Wine Polyphenols Stimulate Superoxide Anion Production to Promote Calcium Signaling and Endothelial-Dependent Vasodilatation

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Summary
The present study was aimed to evaluate the mechanisms involved in the vasorelaxant effects of red wine polyphenol compounds (RWPC) in small mesenteric rat arteries. RWPC produce relaxation in small mesenteric arteries. This relaxant effect was abolished by endothelial denudation, NO-synthase blockade with L-NAME and partial depolarization with KCl or L-NAME plus KCl. Incubation with the reactive oxygen species scavenger, superoxide dismutase (SOD) plus catalase, or inhibition of NAD(P)H-dependent oxidoreductases with diphenyleneiodonium also inhibited RWPC induced vascular relaxation. Application of RWPC elicited a transient increase in intracellular calcium concentration ([Ca²⁺]ᵢ) in bovine aortic endothelial cells (BAEC), which was attenuated by a mixture of SOD and catalase. Incubation of BAEC with RWPC increased the SOD inhibitable production of O₂⁻. These results suggest the involvement of O₂⁻ in the [Ca²⁺]ᵢ increase evoked by RWPC, leading to the activation of enzymes involved in the release of endothelial relaxant factors and subsequent vasodilatation of resistance arteries.

Key words
Red wine polyphenol compounds • Nitric oxide • Calcium • Superoxide anions • Endothelium • Mesenteric artery

Introduction
Epidemiological studies have suggested that the consumption of some dietary factors might reduce the risk of cardiovascular disease. Furthermore, it has been suggested that the beneficial effect of fruits, vegetables and red wine might be attributed to the presence of polyphenol compounds. Indeed, polyphenols possess a multitude of biological activities including an ability to decrease low-density lipoprotein oxidation and to inhibit platelet aggregation (Serafini et al. 1998, Aviram and Fuhrman 2002), to decrease blood pressure and to improve endothelial function (Duarte et al. 2001). These properties could in part explain the beneficial effect of polyphenolic compounds in certain types of cardiovascular diseases. In previous studies, we have reported that red wine polyphenol compounds (RWPC) from two different sources, with a similar pharmacological profile in different blood vessels (RWPC and Provinols™), are able to produce endothelium-dependent relaxation of
the rat thoracic aorta (Andriambeloson et al. 1997, 1999). This effect was mediated by an increase in NO content due to enhancement of NO synthesis rather than protection against its breakdown by oxygen radicals associated with the antioxidant properties of RWPC (Andriambeloson et al. 1997). Furthermore, studies in bovine aortic endothelial cells (BAEC) have shown that an increase in NO-synthase activity is associated with an increase in intracellular calcium concentration ([Ca$$^{2+}$$]) and the activation of tyrosine kinases (Martin et al. 2002). Recently, we have shown that oral treatment with Provinols™ accelerated the regression of blood pressure and prevents the development of cardiovascular remodeling, myocardial fibrosis and aortic stiffness in NO-deficient hypertensive rats. Furthermore, it has been shown that oral administration of Provinols™ produced a decrease of systolic blood pressure in normotensive rats, which was accompanied with an enhanced endothelium-dependent relaxation and induction of gene expression within the arterial wall (Diebolt et al. 2001). This effect probably involved a NO pathway, inasmuch N$$^{0}$$-nitro-L-arginine-methyl-ester (L-NAME) treatment plus RWPC abolished the decrease in blood pressure and the improvement of endothelial function (Bernátová et al. 2002).

However, all these studies were performed in the rat aorta and the mechanism in resistance vessels such as small mesenteric arteries remains to be determined. The aim of the present study was to examine the vasorelaxant effects of RWPC in small mesenteric rat arteries. The influence of reactive oxygen species (ROS) pathway in the endothelial cells on NO production in response to RWPC was also analyzed.

