Red wine polyphenols prevent endothelial dysfunction induced by endothelin-1 in rat aorta: role of NADPH oxidase

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ABSTRACT

RWPs (red wine polyphenols) exert antihypertensive effects and improve endothelial function by reducing the plasma levels of ET-1 (endothelin-1) and the subsequent vascular production of \( \text{O}_2^{•−} \) (superoxide anion). Our present study was designed to evaluate whether RWPs act directly in the vascular wall improving endothelial dysfunction and \( \text{O}_2^{•−} \) production induced by ET-1 and to analyse the compounds responsible for these protective effects. We incubated rat isolated aortic rings in the presence or absence of ET-1 (10 nM) and RWPs (10\(^{−4}\) to 10\(^{−2}\) g/l) or catechin (0.2 \( \mu \)M), epicatechin (10 \( \mu \)M) and resveratrol (0.1 \( \mu \)M). ET-1 reduced the relaxant responses to acetylcholine, increased intracellular \( \text{O}_2^{•−} \) production, NADPH oxidase activity and protein expression of NADPH oxidase subunit p47\(_{\text{phox}}\). All these changes were prevented by RWPs. The preventive effects of RWPs were unaffected by co-incubation with either ICI-182780, an ER (oestrogen receptor) antagonist, or GW9662, a PPAR\(\gamma\) (peroxisome-proliferator-activated receptor \(\gamma\)) antagonist. RWPs inhibited the phosphorylation of the mitogen-activated protein kinase, ERK1/2 (extracellular signal-regulated kinase 1/2), a key regulator of p47\(_{\text{phox}}\) expression in response to ET-1. When the isolated polyphenols were tested, at the concentrations found in 10\(^{−2}\) g/l RWPs, only epicatechin prevented endothelial dysfunction and all biochemical changes induced by ET-1 in the vascular wall. Taken together, these results indicate that RWPs prevent ET-1-induced vascular \( \text{O}_2^{•−} \) production by reducing overexpression of p47\(_{\text{phox}}\) and the subsequent increased NADPH oxidase activity, leading to improvement in endothelial function. The effects of RWPs appear to be independent of ER and PPAR\(\gamma\) activation and are related to ERK1/2 inhibition.

Key words: aortic ring, endothelial dysfunction, endothelin-1, NADPH oxidase, red wine polyphenol.

Abbreviations: ACh, acetylcholine; AngII, angiotensin II; BH4, tetrahydrobiopterin; DAPI, 4′,6-diamidino-2-phenylindole dichlorohydrate; DHE, dihydroethidium; DOCA, deoxycorticosterone acetate; eNOS, endothelial NO synthase; ER, oestrogen receptor; ERK1/2, extracellular signal-regulated kinase 1/2; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NIH, National Institutes of Health; l-NAME, N\(^{\text{G}}\)-nitro-l-arginine methyl ester; \( \text{O}_2^{•−} \), superoxide anion; OCT, optimum cutting temperature; PPAR, peroxisome-proliferator-activated receptor; ROS, reactive oxygen species; RT, reverse transcriptase; RWP, red wine polyphenol; SOD, superoxide dismutase.

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INTRODUCTION

ET-1 (endothelin-1) is a potent endothelial-derived vasoconstrictor peptide playing a crucial role in cardiovascular physiology, and its alterations have been associated with most forms of cardiovascular disease. Experimental models of hypertension such as DOCA (deoxycorticosterone acetate)-salt- or AngII (angiotensin II)-infused rats, atherosclerosis and diabetes display high levels of circulating ET-1 and are associated with endothelial dysfunction [1–4]. Endothelial dysfunction, defined as an imbalance of endothelium-derived vasoactive factors leading to vasoconstriction and structural changes in the vessel wall, is an early event in the pathophysiology of atherosclerosis and hypertension, and it is an independent predictor of poor prognosis [5]. A significant body of evidence indicates that ET-1, through ETA/ETB receptor activation, increases ROS (reactive oxygen species) production in the vasculature [e.g. O2•− (superoxide anion)], resulting in endothelial dysfunction [6–8].

