RESEARCH ARTICLE

The red wine extract-induced activation of endothelial nitric oxide synthase is mediated by a great variety of polyphenolic compounds

Cyril Auger[1], Mehdi Chaabi[2], Eric Anselm[1], Annelise Lobstein[2] and Valérie B. Schini-Kerth[1]

[1] Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France
[2] Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France

Received: December 17, 2009
Revised: January 29, 2010
Accepted: February 8, 2010

Phenolic extracts from red wine (RWPs) have been shown to induce nitric oxide (NO)-mediated vasoprotective effects, mainly by causing the PI3-kinase/Akt-dependent activation of endothelial NO synthase (eNOS). RWPs contain several hundreds of phenolic compounds. The aim of the present study was to identify red wine phenolic compounds capable of activating eNOS in endothelial cells using multi-step fractionation. The red wine phenolic extract was fractionated using Sephadex LH-20 and preparative RP-HPLC approaches. The ability of a fraction to activate eNOS was assessed by determining the phosphorylation level of Akt and eNOS by Western blot analysis, and NO formation by electron spin resonance spectroscopy. Tentative identification of phenolic compounds in fractions was performed by MALDI-TOF and HPLC-MS techniques. Separation of RWPs by Sephadex LH-20 generated nine fractions (fractions A to I), of which fractions F, G, H and I caused significant eNOS activation. Fraction F was then subjected to semi-preparative RP-HPLC to generate ten subfractions (subfraction SF1 to SF10), all of which caused eNOS activation. The active fractions and subfractions contained mainly procyanidins and anthocyanins. Isolation of phenolic compounds from SF9 by semi-preparative RP-HLPC lead to the identification of petunidin-*O*-coumaroyl-glucoside as a potent activator of eNOS.

Keywords:
Endothelial function / Endothelial nitric oxide synthase / Fractionation / Polyphenols / Red Wine

1 Introduction

Several epidemiological studies have shown an inverse relationship between consumption of polyphenol rich food such as fruits, vegetables and mortality due to cardiovascular diseases and cancer [1–3]. In particular, moderate and regular consumption of red wine has been reported to exert beneficial health effects by reducing the risk of cardiovascular diseases [4, 5]. The beneficial effects of these diets have been attributed, in part, to their high content in polyphenolic compounds.

Red wine polyphenols (RWPs) might protect the vascular system by different mechanisms. In particular, they have been shown to prevent oxidation of low-density lipoproteins and to increase the antioxidant capacity of plasma in humans [6, 7]. They can also inhibit platelet adhesion and aggregation, and smooth muscle cell migration and proliferation [8, 9]. Moreover, RWPs have been shown to have anti-atherosclerotic and anti-thrombotic properties [10, 11]. Alternatively, RWPs might protect the vascular system by increasing the endothelial formation of vasoprotective

Correspondence: Dr. Valérie B. Schini-Kerth, UMR CNRS 7213 – Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg–Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch, France
E-mail: valerie.schini-kerth@unistra.fr
Fax: +33-3-68-85-43-13

Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; **eNOS,** endothelial nitric oxide synthase; **ESR,** electron spin resonance; **HBSS,** Hanks balanced salt solution; **NO,** nitric oxide; **RWPs,** red wine polyphenols

factors including nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). Indeed, numerous studies have shown that red wine and grape-derived products induce endothelium-dependent relaxations involving predominantly NO and often also, to some extent, EDHF in isolated blood vessels [12–17]. Moreover, red wine and dealcoholized red wine increase flow-mediated endothelium-dependent vasodilatation in healthy humans [18]. The characterization of the signaling pathway mediating the endothelial formation of NO in response to grape-derived products has indicated a redox-sensitive activation of the Src/PI3-kinase/Akt pathway leading subsequently to the activation of endothelial NO synthase (eNOS) by phosphorylation on Serine 1177 [14, 15]. In addition, it has also been reported that red wine extract and *O*-galloylpunicalagin, a polyphenolic tannin, can induce a transient increase in the calcium signal leading to activation of eNOS possibly *via* the calcium-calmodulin pathway and/or the PI3-kinase/akt pathway in endothelial cells [19–22].

Red wines are complex mixtures of polyphenols containing several hundreds of phenolic compounds including procyanidins and anthocyanins [23]. To identify phenolic compounds, which are able to activate eNOS, the red wine extract was subjected to a multi-step fractionation approach. The red wine extract was separated using Sephadex LH-20 and semi-preparative RP-HPLC. The ability of a fraction to activate eNOS was assessed in cultured coronary artery endothelial cells by determining the level of phosphorylation of Akt and eNOS using Western blot analysis and the formation of NO using electron spin resonance spectroscopy. The polyphenolic compounds in the different fractions were identified using ESI-MS and MALDI-TOF-MS. The present findings indicate that the stimulatory effect of the red wine extract on eNOS is mimicked by a great variety of polyphenolic compounds including mainly procyanidins and anthocyanins. They further identified petunidin-*O*-coumaroyl-glucoside as a potent activator of eNOS.

2 Materials and methods

2.1 Red wine polyphenolic extract

Red wine phenolic extract dry powder was obtained from French red wine (Corbières A.O.C., vintage 2001, composed of a blend of Carignan, Grenache Noir, and Syrah). The extract was provided by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Pr. P.-L. Teissedre (Département d'Oenologie, Bordeaux, France). The extract was prepared and analyzed as previously described [10]. Briefly, phenolic compounds were adsorbed on a preparative column, then alcohol desorbed. The alcoholic eluent was gently evaporated, and the concentrated residue was lyophilized and finely sprayed to obtain the phenolic extract dry powder. One liter of red wine produced 2.9 g of phenolic extract, which contained 471 mg/g of total phenolic compounds expressed as gallic acid equivalent.

2.2 Multi-step fractionation

2.2.1 Fractionation on Sephadex LH-20

The first step of fractionation was done using Sephadex LH-20 (Sigma-Aldrich). Briefly, 50 g of Sephadex powder was swollen for 24 h in water and the suspension was poured into a glass column (25 × 500 mm). Red wine phenolic extract (5 g) was dissolved in a minimum volume of water, filtered to remove solid particles and deposited on the top of the column. The reservoir was filled with water and the flow was adjusted to about 1 mL/min. Elution consisted of a water/methanol stepwise gradient ranging from 0 to 100% of methanol in water with a 10% step, and finally of an aqueous solution of acetone 60%. All individual eluent fractions were collected and concentrated to dryness using a rotary evaporator set at 40°C. Aliquots of each fraction were analyzed by TLC analysis (TLC silica gel 60, Merck) with tert-butanol/acetic acid/water (3:1:1; v/v/v). Spots were detected at 254 and 365 nm and revealed with vanillin-sulfuric acid reagent. After pooling closely related fractions, nine fractions (A to I) were obtained and tested on cultured porcine coronary endothelial cells for phosphorylation of Akt and e NOS (Fig. 1).

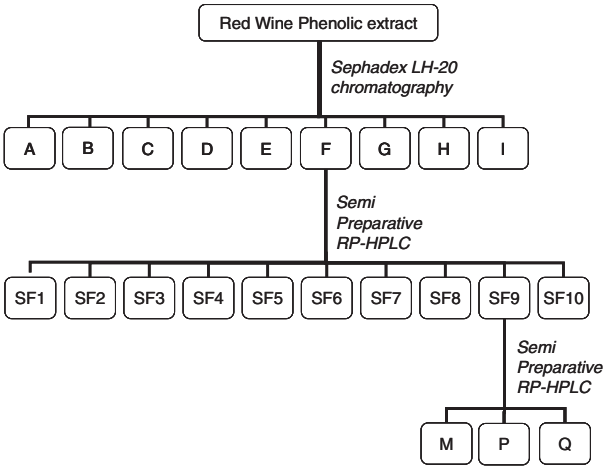


Figure 1. Protocol used for the fractionation of RWPs. The extract was first fractionated into nine fractions (A to I). The active fraction F was then fractionated by semi-preparative RP-HPLC into ten subfractions (SF1-SF10). Thereafter, subfraction 9 was subjected to fractionation by semi-preparative RP-HPLC to isolate three phenolic compounds: malvidin-*O*-coumaroyl-glucoside (M); petunidin-*O*-coumaroyl-glucoside (P); quercetin-glucuronide (Q).