Methods

Tissue preparation

Rats were killed by cervical dislocation and then exsanguinated by carotid artery transection. The small bowel was pinned in a dissecting dish containing physiological salt solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, CaCl$$\text{2}$$ 2.5, MgSO$$\text{4}$$ 1.17, KH$$\text{2}$$PO$$\text{4}$$ 0.4, NaHCO$$\text{3}$$ 14.9 and glucose, 5.5. Branch II or III resistance arteries were cleaned of fat and connective tissue, and a 2 mm long segment was removed. The segment was mounted in a myograph (Mulvany and Halpern 1977) filled with PSS continuously kept at 37 °C and gassed with 95 % O$$\text{2}$$ and 5 % CO$$\text{2}$$ mixture (pH 7.4). Briefly, two tungsten wires (30 µm in diameter) were inserted through the lumen of the vessel. Mechanical activity was recorded isometrically by a force transducer (model DSG BE4, Kistler-Morse) connected to one of the two tungsten wires; the other wire was attached to a support carried by a micromanipulator. A mesenteric artery segment was equilibrated for 30 min before it was passively stretched to an internal diameter that yields a circumference equivalent to 90 % of that given by an internal pressure of 100 mm Hg that requires a load of ~200 mg. The internal diameter of this vessel was 150-200 µm.

After an equilibration period, the small mesenteric arteries were maximally contracted with norepinephrine (10 µM) in order to test their contractile capacity. In some experiments, the endothelium was removed by intraluminal perfusion of a small mesenteric artery with 0.5 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) for 30 s followed by repeated washing with normal PSS. The presence of functional endothelium was assessed in all preparations by the ability of acetylcholine (1 µM) to induce more than 50 % relaxation of vessels maximally precontracted with norepinephrine. Vessels were considered to be denuded of functional endothelium when there was no relaxation response to acetylcholine.

Experimental protocol

Pharmacological mechanism of RWPC actions was investigated in rat mesenteric arteries prepared for in vitro studies. RWPC-induced relaxation was investigated in arteries with or without functional endothelium. Arteries were precontracted to the same tension with norepinephrine (i.e. at 80 % of maximal response obtained in vessels with or without functional endothelium by 3 or 1 µM, respectively). When the contraction reached a steady state, increasing concentration of RWPC (10$$^{-2}$$-10$$^{-2}$$ g l$$^{-1}$$) was added cumulatively. All the following experiments were carried out in arteries with intact endothelium.

In order to characterize the involvement of NO and cyclo-oxygenase products in the RWPC-induced relaxation, arteries with functional endothelium were exposed to the NO-synthase inhibitor, L-NAME (300 µM), or the cyclo-oxygenase inhibitor, indomethacin (10 µM). The involvement of endothelial derived hyperpolarizing factor (EDHF) was studied using PSS containing 25 mM KCl, and PSS containing 25 mM KCl plus L-NAME (300 µM). The concentration of norepinephrine was adjusted in order to obtain the same level of precontraction (i.e. it was reduced to 1 µM when
L-NAME plus 25 mM KCl-PSS were applied in the bath. To test the involvement of mono-oxygenase as sources of ROS implicated in the RWPC-induced relaxation, concentration-response curves to RWPC were performed in small mesenteric arteries with functional endothelium in the presence of a NAD(P)H-dependent oxidoreductase inhibitor, diphenyleneiodonium (DPI, 10 µM). The effect of ROS on RWPC-induced relaxation was studied using O$_2^\cdot$ scavengers, superoxide dismutase (SOD, 100 U ml$^{-1}$) plus catalase (100 U ml$^{-1}$).

The involvement of pertussis toxin (PTX)-sensitive G protein was investigated in RWPC-induced relaxation of small mesenteric arteries. Mesenteric segments were incubated for 12 h at 37 °C in minimum essential medium, containing 10 % fetal calf serum and PTX (300 ng ml$^{-1}$) that were gassed with 95 % O$_2$ and 5 % CO$_2$ mixture. In control experiments, vessels were incubated with PTX-free medium under the same condition. After this incubation period, tissues were mounted to record mechanical activity as described previously. The presence of endothelium was verified in all preparations.