The major source of intracellular ROS in vascular cells is NADPH oxidase, a multisubunit enzymatic complex, which comprises two membrane-bound subunits, Nox (Nox-1, Nox-2 or gp91phox, Nox-4 or Nox-5) and p22phox, which are regulated by cytoplasmic subunits such as p47phox, p67phox and a low-molecular-mass G-protein (rac1 or rac2) [9]. ET-1 mediates vascular O2•− production via activation of NADPH oxidase, xanthine oxidase and mitochondria. The oxidative pathway is amplified by O2•−-induced eNOS (endothelial NO synthase) uncoupling leading to further ROS generation, which ultimately leads to endothelial dysfunction [8,10,11]. Moreover, we have found recently that ET-1 induces NADPH-oxidase-driven O2•− generation through an increase in p47phox protein expression [11]. The signalling pathway for this effect involves sequential activation of PKC (protein kinase C), c-Src and ERK1/2 (extracellular signal-regulated kinase 1/2) [12].

Moderate alcohol consumption is associated with a significant reduction of cardiovascular mortality in humans [13]. Some studies indicate that wine may be more protective than other liquors such as beer or spirits [14,15]. A meta-analysis of 26 studies on more than 200,000 individuals revealed that when compared with abstainers, cardiovascular risk was reduced by 22% in beer drinkers and by 32% in wine drinkers [16]. This and other studies suggest that wine may contain non-alcoholic compounds that, in addition to ethanol, protect against atherosclerotic vascular disease. Consistent with this idea, the relatively high wine intake in France is thought to account for the lower mortality from CHD (coronary heart disease), which is reduced by 50% compared with other European countries and the U.S.A., despite similar intakes of saturated fats [17,18]. However, the molecular basis of this phenomenon, commonly known as the ‘French paradox’, remains unknown.

RWP (red wine polyphenols) reduce cardiovascular risk factors such as hypertension and endothelial dysfunction. In fact, chronic administration of RWP reduces blood pressure and improves endothelial function in DOCA-salt rats, a low-renin model of hypertension in which ET-1 plays a predominant role [19,20]. In these hypertensive rats, RWP prevents vascular oxidative stress and inhibit NADPH oxidase activity [20]. Similarly, in Zucker rats, RWP reduced O2•− release via decreased expression of the NADPH oxidase membrane subunit Nox1 [21]. However, it is not clear whether these protective effects of RWP are a result of decreased ET-1 synthesis [20] and/or interference with ET-1 signalling pathways to lead oxidative stress in the vascular wall. Furthermore, the main compounds of RWP extract responsible for these protective effects are unknown.

Given the key role of ET-1 and oxidative stress in cardiovascular disease, we have investigated the mechanisms involved in the protective effects of a dietary mixture of RWPs in ET-1-induced vascular oxidative response and endothelial dysfunction in isolated rat aortic segments. We also tested the effects of the three main monomeric polyphenols (catechin, epicatechin and resveratrol) included in this RWP extract.

MATERIALS AND METHODS

Ethics

All of the procedures conform to the Guide for the Care and Use of Laboratory Animals [NIH (National Institutes of Health) publication no. 85–23, revised 1996] and were approved by our Institutional Committee for the ethical care of animals.

Isolation and incubation of rat aortic rings

Experiments were conducted in male Wistar rats obtained from Harlan Laboratories weighing 200–250 g. The descending thoracic aortae were dissected and cut into rings. Rings were incubated in Krebs solution (118 mM NaCl, 4.75 mM KCl, 25 mM NaHCO3, 1.2 mM MgSO4, 2 mM CaCl2, 1.2 mM KH2PO4 and 11 mM glucose, pH 7.4) containing an antibiotic/antimycotic mixture (penicillin, gentamicin and anfotericin B) for 4 h in a cell culture incubator in the absence or presence of ET-1 (10 nM) and in the presence of vehicle (DMSO 0.1%), RWP (10−4, 10−3 and 10−2 g/l), apocynin (10−4 M), catechin (0.2 μM), epicatechin (10 μM) or resveratrol (0.1 μM). The concentration of RWP used in the present study has been found to produce the maximal endothelium-dependent relaxation in rat vascular tissues [22] and was comparable with the concentration of polyphenols present in the plasma with a dose of RWP of 20 mg/kg of body weight when used in in vivo experiments [21]. Aortae were immediately used for O2•− production in the vasculature [e.g. O2•− (superoxide anion)].
production or contractile tension recording or frozen in liquid nitrogen and stored at −80 °C for Western blotting or included in OCT (optimum cutting temperature) compound and then frozen in liquid nitrogen and stored at −80 °C for intracellular O$_2^{•-}$ production analysis.