2.2.2 Semi-preparative RP-HPLC

Fraction F was further fractionated using semi-preparative RP-HPLC system comprising a Gilson 305 pump and a Gilson 115 UV detector (Gilson International-France, Roissy, France) fitted with a column C18-Nucleodur®, 250 × 21 mm; 10 μm, (Macherey-Nagel, Hoerdt, France) eluted with 0.01% aqueous formic acid (phase A) and ACN (phase B) at a flow rate of 14 mL/min following the conditions: 90% of A for 5 min, from 90 to 50% A for 40 min and to 100% B for 5 min, followed by washing and reconditioning of the column. Subfractions were monitored at 280 nm and each individual large peak (SF1 to SF10) was collected separately (Fig. 1). All ten subfractions (SF1 to SF10) were reduced to dryness using a rotary evaporator set at 30°C.

Individual compounds in SF9 were isolated by semi-preparative RP-HPLC using the same system as above with elution condition as follow: 90% A for 15 min, from 90 to 50% A for 40 min, 50% A for 15 min, 100% B for 10 min, followed by washing and reconditioning of the column. The three resulting subfractions (M, P and Q) were reduced to dryness using a rotary evaporator set at 30°C.

2.3 Analysis of phenolic compounds

2.3.1 Analytical RP-HPLC

All fractions and subfractions were analyzed using an analytical RP-HPLC system comprising a Varian 9010 pump and a Varian Prostar® DAD detector (Varian, Les Ulis, France) fitted with a column C18-Nucleodur®, 250 × 4.6 mm; 5 μm (Macherey-Nagel, Hoerdt, France).

2.3.2 LC-MS

Identification of phenolic compounds in subfractions was carried out by LC-MS technique on an Agilent HPLC chain fitted with a polaris column and coupled to a HCT ultra mass spectrometer (Bruker daltonics). All analyses were done in both positive and negative ionization using data dependent MS^2.

2.3.3 MALDI-TOF

The phenolic compounds of the active fractions were identified by means of MALDI-TOF analysis. Samples were prepared following the method described by Krueger *et al.* using *trans*-3-indoleacrylic acid as a matrix [24]. Briefly, dry samples of each fraction were dissolved in 80% acetone at 18 mg/mL. The matrix solution was then obtained by adding 100 μL of sample solution to 5 mg of *trans*-3-indoleacrylic acid, and 0.25 μL of this matrix solution was deposited on the target. MALDI-TOF mass spectra were collected on an Ultraflex I mass spectrometer (Bruker Daltonics) using positive mode spectra in the linear and reflectron mode. Spectra were calibrated using bradykinin (1060.6 MW) and glucagon (3483.8 MW). Tentative identification of phenolic compounds was based on the mass spectra and fragmentation compared to existing published compounds [24–27].

2.3.4 Culture of coronary artery endothelial cells

Pig hearts were collected from the local slaughterhouse and left circumflex coronary arteries were excised, cleaned of loose connective tissue and flushed with PBS without calcium to remove remaining blood. Endothelial cells were isolated by collagenase treatment (type I, Worthington, 1 mg/mL for 12 min at 37°C), and cultured in culture dishes containing medium MCDB 131 (Invitrogen) with 15% fetal calf serum supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 μg/mL), and L-glutamine (2 mM) (all from Cambrex), and grown for 48–72 h. All experiments were performed with confluent cultures of cells used at first passage. Cells were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin (QBiogene) for 6 h prior to treatment.

2.3.5 Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)). Total proteins (25 μg) were separated on 12% SDS-polyacrylamide (Sigma) gels at 100 V for 2 h. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% bovine serum albumin, Tris-buffered saline solution (Biorad) and 0.1% Tween-20 (Sigma) (TBS-T) for 1 h. For detection of proteins, membranes were incubated with the respective primary antibody (eNOS, BD Transduction Laboratories; p-Akt Ser473 and p-eNOS Ser1177, Cell Signaling Technology; dilution of 1:1000) overnight at 4°C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, dilution of 1:5000; Cell Signaling Technology) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham). Ponceau staining was performed to verify the quality of the transfer and equal amounts of proteins in each lane.

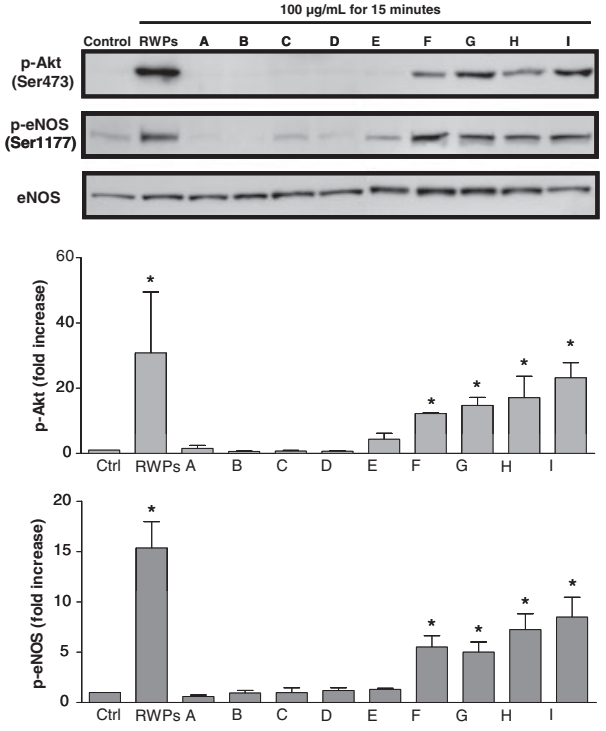


Figure 2. Fractions F to I from RWPs induce phosphorylation of Akt at Ser473 and eNOS at Ser1177 in cultured endothelial cells. Endothelial cells were exposed to RWPs or a test fraction (100 µg/mL) for 15 min at 37°C. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. (A) Representative immunoblots; (B) and (C) corresponding cumulative data. $n = 3$ different experiments. $^{*}p < 0.05$ *versus* control, ♯ $p > 0.05$ *versus* RWPs.

2.4 Determination of NO formation in endothelial cells

Determination of NO formation was assessed by electron spin resonance spectroscopy (ESR) after formation of [Fe(II)NO $(DETC)_2$], a paramagnetic DETC iron complex with NO, in cultured endothelial cells. The ESR methodology was used as reported previously with minor modifications [28, 29]. Confluent cultures of endothelial cells were washed twice with Hank's balanced salt solution (HBSS) buffered with 10 mM HEPES, and then they were incubated in a HBSS–HEPES solution in the presence of bovine serum albumin (20.5 mg/mL), 1.5 mM $CaCl_2$, 0.3 mM L-arginine for 30 min at 37°C in presence or absence of N^{ω}-nitro-L-arginine (300 µM), a specific inhibitor of endothelial NO synthase. Spin trap chemicals $FeSO_4$ (0.8 mM) and DETC (1.6 mM) were rapidly mixed to obtain a colloid form [Fe(II)$(DETC)_2$], which was added to endothelial cells at a final concentration of 0.4 mM. After 5 min, the endothelial formation of NO was induced by addition of either RWPs, fraction F or subfraction P (100 µg/mL) for 30 min, in presence or absence of N^{ω}-nitro-L-arginine (300 µM). Thereafter, dishes were placed on ice, and the incubation medium was removed before addition of 0.2 mL of the HBSS–HEPES buffer. Cells were then scraped, and the cell suspension was collected in a calibrated tube. Tubes were rapidly frozen at 77 K for ESR measurements. ESR measurements were performed on an MS100 spectrometer (Magnettech) under the following conditions: temperature 77 K, microwave frequency 9.34 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 1 mT. The third component of the ESR signal was used for relative comparison of the concentration of NO trapped in each sample.