All the inhibitors were used at maximally active concentrations and were incubated with the tissue for 15 min prior the precontraction with norepinephrine.

**Cultured bovine aortic endothelial cells (BAEC)**

Bovine aortic endothelial cells (BAEC) were isolated from bovine aortae and placed as described previously by Kessler et al. (1995). Cells were cultured in plastic flasks using as culture medium a mixture of DMEM and Ham’s F12 mediums (50/50) supplemented (to final concentration) with 10 % heat inactivated fetal calf serum, 2 mM L-glutamine, 100 mg ml$^{-1}$ heparin, 10 000 U ml$^{-1}$ penicillin, 10 000 U ml$^{-1}$ streptomycin, 10 µM vitamin C. The cultures were maintained at 37 °C in a 5 % CO$_2$ humified incubator. Cells were used for Ca$^{2+}$ measurement and for O$_2^\cdot$ quantification after the 1st or 2nd passages in the confluence state.

**Measurement of [Ca$^{2+}$]i**

[Ca$^{2+}$], measurement was performed using the fluorescent Ca$^{2+}$-sensitive probe, Fura-2, with a dual excitation wavelength fluorometer (Hitachi, F-2000). Cells were washed and incubated with Fura-2/AM (5 µM) for 1 h at room temperature in PSS with the following composition in mM: NaCl 119; KCl 4.75; CaCl$_2$ 1.25; MgSO$_4$ 1.2; KH$_2$PO$_4$ 1.2; NaHCO$_3$ 25; glucose 5 and HEPES 20 at pH 7.4. Cells were then washed twice with phosphate-buffered saline (PBS) Ca$^{2+}$/Mg$^{2+}$-free and dispersed using 1 % trypsin. After 5 min centrifugation at 60xg, cells were again washed and suspended in PSS (2 x 10$^6$ cells ml$^{-1}$). Then, the cells were transferred into quartz cuvette (final volume of 2.5 ml), continuously stirred and maintained at 37 °C. Fluorimetric readings were performed with F-2000 Hitachi spectrofluorimetric system, with excitation alternating between 340 and 380 nm (10 Hz) and emission at 510 nm. In each preparation, the maximum and minimum fluorescence values were sequentially determined by the addition of 10 µM ionomycin in the presence of 2 mM Ca$^{2+}$, followed by the addition of 10 mM EGTA at pH 8.

Ca$^{2+}$ response to RWPC was achieved at the concentration producing maximal endothelium-dependent relaxation in mesenteric segments (10$^{-2}$ g l$^{-1}$). The role of extracellular O$_2^\cdot$ and NAD(P)H oxidase pathways was also studied using SOD (100 U ml$^{-1}$) plus catalase (100 U ml$^{-1}$) or DPI (10 µM), respectively.

**Measurement of O$_2^\cdot$ production**

O$_2^\cdot$ production was measured as SOD inhibitable reduction of cytochrome C. BAEC were preincubated in PSS for 15 min at 37 °C, washed once and incubated with PSS containing cytochrome C (1 mg ml$^{-1}$), in the presence or absence of SOD (200 U ml$^{-1}$) and with or without RWPC (10$^{-2}$ g l$^{-1}$). After 60 min incubation, the mediums were removed from all dishes, placed on ice, and their absorbances were immediately read at 550 nm against PSS. In parallel, for assessment of the role of Ca$^{2+}$ in the O$_2^\cdot$ production, the same protocol was performed by removing CaCl$_2$ from PSS. Cell-free dishes were used to test the effect of the RWPC on the reduction of cytochrome C. O$_2^\cdot$ specific reduction of cytochrome C was expressed as the difference in absorbance between cells incubated with or without SOD using an extinction coefficient of 21 mM$^{-1}$ cm$^{-1}$.