**Contractile tension recording**

Aortic rings, previously incubated as mentioned above, were mounted in organ chambers by means of two L-shaped stainless steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer coupled to a signal amplifier (Dynamometer UFI; Cibertec) and connected to a computer via an A/D interface. Contractile tension was recorded by a PowerLab 800 (AD Instruments, Cibertec), as previously described [23]. The chamber was filled with Krebs solution at 37 °C and gassed with 95% O$_2$ and 5% CO$_2$. Rings were stretched to 2 g of tension and equilibrated for 90–120 min. During this period, Krebs solution was changed every 30 min, and rings were re-stretched as needed to maintain a final tension of 2 g. After equilibration, arteries were stimulated with phenylephrine (1 μM or 0.1 μM, in control or ET-1-treated aortic rings, respectively, to obtain similar level of precontraction), and a concentration–response curve was constructed by cumulative addition of ACh (acetylcholine). In some experiments, endothelium-independent responses to SNP (sodium nitroprusside) were also performed in the dark in rings pre-contracted with 1 μM phenylephrine. To evaluate the participation of endothelial NO in endothelial dysfunction induced by ET-1, several aortic rings were incubated with or without the eNOS inhibitor L-NAME (N$^{G}$-nitro-L-arginine methyl ester) (100 μM) in organ chambers for 30 min before the addition of phenylephrine (10$^{-9}$–10$^{-4}$ M).

**In situ detection of vascular O$_2^{•-}$ production**

DHE (dihydroethidium) was used to localize O$_2^{•-}$ in vessel sections in situ. DHE enters the cells and is oxidized by O$_2^{•-}$ to yield ethidium, which binds to DNA to produce bright red fluorescence. Aortic rings, incubated previously as described above, were cryopreserved by incubation with PBS (0.1 M) containing 30% sucrose for 1–2 h, included in OCT compound, frozen, and 10-μm cross-sections were obtained in a cryostat (HMS500 OM; Microm International). Sections were incubated in a humidified chamber for 30 min in Hepes-buffered solution (130 mM NaCl, 5 mM KCl, 1.2 mM MgCl$_2$; 10 mM glucose and 10 mM Hepes, pH 7.3 with NaOH) at 37 °C. Then, the sections were incubated further for 30 min in Hepes solution containing DHE (10 μM) in the dark. Preparations were counterstained with the nuclear stain DAPI (4′,6-diamidino-2-phenylindol dichlorohydrate; 0.3 μM) for 5 min at 37 °C in the dark, washed and mounted with a coverslip. Some sections from control rings were incubated for 30 min with the O$_2^{•-}$ intracellular scavenger tiron (10 μM) before DHE. Four sections of each preparation were examined on a fluorescence microscope (Leica DM IRB) using a ×40 objective with the following pairs of excitation/emission filters: 340–380/425 nm long pass to visualize DAPI blue fluorescence, BP450–490/515 nm long pass to visualize the green autofluorescence of elastin and 545–580/610–75 nm long pass to visualize ethidium red fluorescence. Sections were photographed with a Leica DC300F colour digital camera, and images were saved for off-line analysis. Microscope and camera settings were kept constant for all preparations. Ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32); NIH, http://rsb.info.nih/ij/). O$_2^{•-}$ production was estimated from the ratio of ethidium/DAPI fluorescence [24].

**NADPH oxidase activity**

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described [24]. Aortic rings were incubated for 30 min at 37 °C in Hepes-containing physiological salt solution [119 mM NaCl, 20 mM Hepes, 4.6 mM KCl, 1 mM MgSO$_4$, 0.15 mM Na$_2$HPO$_4$, 0.4 mM KH$_2$PO$_4$, 1 mM NaHCO$_3$, 1.2 mM CaCl$_2$ and 5.5 mM glucose (pH 7.4)]. Aortic production of O$_2^{•-}$ was stimulated by addition of NADPH (100 μM). Rings were then placed in tubes containing physiological salt solution, with or without NADPH, and lucigenin was injected automatically at a final concentration of 5 μM to avoid known artifacts when used at higher concentrations. NADPH oxidase activity was determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507; Berthold) at 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as RLU (relative luminescence units)/min per mg of dry aortic tissue.

