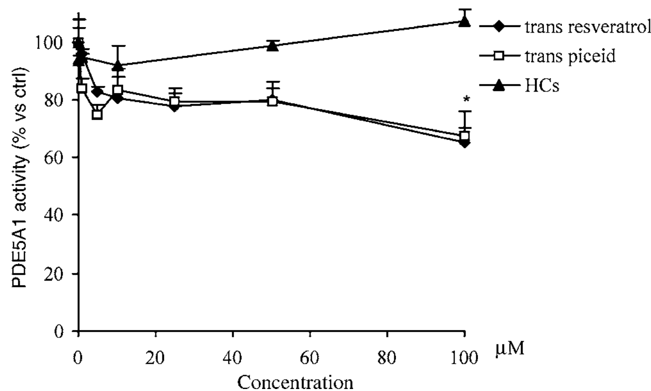


Figure 4. Effect of *trans*-resveratrol, *trans*-piceid, and HCs on PDE5A1 activity. Results are expressed as percent of control activity ± SD. Control enzyme activity was 51.9 ± 9.1 pmol min^{-1} (mg of protein)$^{-1}$ (*, $p < 0.05$).

this order: malvidin > peonidin = delphinidin > petunidin > pelargonidin = cyanidin.

Because wine contains also stilbenes (*trans*-resveratrol and *trans*-piceid) and HCs, it was also investigated whether both of these classes of compounds could affect PDE5A1 activity. As shown in **Figure 4**, PDE5A1 was not inhibited by either compound. Only a minor effect (−30% vs control) was seen with 100 μM *trans*-resveratrol and *trans*-piceid.

The results reported in this paper demonstrate that red wine and polyphenols occurring in red grapes inhibit human cGMP-specific PDE. Among the different classes of phenols, only ACs seem to be the active constituents, because seed extracts (mainly containing PAs), stilbenes, and HCs showed no effect. Our results agree with previous observations that red wine-derived phenolic compounds caused relaxation of aortic rings in the absence of functional endothelium, although at higher concentrations compared to those necessary when endothelium was present (*14*). It is remarkable that ACs have an effect comparable to that of the well-known specific PDE5 inhibitor zaprinast (11.6 vs 9.8 μM, respectively). Malvidin-3-*O*-β-glucoside was found to be the most active compound; however, none of the single compounds accounted for the total activity of the mixture.

This work describes for the first time the in vitro inhibition of human PDE5A1 activity by polyphenols, in particular, ACs from wine and grapes, suggesting that the vasodilating effect might also occur through a process complementary to the increase of NO availability. However, the physiological significance of the findings awaits further verification as to whether (i) ACs reach the site of effect unmodified, after being absorbed through the gastrointestinal tract, and (ii) AC levels in situ attain concentrations compatible with those necessary for PDE inhibition. According to animal and human studies ACs are absorbed in the glycosylated form after oral consumption (*39*−*42*), and the stomach seems to be the site of absorption for ACs (*29*, *43*). Circulating AC levels in general are low (*44*) due to limited bioavailability and rapid distribution, as shown for blueberry ACs (*45*). Furthermore, plasma levels had been evaluated only after single dosing of wine or fruit extracts, and it is unknown which levels ACs attain when wine or fruit is consumed daily. The amount of ACs in the vascular wall has not been examined as well. Pharmacological dosing, as might occur when dietary polyphenols are consumed as supplements, might lead to AC levels compatible with the range at which anthocyanins exert vasorelaxation through PDE inhibition.

ABBREVIATIONS USED

PDE, phosphodiesterase; ACs, anthocyanins; HCs, hydroxycinnamates; PAs, proanthocyanidins; NO, nitric oxide; HPLC, high-performance liquid chromatography; SMC, smooth muscle cell.

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LITERATURE CITED

(1) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. Wine as a biological fluid: history, production, and role in disease prevention. *J. Clin. Lab. Anal.* **1997**, *11* (5), 287−313.