**Drugs**

RWPC was from Dr M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France). RWPC polyphenol contents were already described previously (Andriambeloson et al. 1997).

Acetylcholine, catalase, CHAPS, cytochrome C, DPI, indomethacin, ionomycin, L-NAME, norepinephrine, PTX, and SOD were purchased from Sigma Chemical Co. (Grenoble, France). Stock solutions were prepared in distilled water (Q10, Millipore) except...
for indomethacin (dissolved in 5% NaHCO₃) and RWPC (in 10% ethanol).

**Statistical analysis**

Relaxations are expressed as a percentage of the level of precontraction. All results are expressed as mean ± S.E.M. of n experiments. ANOVA was used for statistical analysis. P<0.05 was considered statistically significant.

**Results**

**Relaxant effects of RWPC**

Relaxant effects of RWPC in a small mesenteric artery of the rat are represented in Figure 1. RWPC produce a concentration-dependent relaxation in small mesenteric arteries with functional endothelium. pD₂ values and maximum relaxation (E_max) were 2.1±0.5 x 10⁻⁴ g l⁻¹ and 63.0±6.0 %, n=18, respectively. However, in the absence of a functional endothelium, the relaxation induced by RWPC was completely abolished. All the following experiments were performed in small mesenteric arteries in the presence of a functional endothelium. Blockade of endothelial NO-synthase by L-NAME (300 µM), partial depolarization with 25 mM KCl or L-NAME plus KCl (25 mM) abolished RWPC-induced endothelium-dependent vasorelaxation. The presence of a cyclooxygenase inhibitor, indomethacin (10 µM) did not alter RWPC-induced relaxation (Fig. 2).

The presence of ROS scavengers, SOD plus catalase (both 1000 U ml⁻¹) slightly but significantly reduced the relaxation induced by RWPC (Fig. 3a). Inhibition of NAD(P)H-dependent oxidoreductases by DPI (10 µM) induced a significant decrease of the response to RWPC in rat mesenteric arteries (Fig. 3b). The treatment with PTX for 24 h did not alter the contraction elicited by norepinephrine in mesenteric segments. The RWPC-induced relaxation was not affected by PTX treatment (Fig. 3c).

**Ca²⁺ response to RWPC and role of O₂⁻**

On-line recordings of Ca²⁺ response induced by RWPC were studied (Fig. 4a). After stabilization of [Ca²⁺], for a few minutes, a mean basal value of 166.4±7.3 nM was measured in the presence of 1.25 mM of extracellular Ca²⁺. Application of RWPC (10⁻² g l⁻¹) elicited a transient increase in [Ca²⁺], which reached a maximum within 40-50 s (307.7±24.1 nM) and returned slowly to a sustained level (204.1±25.4 nM) within 150 s. RWPC (10⁻² g l⁻¹) was not able to produce an increase of [Ca²⁺] in cultured aortic smooth muscle cells (data not shown). Figure 4a shows that the mixture of SOD and catalase attenuated the Ca²⁺ rise induced by RWPC suggesting a role of oxygen radicals in the mechanism of action of RWPC. In contrast, the inhibitor of NAD(P)H-oxidase (DPI) did not affect the Ca²⁺ rise induced by RWPC (Fig. 4a) suggesting that the stimulation of such an enzyme is unlikely to participate in the effect of RWPC. In addition, incubation of BAEC with RWPC elicited a significant increase in the production of O₂⁻ in the presence of 1.25 mM of Ca²⁺ in the PSS as indicated by SOD-inhibitable reduction of cytochrome C. Removal
of Ca\(^{2+}\) from the PSS completely inhibited the increase in O\(_2\) \(^{-}\) production induced by RWPC (Fig. 4b). In cell-free conditions, no O\(_2\) \(^{-}\) was detected after addition of RWPC (data not shown).