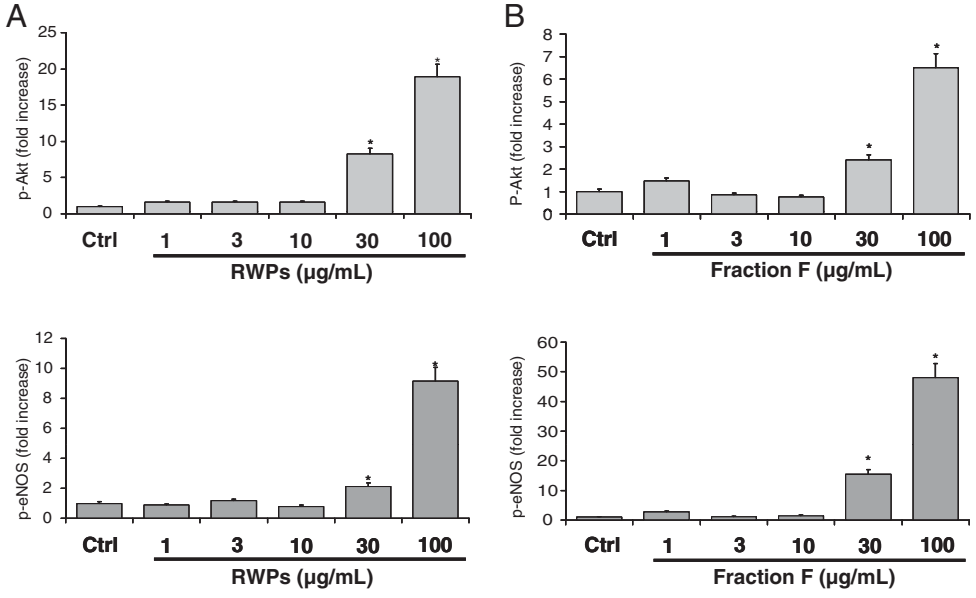


Figure 3. Concentration-response of the stimulating effect of RWPs and fraction F on induction of phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Endothelial cells were exposed to RWPs or a test fraction for 15 min at 37°C with concentrations of 0, 1, 3, 10, 30 or 100 µg/mL. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. Concentration-effect curve for (A) RWPs and (B) fraction F. $n = 3$ different experiments. $^{*}p < 0.05$ *versus* control.

Table 1. Tentative identification of polyphenolic compounds present in the active fractions of RWPs as analyzed by MALDI-TOF

	Ion detected (*m/z*)	Fragments (*m/z*)	Compound ID	Ion type	Ref.
Fraction F	609.6	301	Peonidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	639.5	331	Malvidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	690.5	463	Acetyl-pinotin A	[M+Na]+	[21]
	809	647, 519, 357	Malvidin-3-*O*-glucoside-8-ethyl-(epi)catechin	[M+H]+	[20, 21]
	823.6	639, 493, 331	Malvidin-3-*O*-(coumaroyl)-5-diglucoside	[M+Na]+	[19]
	887.8		Procyanidin trimer	[M+Na]+	[18]
	955.7	665, 357	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-8-ethyl-(epi)catechin	[M+H]+	[20]
	1770.3		Procyanidin pentamer digallate	[M+Na]+	[18]
Fraction G	609.6	301	Peonidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	639.5	331	Malvidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	690.5	463	Acetyl-pinotin A	[M+Na]+	[21]
	755.5	447	Pigment B	[M+H]+	[21]
	785.6	477	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-vinylguaiacol	[M+H]+	[21]
	805.5	643	Malvidin-3-*O*-glucoside-4-vinyl-(epi)catechin	[M+H]+	[20, 21]
	823.6	639, 493, 331	Malvidin-3-*O*-(6″-coumaroyl)-5-diglucoside	[M+Na]+	[19]
	955.7	665, 357	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-8-ethyl-(epi)catechin	[M+H]+	[20]
	1093.3	931	Malvidin-3-*O*-glucoside-4-vinyl-dicatechin	[M+H]+	[20]
	2249		Procyanidin pentamer pentagallate	[M+Na]+	[18]
	2384		Procyanidin hexamer tetragallate	[M+Na]+	[18]
Fraction H	639.5	331	Malvidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	655	331	Malvidin-3-*O*-(6″-caffeoyl)-glucoside and/or Malvidin-3,5-diglucoside	[M+H]+	[19, 20, 21]
	785.7	477	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-vinylguaiacol	[M+H]+	[21]
	823.6	639, 493, 331	Malvidin-3-*O*-(6″-coumaroyl)-5-diglucoside	[M+Na]+	[19]
	805.2	643	Malvidin-3-*O*-glucoside-4-vinyl(epi)catechin	[M+H]+	[20, 21]
	889		Procyanidin trimer	[M+Na]+	[18]
	1093.8	931, 641	Malvidin-3-*O*-glucoside-4-vinyl-di(epi)catechin	[M+H]+	[20]
	1240	931, 641	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-4-vinyl-di(epi)catechin	[M+H]+	[20]
	1465		Procyanidin pentamer	[M+Na]+	[18]
	2041		Procyanidin heptamer	[M+Na]+	[18]
	2202		Procyanidin pentamer pentagallate	[M+H]+	[18]
Fraction I	558.7	355	Acetylvisitin B	[M+H]+	[21]
	599.9		Procyanidin dimer	[M+Na]+	[18]
	639.5	331	Malvidin-3-*O*-(6″-coumaroyl)-glucoside	[M+H]+	[19, 21]
	660.9	331	Malvidin-3-*O*-(6″-coumaroyl)-glucoside	[M+Na]+	[19, 21]
	785.6	477	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-vinylguaiacol	[M+H]+	[21]
	889.6		Procyanidin trimer	[M+Na]+	[18]
	1177.9		Procyanidin tetramer	[M+Na]+	[18]
	1193.9		Trimer digallate	[M+Na]+	[18]
	1273.9	931, 641	Malvidin-3-*O*-(6″-coumaroyl)-glucoside-4-vinyl-di(epi)catechin	[M+Na]+	[20]
	1466.1		Procyanidin pentamer	[M+Na]+	[18]
	1770.3		Procyanidin pentamer digallate	[M+Na]+	[18]

2.5 Chemicals

All solvents and chemicals used were of HPLC grade. Solvents were purchased from Carlo Erba (Val de Reuil, France). Malvidin-3-*O*-glucoside (Oenin) and petunidin chloride were provided by Extrasynthese (France), petunidin-3-*O*-glucoside by Polyphenols (Norway) and resveratrol by Alexis (Coger, Paris, France). All other chemicals were provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France).

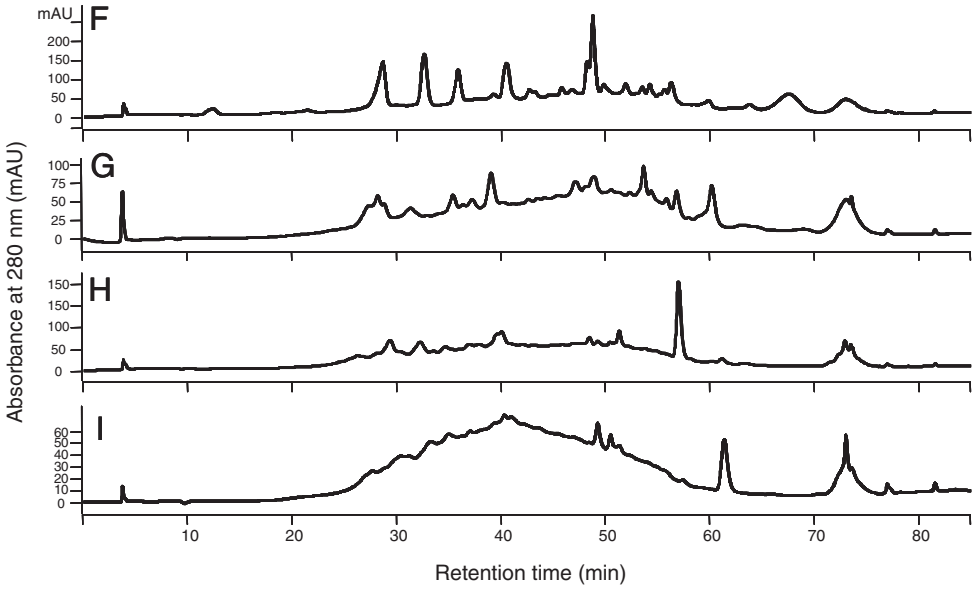


Figure 4. RP-HPLC chromatograms of the active fractions with detection at 280 nm.