(2) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341* (8843), 454−457.

(3) Aviram, M.; Fuhrman, B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 146−161.

(4) Mattivi, F.; Zulian, C.; Nicolini, G.; Valenti, L. Wine, biodiversity, technology, and antioxidants. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 37−56.

(5) Francis, F. J. Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.* **1989**, *28* (4), 273−314.

(6) Fremont, L. Biological effects of resveratrol. *Life Sci.* **2000**, *66* (8), 663−673.

(7) Waterhouse, A. L. Wine phenolics. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 21−36.

(8) Klinge, C. M.; Risinger, K. E.; Watts, M. B.; Beck, V.; Eder, R.; Jungbauer, A. Estrogenic activity in white and red wine extracts. *J. Agric. Food Chem.* **2003**, *51*, 1850−1857.

(9) Demrow, H. S.; Slane, P. R.; Folts, J. D. Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. *Circulation* **1995**, *91* (4), 1182−1188.

(10) Santos-buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds: nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(11) Leighton, F.; Cuevas, A.; Guasch, V.; Perez, D. D.; Strobel, P.; San Martin, A.; Urzua, U.; Diez, M. S.; Foncea, R.; Castillo, O.; Mizon, C.; Espinoza, M. A.; Urquiaga, I.; Rozowski, J.; Maiz, A.; Germain, A. Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans. *Drugs Exp. Clin. Res.* **1999**, *25* (2−3), 133−141.

(12) Perez, D. D.; Strobel, P.; Foncea, R.; Diez, M. S.; Vasquez, L.; Urquiaga, I.; Castillo, O.; Cuevas, A.; San Martin, A.; Leighton, F. Wine, diet, antioxidant defenses, and oxidative damage. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 136−145.

(13) Fitzpatrick, D. F.; Hirschfield, S. L.; Coffey, R. G. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol.* **1993**, *265* (2 Pt 2), H774−H778.

(14) Andriambeloson, E.; Kleschyov, A. L.; Muller, B.; Beretz, A.; Stoclet, J. C.; Andriantsitohaina, R. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br. J. Pharmacol.* **1997**, *120* (6), 1053−1058.

(15) Andriambeloson, E.; Stoclet, J. C.; Andriantsitohaina, R. Mechanism of endothelial nitric oxide-dependent vasorelaxation induced by wine polyphenols in rat thoracic aorta. *J. Cardiovasc. Pharmacol.* **1999**, *33* (2), 248−254.

(16) Li, H.; Forstermann, U. Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* **2000**, *190* (3), 244−254.