**Fig. 3.** Effects of A) superoxide dismutase (SOD, 100 U ml\(^{-1}\)) plus catalase (CAT, 100 U ml\(^{-1}\)), B) diphenyleneiodonium (DPI, 10 µM), C) pertussis toxin (PTX, 300 ng ml\(^{-1}\)) on RWPC-induced relaxation (control) in small mesenteric arteries with endothelium. Results are shown as means ± S.E.M of 6-18 different experiments. * P<0.05 vs control.

**Discussion**

The present study demonstrates that RWPC-induced vascular relaxation in small mesenteric arteries of rats depends on the presence of the endothelium, and mechanisms involving NO-pathway and EDHF, but not cyclo-oxygenase products. Furthermore, it has been shown that SOD plus catalase-sensitive pathways are involved. Interestingly, studies in BAEC also showed that RWCP triggered O\(_2\) \(^{-}\) production and its action on the increase in [Ca\(^{2+}\)], was sensitive to the SOD/catalase system. Altogether, these results suggest the involvement of O\(_2\) \(^{-}\) in the increase in [Ca\(^{2+}\)], implicated in the activation of enzymes responsible for the release of relaxant factors in endothelial cells and the associated vasodilatation of resistance arteries.

**Fig. 4.** A) Original tracing showing RWPC (10\(^{-2}\) g l\(^{-1}\)) -induced increase of intracellular calcium concentration [Ca\(^{2+}\)], in Fura-2 loaded bovine aortic endothelial cells (BAEC), in the absence (control) and presence of diphenyleneiodonium (DPI, 10 µM), or superoxide dismutase (SOD, 100 U ml\(^{-1}\)) plus catalase (CAT, 100 U ml\(^{-1}\)). B) Effects of RWPC (10\(^{-2}\) g l\(^{-1}\)) on O\(_2\) \(^{-}\) production measured as SOD-inhibitable reduction of cytochrome C in BAEC incubated with PSS containing 1.25 mM Ca\(^{2+}\) or PSS Ca\(^{2+}\)-free. Results are shown as means ± S.E.M. of 5-9 different experiments. * P<0.05 vs basal.

The present work demonstrates that RWPC can cause an endothelium-dependent relaxation in small mesenteric arteries of the rat. The effect of RWPC was also almost completely inhibited either by the presence of NO-synthase inhibitor, L-NAME, or in a depolarizing PSS containing 25 mM KCl, that inhibits the hyperpolarization produced by EDHF. However, the presence of the cyclo-oxygenase inhibitor, indomethacin
did not alter RWPC-induced relaxation. These results indicate that NO-synthase pathway and EDHF are probably involved in RWPC-induced relaxation of small mesenteric rat arteries. In general, the L-arginine-NO pathway either partially accounts for or is sufficient to produce full endothelial relaxation in these resistance vessels. Likewise, EDHF alone is able to fully relax small mesenteric arteries. In the present study, it was found that blockade of either NO-pathway or EDHF alone almost entirely prevented endothelial-dependent relaxation of the small mesenteric arteries in response to RWPC. With regard to EDHF, hyperpolarization triggered by RWPC might not be able to produce relaxation by itself. On the other hand, pharmacological blockade of EDHF action using partial KCl depolarization, almost abolished RWPC-induced endothelial vasodilatation in the arteries suggesting that NO alone was unable to account for this effect of RWPC.

Several hypotheses could be advanced to explain these findings: Firstly, the use of KCl depolarization might interact with both EDHF and NO pathways in the small mesenteric arteries. Indeed, NO release has been reported to be inhibited by partial depolarization in endothelial cells of different vascular preparations (Edwards et al. 1998, Qiu and Quilley 2001). Secondly, another factor might be needed for EDHF to promote relaxation, in this case the L-NAME-sensitive pathway. Thirdly, it is possible that EDHF-induced hyperpolarization of endothelial cells is necessary to promote the synthesis and/or liberation of NO in these cells when exposed to RWPC in the same manner as in our previous study performed in small mesenteric arteries from the Lyon strain (Freitas et al. 2003). Nevertheless, the present finding that NO and EDHF are simultaneously required to promote endothelium-dependent relaxation in resistance vessels in response to RWPC suggests the existence of an interaction mechanism between NO and EDHF which is inverse from the reciprocal compensatory role previously described in other vessels in response to other vasodilator agents (Wu et al. 1993, Hwa et al. 1994, Feletou and Vanhoutte 1999).