2.6 Statistical analysis

Values are expressed as means ± SEM. Statistical evaluation was performed with ANOVA for paired data followed by Fischer's protected least significant difference test. Values of $p < 0.05$ were considered statistically significant.

3 Results

Fractionation of the red wine polyphenolic extract using Sephadex LH-20 provided nine major fractions labeled A to I. The ability of the different fractions to cause eNOS activation in cultured endothelial cells was assessed by the phosphorylation level of Akt at Ser473, which has been shown to mediate eNOS activation in response to RWPs and grape-derived products [15, 30], and eNOS at Ser1177, an activator site [31]. Amongst the nine fractions, only fractions F to I were able to significantly induce to a similar level the phosphorylation of both Akt and eNOS in endothelial cells (Fig. 2). The stimulatory effect of fractions H and I was similar to that induced by the whole extract at 100 μg/mL, whereas fractions F and G were slightly but significantly less effective (Fig. 2). In addition, the stimulatory effect of both the active fractions and the whole extract on Akt and eNOS phosphorylation was observed at concentrations of or greater than 30 μg/mL (Fig. 3). Analysis of the four active fractions by MALDI-TOF indicated that they contain predominantly a mixture of anthocyanins and procyanidin oligomers (Table 1).

RP-HPLC profiles indicated that fraction F contained more individual peaks and a smaller unresolved peak at 280 nm than fractions G, H and I (Fig. 4). Therefore, fraction F was selected and further subjected to fractionation using semi-preparative RP-HPLC to yield ten subfractions (SF1 to SF10; Fig. 1). All subfractions significantly induced phosphorylation of both Akt and eNOS in endothelial cells to a similar extent as that induced by the original fraction F (Fig. 5). Analysis of the different subfractions by HPLC-MS indicated that SF1 to SF6 contained predominantly procyanidin dimers, whereas SF7 to SF10 contained mainly monomeric conjugated anthocyanins (Table 2).

To identify active compounds, the three major polyphenolic compounds present in SF9 were isolated using semi-preparative RP-HPLC and their activity was assessed using cultured endothelial cells. Quercetin-*O*-glucuronide and malvidin-*O*-coumaroyl-glucoside did not induce the phosphorylation of Akt and eNOS (Fig. 6). In contrast, petunidin-*O*-coumaroyl-glucoside markedly increased the phosphorylation level of Akt and eNOS to a level similar to that induced by RWPs at 100 μg/mL (Fig. 6).

To identify the role of the coumaroyl-glucoside moiety in the stimulatory effect of the conjugated petunidin, the effect of petunidin-3-*O*-glucoside and petunidin aglycone was determined. In contrast to petunidin-*O*-coumaroyl-glucoside, neither petunidin-3-*O*-glucoside nor the corresponding aglycone were able to induce the phosphorylation of Akt and eNOS (Fig. 7).

Next, the stimulating effect of RWPs, fraction F and petunidin-*O*-coumaroyl-glucoside on the endothelial formation of NO was determined using ESR. RWPs, fraction F and petunidin-*O*-coumaroyl-glucoside significantly increased the endothelial formation of NO (Fig. 8). In addition, the stimulatory effect of polyphenols was abolished by N^{ω}-nitro-L-arginine, a specific inhibitor of eNOS, indicating the involvement of eNOS.

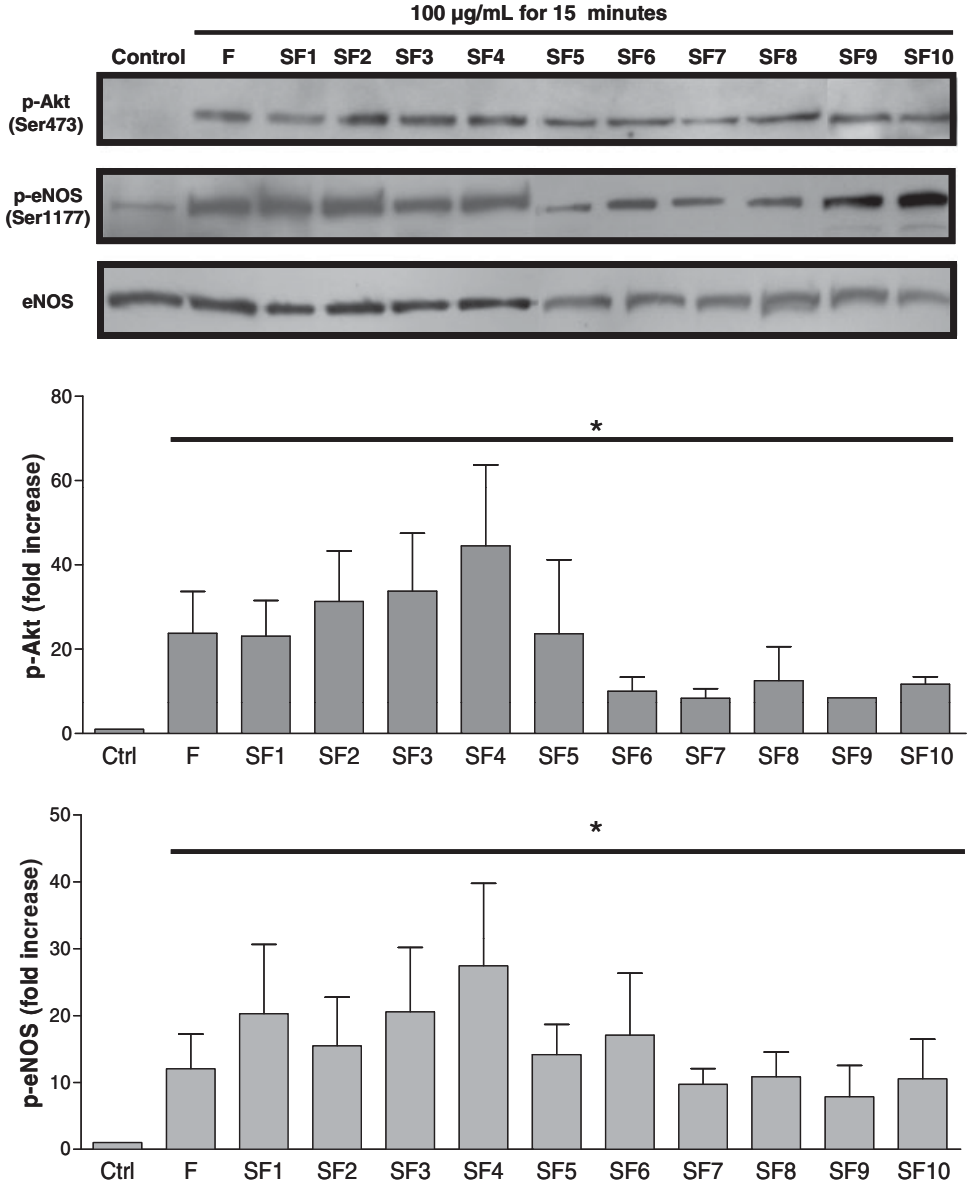


Figure 5. Subfractions isolated from fraction F induce phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Endothelial cells were exposed to fraction F or a test subfraction (100 µg/mL) for 15 min at 37°C. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. Representative immunoblots, and corresponding cumulative data. $n = 5$ different experiments. $^{*}p < 0.05$ *versus* control.