To characterize the involvement of uncoupled eNOS on ET-1-stimulated aortic O$_2^{•-}$ generation, some endothelium-intact aortic rings were then placed in tubes containing physiological salt solution, stimulated with the calcium ionophore A23187 (10 μM), as described previously [11].

**Western blotting analysis**

Aortic homogenates were separate by SDS/PAGE. Proteins were transferred on to PVDF membranes, incubated with primary polyclonal rabbit anti-p47$^{	ext{phox}}$ (SantaCruz Biotechnology), anti-ET$_A$ receptor (SantaCruz Biotechnology) and anti-phosho-eNOS (Ser1177) (Cell Signaling Technology) antibodies and, monoclonal mouse
anti-eNOS (Cell Signalling Technology) or anti-phospho-ERK1/2 (Thr183/Tyr185) (Sigma–Aldrich) antibodies overnight and with the secondary HRP (horseradish peroxidase)-conjugated antibody. Antibody binding was detected by an ECL (enhanced chemiluminescence) system (Amersham Biosciences), and densitometric analysis was performed using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com) [20]. Samples were re-probed for expression of smooth muscle α-actin or ERK1/2. p47phox and ETA protein abundance/α-actin ratio or phospho-ERK1/2:ERK1/2 and phospho-eNOS/eNOS were calculated, and data are expressed as a percentage of the values in control aorta from the same gel.

RT (reverse transcriptase)—PCR

For RT–PCR, total RNA was extracted from aorta by homogenization and converted into cDNA by standard methods. PCR was performed with a Techne Techgene thermocycler (Techne). Initial denaturation was done at 95°C for 3 min and followed by 30–40 cycles of amplification. Each cycle consisted of 1 min of denaturation at 94°C, 45 s of annealing at 60°C for p47phox, 55°C for p22phox, 55°C for caveolin-1 or 63°C for eNOS and 1 min for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow reannealing of amplified products. RT–PCR products were then size-fractionated through a 1.5% agarose gel, and the bands were visualized with ethidium bromide and quantified by densitometric analysis performed on the scanned images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). The sequences for primers for p47phox and p22phox were selected according to the published sequences in GenBank® and were as follows: p47phox (191 bp) sense, 5′-CCCACGGCACAGATTAGAAGC-3′ and antisense, 5′-TGGATTGTCCCTTTGAGTCAGG-3′; and p22phox (220 bp) sense, 5′-GCGGTGTGGACAGAAGTACC-3′ and antisense, 5′-CTTGCGGTAGCTCAATGGG-3′. The sequences for the primers for caveolin-1 and eNOS were as follows: caveolin-1 (304 bp) sense, 5′-TCTACAAGGCCCAAACAAAGG-3′ and antisense, 5′-AGAAAGAAGAGAGTGGCAAAG-3′; and eNOS (164 bp) sense, 5′-CGAGATATCTTCTGGTCAATGGG-3′ and antisense, 5′-GTGGATTGTGCTGCCTCCTAGG-3′. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control for the coamplification. The signals were expressed relative to the intensity of GAPDH in each sample.

Drugs

RWPs dry powder from red wine was provided by Mr D. Ageron (Société Francaise de Distillerie, Vallont Pont d’Arc, France). The content of polyphenols in RWPs has been determined as follows (in mg/g of dry powder): 5.3 catechin, 222.5 epicatechin, dimers (1.9 B1; 21.1 B2; 7.4 B3), 176.3 anthocyanins, 4.7 gallic acid, 15.1 p-coumaric acid and 1.5 resveratrol. All drugs and reagents were from Sigma-Aldrich, except for DAPI which was from Calbiochem. RWPs, apocynin, catechin, epicatechin and resveratrol were initially dissolved in DMSO and all other drugs were in distilled water.

Statistical analysis

Results are means ± S.E.M., and n reflects the number of animals. Statistically significant differences between groups were calculated using a Students’ t test for unpaired observations or using an ANOVA followed by a Newman–Keuls test for multiple comparisons. P < 0.05 was considered statistically significant. Concentration–response curves were fitted to the logistic equation: $E = E_{\text{max}}/(1 + 10^{-(k \cdot [\text{dose}] - pD2)})$, where $E_{\text{max}}$ is the maximal effect, $k$ is a factor which represents the slope of the curve and $pD2$ is the drug concentration exhibiting 50% of the $E_{\text{max}}$ expressed as negative log molar.