Recently, we have reported that RWPC increase [Ca^{2+}]i in BAEC. This requires primarily the presence of extracellular Ca^{2+}, mobilization of Ca^{2+} from intracellular stores and activation of phospholipase C and tyrosine kinase pathways (Martin et al. 2002). Interestingly, the increase in [Ca^{2+}]i, produced by RWPC in BAEC (Martin et al. 2002) and the endothelium-dependent relaxation of small mesenteric arteries elicited by the same compounds are insensitive to pertussis toxin treatment. These results strongly suggest that the involvement of PTX-sensitive G proteins in the effect of RWPC is unlikely.

O_{2}^{-} play a key role in the NO-related bioactivity, because they inactivate radical NO by transforming it to peroxynitrite. However, studies in endothelial cells have shown that O_{2}^{-} induce a rapid rise in [Ca^{2+}]i, (Hirosumi et al. 1988, Franceschi et al. 1990, Dreher et al. 1995, Dreher and Junot 1995), which might activate endothelial NO-synthase and may counteract the O_{2}^{-}-mediated inactivation of NO. This hypothesis raises the physiological importance of the critical balance between NO and O_{2}^{-} in cells. In this study, the involvement of extracellular O_{2}^{-} in RWPC-induced vascular relaxation was examined using the O_{2}^{-} scavengers, SOD plus catalase. Our results showed that the effect of RWPC was partially inhibited in the presence of the O_{2}^{-} scavengers SOD plus catalase, suggesting that production of O_{2}^{-} is implicated in the vasorelaxant effect of RWPC in small mesenteric arteries. Recently, we have reported that the in vivo treatment with RWPC was able to increase acetylcholine-induced vascular relaxation in the rat aorta, and the mechanism involved increased O_{2}^{-} production (Diebolt et al. 2001). Interestingly, the Ca^{2+} signaling elicited by RWPC in BAEC was also reduced by the combination of SOD plus catalase. Furthermore, RWPC could stimulate O_{2}^{-} production of BAEC as indicated by cytochrome C reduction assay. Altogether, these data demonstrate the role of O_{2}^{-} in both the enhancement of Ca^{2+} signaling in BAEC and the endothelial vasodilatation of small mesenteric arteries in response to RWPC. These results are consistent with those reported in the literature showing that O_{2}^{-} formation produced by high glucose concentration enhanced agonist-stimulated Ca^{2+}/NO signaling in porcine aortic endothelial cells (Graier et al. 1996).

In order to determine intracellular sources of O_{2}^{-} implicated, the effect of the NAD(P)H oxidoreductases inhibitor DPI was used. Pretreatment with DPI reduced the relaxation of small mesenteric arteries induced by RWPC, but did not modify the increase of [Ca^{2+}]i in BAEC in response to RWPC. These results suggest a dissociation of DPI effect in the two responses. The lack of effect of DPI on the increase of [Ca^{2+}]i in BAEC in response to RWPC ruled out the involvement of NAD(P)H oxidoreductases as a O_{2}^{-} source activated by RWPC. However, DPI at used concentration (10 µM) has also been reported to inhibit NO synthase, which explain
its inhibitory effect in NO-mediated relaxation induced by RWPC.

In conclusion, the present study shed light on cross-talk between NO and EDHF indicating that both NO and EDHF are required to promote endothelium-dependent relaxation produced by RWPC in mesenteric resistance arteries. This effect of RWPC is associated with an increased O$_2^-$ production in order to promote Ca$^{2+}$ signaling and the release of endothelial factors leading to vasodilatation.

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