4 Discussion

Several *in vivo* and *in vitro* studies have indicated that RWPs and grape-derived products induce endothelium-dependent vasodilatation, in part, by increasing the endothelial formation of NO and also often, to some extent, EDHF, two potent vasoprotective factors, in both humans and experimental animals [16, 17, 32–34]. Red wines are complex mixture, which contain high levels of polyphenols (up to 4 g/L) including several hundreds of different polyphenols such as phenolic acids, anthocyanins and procyanidins [23]. Despite the fact that numerous studies indicate that red wine extract has a beneficial effect on the endothelial function, the active red wine phenolic compounds still remain poorly characterized. Therefore, the aim of the present study was to search for red wine polyphenolic compounds capable of activating eNOS using a three-step fractionation approach. The present findings indicate that several fractions (F to I) derived from the red wine extract and several subfractions derived from active fraction F (SF1 to SF10) are able to significantly induce eNOS activation. The detailed analysis of the active SF indicated that they contain predominantly procyanidins and conjugated anthocyanins. Moreover, the fractionation of SF9 permitted the isolation of two conjugated anthocyanins and the identification in particular of a petunidin-*O*-coumaroyl-glucoside as an eNOS activator as potent as the red wine extract. Thus, the present findings indicate that a great variety of phenolic compounds including anthocyanins and procyanidins contribute to the stimulatory effect of RWPs on the endothelial formation of NO.

Table 2. Identification by HPLC-MS^2 of the main polyphenolic compounds present in active sub-fractions.

	[M+H]+ (*m/z*)	Fragments (*m/z*)	Identification
SF1	611	443, 317	Petunidin-vinylphenol
	867	579, 427, 301	Procyanidin trimer
	595	425, 443, 287, 577	Procyanidin dimer Gallate
SF2	595	425, 443, 287, 577	Procyanidin dimer Gallate
	579	427, 409, 301	Procyanidin dimer
SF3	579	427, 409, 301	Procyanidin dimer
SF4	579	427, 409, 301	Procyanidin dimer
	579	427, 409, 301, 291	Procyanidin dimer
SF5	579	427, 409, 301, 291	Procyanidin dimer
	579	427, 409, 301, 291	Procyanidin dimer
SF6	579	427, 409, 301, 291	Procyanidin dimer
	579	427, 409, 301, 291	Procyanidin dimer
	579	291, 165, 123	Procyanidin dimer
SF7	493	331	Malvidin-*O*-glucoside
	535	331	Malvidin-*O*-acetyl-glucoside
	505	301	Peonidin-*O*-acetyl-glucoside
	809	519, 357	Malvidin-*O*-glucoside-ethyl-(epi)catechin
	639	331	Malvidin-*O*-coumaroyl-glucoside
SF8	493	331	Malvidin-*O*-glucoside
	625	317	Petunidin-coumaroyl-glucoside
	639	331	Malvidin-*O*-coumaroyl-glucoside
SF9	479	303	Quercetin-*O*-glucuronide
	493	331	Malvidin-*O*-glucoside
	625	317	Petunidin-*O*-coumaroyl-glucoside
	639	331	Malvidin-*O*-coumaroyl-glucoside
SF10	625	317	Petunidin-*O*-coumaroyl-glucoside
	609	301	Peonidin-*O*-coumaroyl-glucoside
	639	331	Malvidin-*O*-coumaroyl-glucoside

RWPs have been shown to cause endothelium-dependent NO-mediated relaxations in several types of large arteries and arterioles, and to stimulate the formation of NO in cultured endothelial cells [15, 34, 35]. The stimulatory effect of RWPs on the endothelial formation of NO is mediated by the redox-sensitive activation of the PI3-kinase/Akt pathway leading to the phosphorylation of eNOS on Ser1177 [14, 36, 37]. Indeed, both the RWPs-induced endothelium-dependent relaxations and phosphorylation of eNOS are markedly reduced by membrane permeant analogues of superoxide dismutase and catalase, whereas native SOD and catalase were without effect [14, 36]. Both responses are also significantly reduced by inhibitors of PI3-3kinase and are associated with the phosphorylation of Akt in endothelial cells [14, 36]. In addition, RWPs induced a time-dependent formation of reactive oxygen species predominantly superoxide anions in endothelial cells [36]. The intracellular formation of reactive oxygen species acts as an upstream stimulator of the PI-3 kinase/Akt pathway since the RWPs-induced phosphorylation of Akt is prevented by intracellular antioxidants [14, 36].

To identify polyphenolic compounds capable of activating eNOS, the red wine extract was subjected to fractionation using Sephadex LH-20 gel coupled to an aqueous methanol gradient, allowing a separation based on the relative polarity of the compounds. This first step of fractionation generated nine major fractions, out of which only the last four fractions (F to I) significantly induced eNOS activation as indicated by marked increases in the phosphorylation level of eNOS on Ser1177. In the present experimental conditions, the chromatography of RWPs concentrates small and polar molecules like phenolic acids in the early fractions, whereas the later ones contain predominantly complex forms of phenolic compounds like procyanidins. Indeed, HPLC and MALDI-TOF analysis of the different fractions indicated that the four active fractions are enriched in procyanidin compounds and conjugated anthocyanins. In addition, HPLC analysis also revealed that resveratrol was present in fractions G and H but not in fractions F and I (data not showed). Since fractions F and I caused similar activation of eNOS as fractions G and H, the present findings indicate that polyphenolic compounds besides resveratrol, a well-known activator of eNOS [38, 39], contribute to the stimulatory effect of red wine on eNOS [39].

Thereafter, the fraction F was selected amongst the active ones due to the presence of relatively low levels of unresolved compounds in RP-HPLC, which are technically difficult to isolate. Fraction F subjected to semi-preparative RP-HPLC generated ten major subfractions, all of which caused eNOS activation to a similar level as the original fraction F. Analysis of the different subfractions indicated that SF1 to SF6 contained predominantly procyanidin

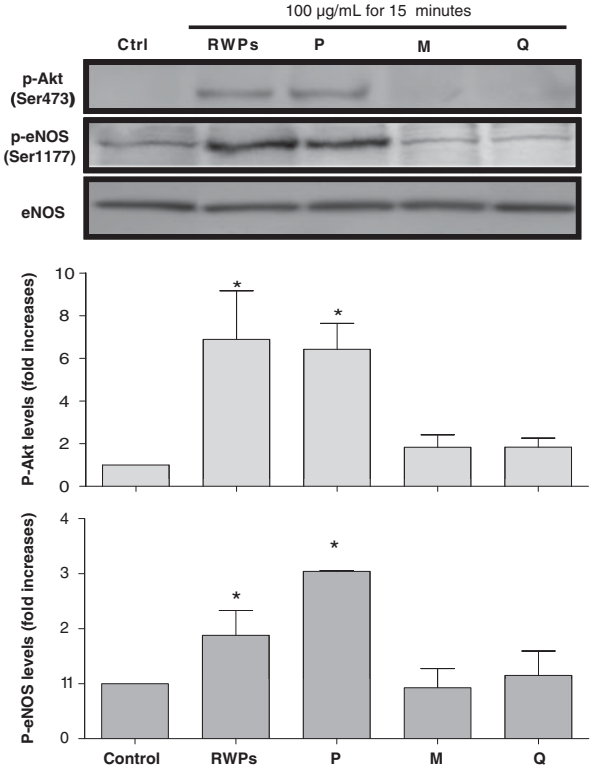


Figure 6. Potential of phenolic compounds isolated from subfraction 9 of RWPs to induce phosphorylation of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Endothelial cells were exposed to RWPs or a phenolic compound (100 µg/mL) for 15 min at 37°C. Thereafter, the level of p-Akt and p-eNOS was determined by Western blot analysis. Representative immunoblots, and corresponding cumulative data. $n = 3$–4 different experiments. $^*p < 0.05$ *versus* control. RWPs: red wine polyphenols; P: petunidin-*O*-coumaroyl-glucoside; M: malvidin-*O*-coumaroyl-glucoside; Q: quercetin-glucuronide.