RESULTS

Effects of RWPs on endothelial dysfunction

Incubation of the aortic rings for 4 h in the absence of ET-1 produced no significant changes in the contractile response to phenylephrine or in the relaxant response to ACh (results not shown). ET-1 led to the development of endothelial dysfunction as indicated by the reduction in the maximal relaxant effect of ACh (Figure 1A, Table 1) and by the increased contractile response to phenylephrine (Figure 1C). Incubation with RWPs for 4 h prevented ET-1-induced endothelial dysfunction in a concentration-dependent manner (Figure 1A) and decreased the contractile response to phenylephrine (Figure 1C) in ET-1-treated arteries. Similarly, the NADPH oxidase inhibitor apocynin also restored the impaired relaxant response to ACh induced by ET-1 (Figure 1B). The relaxant response to ACh was suppressed by NOS inhibition with l-NAME in both control and ET-1-treated aortic rings (results not shown). The concentration–contractile response induced by phenylephrine in intact aortic rings was significantly reduced in aortae incubated with ET-1 compared with control group when the rings were incubated previously with l-NAME, indicating a reduced basal NO formation in ET-1-treated rings. RWPs (10⁻³ g/l) increased this contractile response, suggesting a higher eNOS activity in these vessels (Figure 1D).

In order to determine if stretch could modify the effects of ET-1 on endothelial dysfunction, in another set of experiments, rings were first mounted in the organ chambers and then incubated with or without
ET-1 for 4 h. Afterwards, after removing ET-1, the relaxant response to ACh was analysed in phenylephrine pre-contracted rings. Under these more physiological experimental conditions, ET-1 incubation also impaired the vasodilatation induced by ACh compared with control rings (maximal effect, 35.2 ± 4.6 compared with 9.2 ± 5.1 % respectively, n = 6; *P < 0.01).

No differences were observed in the endothelium-independent relaxant responses to the endothelium-independent vasodilator sodium nitroprusside in arteries from vehicle-, RWPs-, ET-1- and ET-1–RWPs-treated rings (Figure 2).

**Effects of RWPs on O$_2^{•−}$ production**

In order to characterize O$_2^{•−}$ production and localize it within the vascular wall, ethidium red fluorescence was analysed in sections of aorta incubated with DHE, which is converted into ethidium by O$_2^{•−}$-induced oxidation. The nuclear staining was almost suppressed by the O$_2^{•−}$-intracellular scavenger tiron. Positive red nuclei could be observed in adventitial, medial and endothelial cells (Figure 3A). Red fluorescence was quantified, and the data were normalized by the blue fluorescence of the nuclear stain DAPI (Figure 3B). At 4 h, ET-1 induced an increase in O$_2^{•−}$ production, which was evident in all layers of the vessel. RWPs (10$^{-3}$ g/l) prevented ET-1-induced increase in DHE staining (Figures 3A and 3B).

**Effects of RWPs on NADPH oxidase activity and uncoupled eNOS**

NADPH increases lucigenin luminescence in normal aortic rings, which was almost abolished by prior incubation for 30 min with the flavoprotein inhibitor DPI (diphenyleneiodonium; 10 μM) (results not shown). NADPH oxidase activity was increased in aortic rings incubated with ET-1 compared with control rings.

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**Figure 1** Effects of RWPs on ET-1-induced endothelial dysfunction

Endothelium-intact aortic rings were incubated for 4 h with vehicle (DMSO 0.1 %) or (A) RWPs (10$^{-4}$, 10$^{-3}$ and 10$^{-2}$ g/l) or (B) apocynin (10$^{-4}$ M) and with or without ET-1 (10 nM), then stimulated with phenylephrine, and a concentration–response curve to ACh (0.001–10 μM) was constructed in a cumulative fashion. Concentration–response curves of phenylephrine obtained in aortic rings in the absence (C) or in the presence (D) of L-NAME (100 μM), added to the organ chamber 30 min before the addition of phenylephrine. Results are means ± S.E.M., n = 5–9, *P < 0.05 and **P < 0.01 compared with control rings, #P < 0.05 and ##P < 0.01 compared with ET-1-treated rings.
Apocynin (100 μM), a specific intracellular inhibitor of NADPH oxidase, which prevents the translocation of p47^phox and p67^phox subunits from cytoplasm to membrane and the assembly of NADPH oxidase [25], inhibited the increased ET-1-induced NADPH oxidase activity. Similarly, RWPs, in a concentration-dependent manner, prevented the increase in NADPH oxidase activity induced by ET-1 (Figure 4A).