(17) Diebolt, M.; Bucher, B.; Andriantsitohaina, R. Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. *Hypertension* **2001**, *38* (2), 159−165.
(18) Burns, J.; Gardner, P. T.; O'Neil, J.; Crawford, S.; Morecroft, I.; McPhail, D. B.; Lister, C.; Matthews, D.; MacLean, M. R.; Lean, M. E.; Duthie, G. G.; Crozier, A. Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *J. Agric. Food Chem.* **2000**, *48*, 220−230.
(19) Andriambeloson, E.; Magnier, C.; Haan-Archipoff, G.; Lobstein, A.; Anton, R.; Beretz, A.; Stoclet, J. C.; Andriantsitohaina, R. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.* **1998**, *128* (12), 2324−2333.
(20) Fitzpatrick, D. F.; Bing, B.; Maggi, D. A.; Fleming, R. C.; O'Malley, R. M. Vasodilating procyanidins derived from grape seeds. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 78−89.
(21) Rybalkin, S. D.; Rybalkina, I. G.; Feil, R.; Hofmann, F.; Beavo, J. A. Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J. Biol. Chem.* **2002**, *277* (5), 3310−3317; Epub Nov 26, 2001.
(22) Beavo, J. A. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* **1995**, *75* (4), 725−748.
(23) Koyama, H.; Bornfeldt, K. E.; Fukumoto, S.; Nishizawa, Y. Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. *J. Cell Physiol.* **2001**, *186* (1), 1−10.
(24) Lin, C. S.; Lau, A.; Tu, R.; Lue, T. F. Expression of three isoforms of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in human penile cavernosum. *Biochem. Biophys. Res. Commun.* **2000**, *268* (2), 628−635.
(25) Mattivi, F.; Reniero, F.; Korhammer, S. Isolation, characterization, and evolution in red wine vinification of resveratrol monomers. *J. Agric. Food Chem.* **1995**, *43*, 1820−1823.
(26) Rigo, A.; Vianello, F.; Clementi, G.; Rossetto, M.; Scarpa, M.; Vrhovsek, U.; Mattivi, F. Contribution of proanthocyanidins to the peroxy radical scavenging capacity of some Italian red wines. *J. Agric. Food Chem.* **2000**, *48*, 1996−2002.
(27) Vrhovsek, U.; Mattivi, F.; Waterhouse, A. L. Analysis of red wine phenolics: comparison of HPLC and spectrophotometric methods. *Vitis* **2001**, *40* (2), 87−91.
(28) Rossetto, M.; Vanzani, P.; Zennaro, L.; Mattivi, F.; Vrhovsek, U.; Scarpa, M.; Rigo, A. Stable free radicals and peroxyl radical trapping capacity in red wines. *J. Agric. Food Chem.* **2004**, *52*, 6151−6155.
(29) Passamonti, S.; Vrhovsek, U.; Mattivi, F. The interaction of anthocyanins with bilitranslocase. *Biochem. Biophys. Res. Commun.* **2002**, *296* (3), 631−636.
(30) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(31) Kincaid, R. L.; Manganiello, V. C. Assay of cyclic nucleotide phosphodiesterase using radiolabeled and fluorescent substrates. *Methods Enzymol.* **1988**, *159*, 457−470.
(32) De Lean, A.; Munson, P. J.; Rodbard, D. Simultaneous analysis of families of sigmoidal curves: applications to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **1978**, *235*, E97−E102.
(33) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36* (3), 781−784.
(34) Souquet, J.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43* (2), 509−512.
(35) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348−5355.
(36) Ferretti, C.; Magistretti, M. J.; Robotti, A.; Ghi, P.; Genazzani, E. *Vaccinium myrtillus* anthocyanosides are inhibitors of cAMP and cGMP phosphodiesterases. *Pharmacol. Res. Commun.* **1988**, *20* (Suppl. II), 150.
(37) Matsumoto, H.; Nakamura, Y.; Tachibanaki, S.; Kawamura, S.; Hirayama, M. Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. *J. Agric. Food Chem.* **2003**, *51*, 3560−3563.
(38) Virmaux, N.; Bizec, J. C.; Nullans, G.; Ehret, S.; Mandel, P. Modulation of rod cyclic GMP-phosphodiesterase activity by anthocyanidin derivatives. *Biochem. Soc. Trans.* **1990**, *18* (4), 686−687.
(39) Cao, G.; Muccitelli, H. U.; Sanchez-Moreno, C.; Prior, R. L. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73* (5), 920−926.
(40) Cao, G.; Prior, R. L. Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clin. Chem.* **1999**, *45* (4), 574−576.
(41) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546−1551.
(42) Nielsen, I. L.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. Absorption and excretion of black currant anthocyanins in humans and watanabe heritable hyperlipidemic rabbits. *J. Agric. Food Chem.* **2003**, *51*, 2813−2820.
(43) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. *FEBS Lett.* **2003**, *544* (1−3), 210−213.
(44) Dell'Agli, M.; Busciala, A.; Bosisio, E. Vascular effects of wine polyphenols. *Cardiovasc. Res.* **2004**, *63* (4), 593−602.
(45) Morazzoni, P.; Livio, S.; Scilingo, A.; Malandrino, S. *Vaccinium myrtillus* anthocyanosides pharmacokinetics in rats. *Arzneim.-Forsch.* **1991**, *41*, 128−131.

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