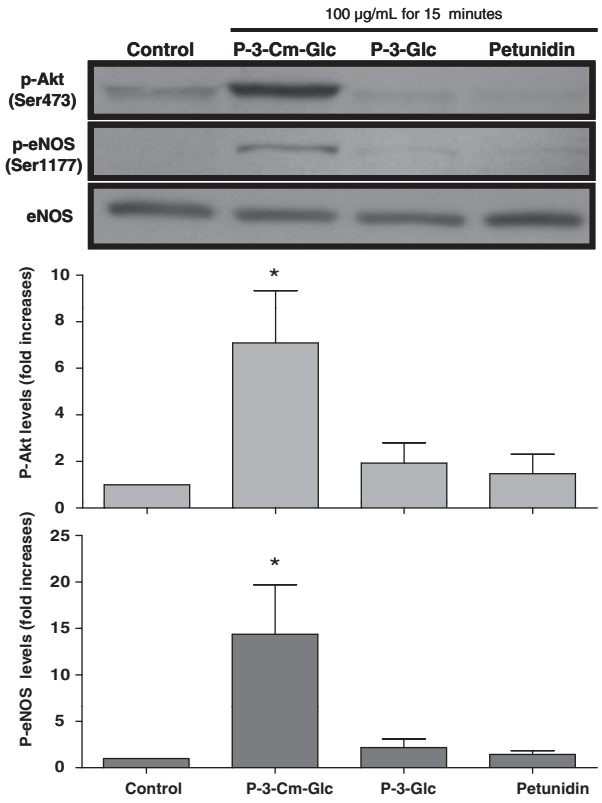


Figure 7. Effect of petunidin and petunidin conjugates on the phosphorylation level of Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Endothelial cells were exposed for 15 min at 37°C and the level of p-Akt and p-eNOS was determined by Western blot analysis. Representative immunoblots, and corresponding cumulative data. $n = 3$–4 different experiments. $^*p < 0.05$ *versus* control. P-Cm-Glc: petunidin-*O*-coumaroyl-glucoside; P-3-Glc: petunidin-3-*O*-glucoside; Petunidin: petunidin aglycone.

dimers, whereas SF7 to SF10 conjugated anthocyanins. In addition, the fractionation of the red wine extract using a different method has also previously shown that the vasorelaxant effect of RWPs is mainly associated with fractions enriched in oligomeric procyanidins or monomeric anthocyanidins [13].

The present findings indicate that procyanidin dimers are potent activators of eNOS. This observation is consistent with previous ones showing that procyanidin oligomers isolated from grape seeds caused endothelium-dependent relaxations in rat aortic rings [40]. They are also consistent with the fact that ingestion of a procyanidin-rich purple grape juice improved the endothelial function in patients with coronary artery disease and reduced blood pressure in hypertensive patients [41–43]. In addition, the inhibitory effect of red wine on the endothelial formation of endothelin 1, a potent endothelium-derived vasocontracting factor, is correlated to their procyanidins content in cultured endothelial cells [44, 45]. Moreover procyanidin oligomers have been identified in blood and urine in intact form following ingestion of a procyanidin-rich grape seed extract by humans and rats, indicating that these polyphenolic compounds are absorbed in the small intestine and, thus, are likely to reach the endothelium where they could exert their beneficial effect [46, 47].

Although procyanidins mediate eNOS activation in response to SF1 to SF6, eNOS activation induced by SF7 to SF10 is mediated predominantly by conjugated anthocyanins. Indeed, HPLC-MS analysis of SF7 to SF10 revealed the presence of 3–5 major conjugated anthocyanins. The isolation of the three major conjugated anthocyanins from SF9 and the evaluation of their biological activity indicated that petunidin-*O*-coumaroyl-glucoside caused strong activation of eNOS whereas malvidin-*O*-coumaroyl-glucoside and quercetin-*O*-glucuronide were without effect. Interestingly, malvidin-*O*-coumaroyl-glucoside differs structurally from petunidin-*O*-coumaroyl-glucoside only by the presence of a methoxyl rather than a hydroxyl function in position 5′ on the B ring (Fig. 9). Thus, the free hydroxyl

group on position 5′ of the B ring seems critically necessary for the activation of eNOS. Previous studies have also shown that methylation of hydroxyl substituents in a grape seed extract abolished its vasorelaxant effect in aortic rings from rabbit [48].

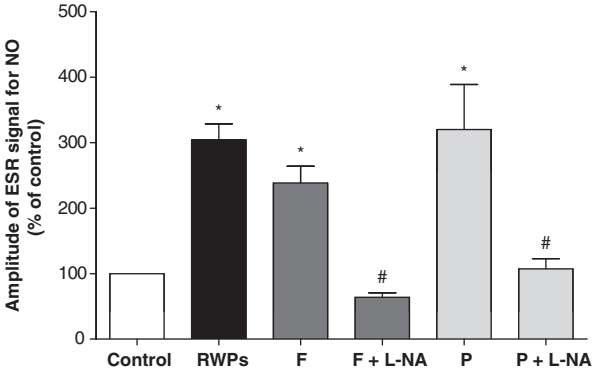
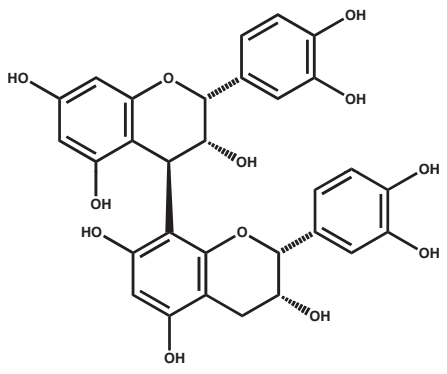


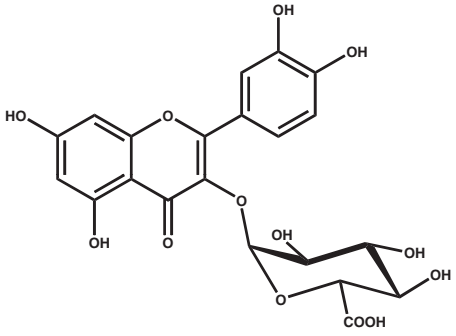
Figure 8. Red wine polyphenols stimulate the formation of NO in cultured endothelial cells as assessed by electron spin resonance spectroscopy. Cells were exposed either red wine polyphenols (RWPs), fraction F or petunidin-*O*-coumaroyl-glucoside (100 μg/mL) for 30 min, in presence or absence of N^{ω}-nitro-L-arginine (L-NA; 300 μM), an inhibitor of eNOS. $n = 3$ different experiments. $^{*}p < 0.05$ *versus* control. $^{\sharp}p < 0.05$ *versus* corresponding inducer.

The anthocyanins are mainly found conjugated to sugar and/or to organic acid. Moreover, the majority of identified anthocyanins are conjugated through an *O*-conjugation on position 3 of the C ring [49]. In this study, we have isolated two conjugated anthocyanins from subfraction SF9 and MS analysis indicated the presence of malvidin and petunidin both conjugated to coumaroyl and hexoside moeties. The MS/MS data showed the presence of two fragment peaks for each compound respectively for the aglycon and the glucoside anthocyanin (data not shown), indicating that the coumaroyl moiety is linked rather to the glucoside moiety than directly to the aglycone as reported previously by Wu and Prior [25]. Therefore, the two compounds have been tentatively identified as malvidin-3-*O*-(6″coumaroyl)-glucoside and petunidin-3-*O*-(6″coumaroyl)-glucoside; these two anthocyanins have previously been observed in red wine and grape [25, 27, 50].

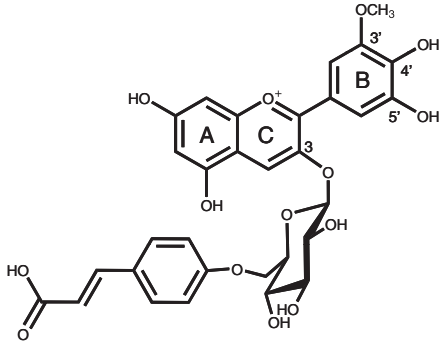
In addition, the present findings suggest that the chemical moiety in position 3 of the C ring has a determinant role for the activation of eNOS. Indeed, the isolated compound tentatively identified as petunidin-3-*O*-(6″-coumaroyl)-glucoside strongly activated eNOS whereas no such effect was observed with petunidin-3-*O*-glucoside and corresponding aglycone. Although the precise role of the coumaroyl remains unclear, it might probably act by stabilizing the petunidin structure by intramolecular copigmentation [51–53].