ET-1 also increased aortic O_2•− generation, measured by lucigenin-enhanced chemiluminescence, in rings stimulated with the calcium ionophore A23187, as a result of uncoupled eNOS activation. This increased O_2•− production was suppressed by l-NAME (100 μM), endothelial denudation (results not shown) and by co-incubation with RWPs (10^{-3} g/l) (Figure 4B).

**Effects of RWPs on ET_A, p47^phox, p22^phox, eNOS and caveolin-1 gene and protein expression**

No changes in ET_A protein expression were observed after ET-1 and ET-1 plus RWPs (10^{-3} g/l) incubation, compared with control rings (Figure 5A). In aortic segments, incubation with ET-1 for 4 h increased both gene and protein expression of p47^phox (Figures 5C and 5D) without affecting p22^phox (Figure 5B), eNOS (Figure 6A) and caveolin-1 (Figure 6B) gene expression, as measured by RT–PCR. Co-incubation with RWPs (10^{-3} g/l) prevented ET-1-induced overexpression of p47^phox. No differences were observed in eNOS phosphorylation among groups (Figure 6C).

**Role of ER (oestrogen receptor), PPAR (peroxisome-proliferator-activated receptor) and ERKs in the effects of RWPs**

The protective effects of RWPs in endothelial function have been attributed to ERα activation [26]. To test the involvement of ERα in the improvement of RWPs of the endothelial dysfunction induced by ET-1, we incubated the rings in the presence of the non-selective ER antagonist ICI-182780 (1 μM) [27]. This agent did not modify the effect of RWPs (10^{-2} g/l) in ACh-induced relaxation study (Figure 7A).

In order to determine whether PPARγ activation is involved in the protective effects of RWPs in endothelial dysfunction induced by ET-1, aortic rings were coincubated with the PPARγ antagonist GW9662 (1 μM) [28] in the presence of ET-1 and RWPs (10^{-2} g/l). RWPs’ improvement of ET-1-induced impaired ACh relaxation was unaffected by GW9662 (Figure 7B). ERK1/2 has been recently involved in the ET-1 signalling pathway to increase NADPH oxidase-driven O_2•− production in vascular wall [12]. In our experiments, aortic incubation with ET-1 for 1 h induced ERK1/2 phosphorylation, which was prevented by coincubation with RWPs (10^{-3} g/l) (Figure 7C).
Figure 3  Effects of RWPs on ET-1-induced vascular $O_2^{•−}$ production measured by DHE fluorescence
(A) Upper panel shows arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by $O_2^{•−}$ and lower pictures show blue fluorescence of the nuclear stain DAPI. (B) Values of red ethidium fluorescence normalized to blue DAPI fluorescence. Vehicle was DMSO (0.1 %) and RWPs (10$^{-3}$ g/l). Results are means ± S.E.M. of four sections obtained from each ring ($n = 4$). **$P < 0.01$ compared with control (Cont) rings; #$P < 0.05$ compared with ET-1-treated rings.

Figure 4  Effects of RWPs on ET-1-stimulated NADPH activity and eNOS uncoupling determined by lucigenin luminescence
Aortic rings with intact endothelium were incubated for 4 h either with vehicle (0.1 % DMSO), RWPs (10$^{-4}$, 10$^{-3}$ and 10$^{-2}$ g/l) or apocynin (100 μM) in the presence or in the absence of ET-1 (10 nM). (A) NADPH oxidase activity stimulated by NADPH (100 μM), and (B) $O_2^{•−}$ production stimulated by the calcium ionophore A23187 (10 μM). Results are means ± S.E.M., $n = 5−12$. *$P < 0.05$ and **$P < 0.01$ compared with control rings; #$P < 0.05$ and ###$P < 0.01$ compared with ET-1-treated rings.