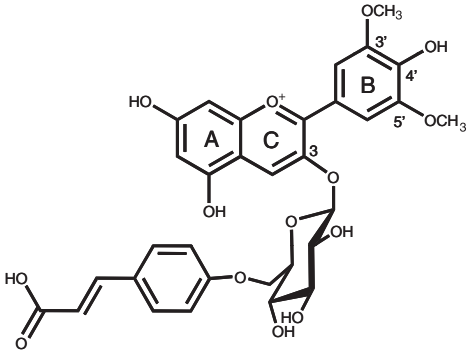
Procyanidin dimer B2



Quercetin-3-*O*-glucuronide



Petunidin-3-*O*-(6“-coumaroyl)-glucoside



Malvidin-3-*O*-(6“-coumaroyl)-glucoside

Figure 9. Structures of several phenolic compounds identified in active subfractions of RWPs.

Although the present study has identified active polyphenolic compounds from a red wine extract, the bioavailability of these compounds remains unclear. Indeed, recent studies have indicated that while procyanidins oligomers are detected in blood and urine in intact form [46], the absorption of these compounds seems to be limited. Moreover, human plasma levels of procyanidins reach the nanomolar range after ingestion of cocoa [54] or grape seed [47]. Similarly, anthocyanins seem also to have a low absorption and bioavailability [55]. Moreover and in contrary to procyanidins, which are mainly absorbed and excreted in intact form, anthocyanins can undergo metabolization by intestinal and hepatic cells to generate glucuronides, methyl and sulfo-conjugates [56, 57]. In addition to the absorbed procyanidins and anthocyanins, a growing number of studies indicate that unabsorbed compounds reach the large intestine where they can be catabolized by the microflora to small ring fission products, which could be directly absorbed [58]. *In vitro* studies using human fecal microflora in fermentation reactor indicated that various small phenolic acids, such as hydroxycarboxylic acids, hydroxyphenylacetic acids or phenyl-alkyl acids are derived from monomeric flavan-3-ols [59, 60], procyanidin dimers [61] and anthocyanins [62–64]. These small phenolic compounds could, in turn, be absorbed in the large intestine and reach the bloodstream. Moreover, Tsuda *et al.* has shown that after oral absorption of cyanidin-3-glucoside by rats, protocatechuic acid is found in plasma at a concentration 8 times higher than that of the parent compound [62]. Similarly, Stoupi *et al.* have reported that after oral absorption of radiolabeled procyanidin dimer B2, 63% of the radioactivity was excreted by urine [65]. These studies suggest that despite the apparent low absorption of the flavonoids, the smaller phenolic acid catabolites seem to be efficiently absorbed in the large intestine, increasing strongly the bioavailability of flavonoid compounds. However, little is known regarding the biological activity of the circulating phenolic acids resulting from the fermentation and, in particular, whether they can affect the endothelial function.

In conclusion, the present findings indicate that the stimulating effect of red wine on the endothelial formation of NO is mediated by a great variety of polyphenolic compounds including procyanidin oligomers and conjugated anthocyanins. Amongst conjugated anthocyanins, petunidin-*O*-coumaroyl-glucoside was identified as a highly active red wine polyphenol. In addition, the present findings also indicate a key role for the hydroxylation status of the B ring and the presence of acid-conjugated glucoside moiety on the C ring of the anthocyanin structure.

This study was supported in part by the Office National Interprofessionnel des Fruits, des Légumes, des Vins et de l'Horticulture (Action Vin et Santé, France).

The authors have declared no conflict of interest.

5 References

[1] Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B. *et al.*, Dietary flavonoids and cancer risk in the Zutphen Elderly Study. *Nutr.Cancer* 1994, *22*, 175–184.

[2] Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B. *et al.*, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993, *342*, 1007–1011.

[3] Sofi, F., Cesari, F., Abbate, R., Gensini, G. F. *et al.*, Adherence to Mediterranean diet and health status: meta-analysis. *BMJ* 2008, *337*, a1344

[4] Renaud, S., de Lorgeril, M., Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992, *339*, 1523–1526.

[5] St Leger, A. S., Cochrane, A. L., Moore, F., Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet* 1979, *1*, 1017–1020.

[6] Serafini, M., Laranjinha, J. A., Almeida, L. M., Maiani, G., Inhibition of human LDL lipid peroxidation by phenol-rich beverages and their impact on plasma total antioxidant capacity in humans. *J. Nutr. Biochem.* 2000, *11*, 585–590.

[7] Serafini, M., Maiani, G., Ferro-Luzzi, A., Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.* 1998, *128*, 1003–1007.

[8] Middleton, E., Jr., Kandaswami, C., Theoharides, T. C., The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 2000, *52*, 673–751.

[9] Iijima, K., Yoshizumi, M., Hashimoto, M., Kim, S. *et al.*, Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene. *Circulation* 2000, *101*, 805–811.

[10] Auger, C., Caporiccio, B., Landrault, N., Teissedre, P. L. *et al.*, Red wine phenolic compounds reduce plasma lipids and apolipoprotein B and prevent early aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters (*Mesocricetus auratus*). *J. Nutr.* 2002, *132*, 1207–1213.

[11] Wollny, T., Aiello, L., Di, T. D., Bellavia, V. *et al.*, 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*, 747–755.

[12] Auger, C., Gerain, P., Laurent-Bichon, F., Portet, K. *et al.*, Phenolics from commercialized grape extracts prevent early atherosclerotic lesions in hamsters by mechanisms other than antioxidant effect. *J. Agric. Food Chem.* 2004, *52*, 5297–5302.

[13] Andriambeloson, E., Magnier, C., Haan-Archipoff, G., Lobstein, A. *et al.*, Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.* 1998, *128*, 2324–2333.

[14] Anselm, E., Chataigneau, M., Ndiaye, M., Chataigneau, T. *et al.*, Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. *Cardiovasc. Res.* 2007, *73*, 404–413.

[15] Ndiaye, M., Chataigneau, M., Lobysheva, I., Chataigneau, T. *et al.*, Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. *FASEB J.* 2005, *19*, 455–457.

[16] Ndiaye, M., Chataigneau, T., Andriantsitohaina, R., Stoclet, J. C. *et al.*, Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. *Biochem. Biophys. Res. Commun.* 2003, *310*, 371–377.

[17] Fitzpatrick, D. F., Hirschfield, S. L., Coffey, R. G., Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol. Heart Circ. Physiol.* 1993, *265*, H774–H778.

[18] Hashimoto, M., Kim, S., Eto, M., Iijima, K. *et al.*, Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery. *Am. J. Cardiol.* 2001, *88*, 1457–1460.

[19] 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*, 248–254.

[20] Chen, L. G., Liu, Y. C., Hsieh, C. W., Liao, B. C. *et al.*, Tannin 1-alpha-O-galloylpunicalagin induces the calcium-dependent activation of endothelial nitric-oxide synthase via the phosphatidylinositol 3-kinase/Akt pathway in endothelial cells. *Mol. Nutr. Food Res.* 2008, *52*, 1162–1171.

[21] Martin, S., Andriambeloson, E., Takeda, K., Andriantsitohaina, R., Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production. *Br. J. Pharmacol.* 2002, *135*, 1579–1587.

[22] Duarte, J., Andriambeloson, E., Diebolt, M., Andriantsitohaina, R., Wine polyphenols stimulate superoxide anion production to promote calcium signaling and endothelial-dependent vasodilatation. *Physiol. Res.* 2004, *53*, 595–602.

[23] German, J. B., Walzem, R. L., The health benefits of wine. *Annu. Rev. Nutr.* 2000, *20*, 561–593.

[24] Krueger, C. G., Dopke, N. C., Treichel, P. M., Folts, J. *et al.*, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.* 2000, *48*, 1663–1667.

[25] Wu, X., Prior, R. L., Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.* 2005, *53*, 2589–2599.

[26] Pati, S., Losito, I., Gambacorta, G., La, N. E. *et al.*, Simultaneous separation and identification of oligomeric procyanidins and anthocyanin-derived pigments in raw red wine by HPLC-UV-ESI-MSn. *J. Mass Spectrom.* 2006, *41*, 861–871.

[27] de Villiers, A., Vanhoenacker, G., Majek, P., Sandra, P., Determination of anthocyanins in wine by direct injection liquid chromatography-diode array detection-mass spectrometry and classification of wines using discriminant analysis. *J. Chromatogr. A* 2004, *1054*, 195–204.

[28] Ullrich, V., Bachschmid, M., Superoxide as a messenger of endothelial function. *Biochem. Biophys. Res. Commun.* 2000, *278*, 1–8.

[29] Kleschyov, A. L., Munzel, T., Advanced spin trapping of vascular nitric oxide using colloid iron diethyldithiocarbamate. *Methods Enzymol.* 2002, *359*, 42–51.

[30] Edirisinghe, I., Burton-Freeman, B., Kappagoda, C. T., Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract. *Clin. Sci.* 2008, *114*, 331–337.

[31] Mount, P. F., Kemp, B. E., Power, D. A., Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J. Mol. Cell Cardiol.* 2007, *42*, 271–279.

[32] Flesch, M., Schwarz, A., Bohm, M., Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. *Am. J. Physiol.* 1998, *275*, H1183–H1190.

[33] Andriambeloson, E., Kleschyov, A. L., Muller, B., Beretz, A. *et al.*, Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br. J. Pharmacol.* 1997, *120*, 1053–1058.

[34] Stoclet, J. C., Kleschyov, A., Andriambeloson, E., Diebolt, M. *et al.*, Endothelial no release caused by red wine polyphenols. *J. Physiol. Pharmacol.* 1999, *50*, 535–540.

[35] Leikert, J. F., Rathel, T. R., Wohlfart, P., Cheynier, V. *et al.*, Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. *Circulation* 2002, *106*, 1614–1617.

[36] Ndiaye, M., Chataigneau, T., Chataigneau, M., Schini-Kerth, V. B., Red wine polyphenols induce EDHF-mediated relaxations in porcine coronary arteries through the redox-sensitive activation of the PI3-kinase/Akt pathway. *Br. J. Pharmacol.* 2004, *142*, 1131–1136.

[37] Madeira, S. V., Auger, C., Anselm, E., Chataigneau, M. *et al.*, eNOS activation induced by a polyphenol-rich grape skin extract in porcine coronary arteries. *J. Vasc. Res.* 2009, *46*, 406–416.

[38] Wallerath, T., Poleo, D., Li, H., Forstermann, U., Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects. *J. Am. Coll. Cardiol.* 2003, *41*, 471–478.

[39] Wallerath, T., Li, H., Godtel-Ambrust, U., Schwarz, P. M. *et al.*, A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. *Nitric Oxide* 2005, *12*, 97–104.

[40] Fitzpatrick, D. F., Fleming, R. C., Bing, B., Maggi, D. A. *et al.*, Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. *J. Agric. Food Chem.* 2000, *48*, 6384–6390.

[41] Park, Y. K., Kim, J. S., Kang, M. H., Concord grape juice supplementation reduces blood pressure in Korean hypertensive men: double-blind, placebo controlled intervention trial. *Biofactors* 2004, *22*, 145–147.

[42] Chou, E. J., Keevil, J. G., Aeschlimann, S., Wiebe, D. A. *et al.*, Effect of ingestion of purple grape juice on

endothelial function in patients with coronary heart disease. *Am. J. Cardiol.* 2001, *88*, 553–555.

[43] Stein, J. H., Keevil, J. G., Wiebe, D. A., Aeschlimann, S. *et al.*, Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation* 1999, *100*, 1050–1055.

[44] Corder, R., Mullen, W., Khan, N. Q., Marks, S. C. *et al.*, Oenology: red wine procyanidins and vascular health. *Nature* 2006, *444*, 566

[45] Corder, R., Douthwaite, J. A., Lees, D. M., Khan, N. Q. *et al.*, Endothelin-1 synthesis reduced by red wine. *Nature* 2001, *414*, 863–864.

[46] Tsang, C., Auger, C., Mullen, W., Bornet, A. *et al.*, The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br. J. Nutr.* 2005, *94*, 170–181.

[47] Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K. *et al.*, Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci. Biotechnol. Biochem.* 2003, *67*, 1140–1143.

[48] Edirisinghe, I., Burton-Freeman, B., Kappagoda, C. T., Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract. *Clin. Sci.* 2008, *114*, 331–337.

[49] Clifford, M. N., Anthocyanins – nature, occurrence and dietary burden. *J. Sci. Food Agric.* 2000, *80*, 1063–1072.

[50] Wang, H., Race, E. J., Shrikhande, A. J., Characterization of anthocyanins in grape juices by ion trap liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* 2003, *51*, 1839–1844.

[51] Giusti, M. M., Rodriguez-Saona, L. E., Wrolstad, R. E., Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* 1999, *47*, 4631–4637.

[52] Figueiredo, P., Elhabiri, M., Saito, N., Brouillard, R., Anthocyanin intramolecular interactions. A new mathematical approach to account for the remarkable colorant properties of the pigments extracted from *Matthiola incana*. *J. Am. Chem. Soc.* 1996, *118*, 4788–4793.

[53] Brouillard, R., Origin of the exceptional colour stability of the Zebrina anthocyanin. *Phytochemistry* 1981, *20*, 143–145.

[54] Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y. *et al.*, Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am. J. Clin. Nutr.* 2002, *76*, 798–804.

[55] Manach, C., Williamson, G., Morand, C., Scalbert, A. *et al.*, Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 2005, *81*, 230S–242S.

[56] Aprikian, O., Duclos, V., Guyot, S., Besson, C. *et al.*, Apple pectin and a polyphenol-rich apple concentrate are more effective together than separately on cecal fermentations and plasma lipids in rats. *J. Nutr.* 2003, *133*, 1860–1865.

[57] Mullen, W., Edwards, C. A., Serafini, M., Crozier, A., Bioavailability of pelargonidin-3-O-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream. *J. Agric. Food Chem.* 2008, *56*, 713–719.

[58] Del Rio, D., Costa, L. G., Lean, M. E., Crozier, A., Polyphenols and health: What compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* 2010, *20*, 1–6.

[59] Deprez, S., Brezillon, C., Rabot, S., Philippe, C. *et al.*, Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* 2000, *130*, 2733–2738.

[60] van't Slot, G., Humpf, H. U., Degradation and metabolism of catechin, epigallocatechin-3-gallate (EGCG), and related compounds by the intestinal microbiota in the pig cecum model. *J. Agric. Food Chem.* 2009, *57*, 8041–8048.

[61] Appeldoorn, M. M., Venema, D. P., Peters, T. H., Koenen, M. E. *et al.*, Some phenolic compounds increase the nitric oxide level in endothelial cells *in vitro*. *J. Agric. Food Chem.* 2009, *57*, 7693–7699.

[62] Tsuda, T., Horio, F., Osawa, T., Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. *FEBS Lett.* 1999, *449*, 179–182.

[63] Fleschhut, J., Kratzer, F., Rechkemmer, G., Kulling, S. E., Stability and biotransformation of various dietary anthocyanins *in vitro*. *Eur. J. Nutr.* 2006, *45*, 7–18.

[64] Aura, A. M., Martin-Lopez, P., O'Leary, K. A., Williamson, G. *et al.*, *In vitro* metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* 2005, *44*, 133–142.

[65] Stoupi, S., Williamson, G., Viton, F., Barron, D. *et al.*, *In vivo* bioavailability, absorption, excretion, and pharmacokinetics of [14C]procyanidin B2 in male rats. *Drug Metab. Dispos.* 2010, *38*, 287–291.