Effects of isolated polyphenols in functional and biochemical changes induced by ET-1

When we tested the effects of catechin, epicatechin and resveratrol in the endothelial dysfunction induced by ET-1, only epicatechin improved the relaxant response induced by ACh in phenylephrine-contracted rings (Figure 8A). Moreover, epicatechin 10 μM prevented the increased $O_2^{•−}$ production (Figure 8B), NADPH oxidase activity (Figure 8C), p47$^{phox}$ expression (Figure 8D) and ERK1/2
Figure 5  Effects of RWPs on the expression of ET<sub>A</sub> receptor, p22<sub>phox</sub> and p47<sub>phox</sub>

Gene expression of p22<sub>phox</sub> (B) and p47<sub>phox</sub> (C) was measured by RT–PCR, and protein expression of ET<sub>A</sub> (A) and p47<sub>phox</sub> (D) was measured by Western blot in aortic rings incubated for 4 h either with vehicle (0.1 % DMSO) or RWPs (10<sup>−3</sup> g/l) in the presence or in the absence of ET-1 (10 nM). Results are means ± S.E.M. (<i>n</i> = 4–6) of densitometric values normalized to the corresponding RT–PCR products of GAPDH (B,C) or to the corresponding α-actin (A,D), and are expressed as a percentage of control. *<i>P</i> < 0.05 and **<i>P</i> < 0.01 compared with control (Cont) rings; #<i>P</i> < 0.05 compared with ET-1-treated rings.

Figure 6  Effects of RWPs on the expression of eNOS, caveolin and eNOS phosphorylation

Aortic eNOS (A) and caveolin-1 (Cav-1) (B) gene expression measured by RT–PCR, and protein expression of phospho-eNOS (Ser<sup>1177</sup>) (C) measured by Western blot in aortic rings incubated for 4 h either with vehicle (0.1 % DMSO) or RWPs (10<sup>−3</sup> g/l) in the presence or in the absence of ET-1 (10 nM). Results are means ± S.E.M. (<i>n</i> = 4–6) of densitometric values normalized to the corresponding RT–PCR products of GAPDH (A,B) or to the corresponding eNOS (C), and are expressed as a percentage of control.
phosphorylation (Figure 8E) induced by ET-1 in aortic rings.

DISCUSSION

ET-1 is a well-known trigger for increased vascular oxidative stress, and the subsequent O$_2$•⁻-driven NO inactivation plays a major role in the genesis of clinical endothelial dysfunction in a variety of pathophysiological conditions, including atherosclerosis, hypertension and diabetes [29]. The present study supports the hypothesis that ET-1 mediates vascular O$_2$•⁻ production via activation of NADPH oxidase leading to endothelial dysfunction. In this study, we show for the first time that ET-1-induced endothelial dysfunction can be prevented by RWPs, independently of their possible interference with ET-1 production. Moreover, these RWPs also prevented the ET-1-induced increase in vascular O$_2$•⁻ production from both NADPH oxidase, through down-regulation of p47$^{phox}$ expression, and from uncoupled eNOS. These protective effects seemed to be independent of both ER$\alpha$ and PPAR$\gamma$ activation but related to ERK1/2 inhibition.

Endothelial dysfunction is present in several experimental models in which ET-1 plays a predominant pathophysiological role such as (i) the DOCA-salt hypertensive rats, a low rennin–angiotensin model of hypertension [7], (ii) streptozotocin-induced diabetic rats [30], (iii) ET-1-infused rats [31], and (iv) transgenic mice overexpressing human preproET-1 specifically in blood vessel endothelium [32]. The fact that ET-1 can also induce endothelial dysfunction in vitro in rat aorta [8,11,33] (present results) indicates that these changes are due to direct effects of ET-1 on the vessel wall, independent of circulating hormones, neurogenic mechanisms or changes in arterial pressure. In our experiments, endothelial dysfunction was indicated by the reduced relaxant response after eNOS activation induced by ACh and by the increased vasoconstrictor response to phenylephrine. Chronic RWPs reduce blood pressure and improve endothelial function in DOCA-salt rats [20]. These protective effects have been related to their antioxidant properties reducing systemic and vascular oxidative stress [19,20]. However, it was unclear whether the effects on endothelial function were due to a direct effect on the vessel wall, secondary to the blood pressure-lowering effect or driven by neurohumoral mechanisms of RWPs. In fact, RWPs reduced ET-1 plasma levels in DOCA-salt rats, without affecting control animals. The present results show that RWPs are also effective in vitro, increasing the ACh-induced relaxation and reducing the phenylephrine-induced contraction in aortic rings exposed to ET-1. Moreover, it should be noted that the effective concentration of RWPs was as low as $10^{-4}$ g/l, indicating that this effect appears to be physiologically relevant [34]. The effect of ET-1 was observed after 4 h of incubation even when ET-1 was absent during the challenge with phenylephrine or ACh. Similarly, RWPs were present during the exposure to ET-1 but absent during the endothelial function tests. These slow and persistent changes induced by ET-1 are consistent with the involvement of changes in gene expression of O$_2$•⁻-generating systems, such as NADPH oxidase subunits. Moreover, the increased p47$^{phox}$ protein expression induced by ET-1 was inhibited by the ETA receptor antagonist BQ123, but not by the ETB receptor antagonist, BQ788 [12]. It is possible that the preventive effects of RWPs in the endothelial dysfunction induced by ET-1 were a result of the change in ETA receptor
Figure 8  Epicatechin displays similar protective effects to RWPs

(A) Endothelium-intact aortic rings were incubated for 4 h with vehicle (0.1% DMSO) or ET-1 (10 nM) or ET-1 (10 nM) plus catechin (ET1-cat) (0.2 μM), or epicatechin (ET1-epi) (10 μM) or resveratrol (ET1-res) (0.1 μM), then stimulated with phenylephrine, and a concentration—response curve to ACh (0.01−10 μM) was carried out in a cumulative fashion. Effects of epicatechin (epi, 10 μM) on the increase in O_{2}^{•−} production measured by DHE fluorescence (B), NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (C), p47^{phox} gene expression measured by RT−PCR (D) and phosphorylation of ERK1/2 by Western blot (E) in ET-1-treated aortic rings. Results are means ± S.E.M., n = 5−7. *P < 0.05 and **P < 0.01 compared with control rings; #P < 0.05 and ##P < 0.01 compared with ET-1-treated rings.

expression. However, this possibility was ruled out because we found that protein expression of ET_{A} receptor was unaltered by RWPs.

The most characteristic feature of endothelial dysfunction is a diminished bioactivity of endothelium-derived NO. In the rat aorta, endothelium-dependent vasodilatation relies almost entirely on the endothelial release of NO. Several potential mechanisms would be involved in the RWP-induced increase in endothelium-derived NO responses, such as changes in the activity and/or expression of eNOS, changes in the vascular levels of O_{2}^{•−} and, thus, O_{2}^{•−}-driven NO inactivation and changes in the sensitivity to the NO/cGMP pathway in vascular smooth muscle cells. Because the responses to nitroprusside were not modified by RWPs, the third potential mechanism can be ruled out. Reduced NO synthesis associated with endothelial dysfunction may be caused by impaired expression of eNOS, post-translational modification of the enzyme (e.g. phosphorylation or fatty acid modifications), interactions with hsp90 (heat-shock protein 90) and caveolin or suboptimal concentrations of the substrate L-arginine or the cofactor BH_{4} (tetrahydrobiopterin). We found that mRNA of eNOS and their negative regulator caveolin-1 in aorta was not altered by ET-1 or co-incubation with RWPs. Moreover, RWPs did not increase eNOS phosphorylation. However, eNOS activity, indirectly measured by phenylephrine contraction in the presence of L-NAME, was reduced by ET-1 and restored by RWPs. These data are consistent with the improvement of eNOS uncoupling induced by RWPs.

Excess of O_{2}^{•−} generation is critically involved in the breakdown of NO associated with endothelial dysfunction in aortic rings from DOCA-salt rats [7,35,36] and
rings incubated with ET-1 [8,11,33]. In our experiments, the presence of SOD (superoxide dismutase) in the organ chamber improved the relaxant response induced by ACh in aortic rings exposed to ET-1. Similarly, Kamata et al. [33] found that co-incubation of aortic rings with ET-1 and poly(ethylene glycol) SOD, a cell-permeant \( O_2^{*−} \) scavenger, completely prevented the impairment in endothelium-dependent relaxation in response to ACh induced by ET-1. In our study, apocynin, which selectively inhibits NADPH oxidase activity, reversed the impaired relaxant response to ACh in ET-1-treated rings. These results showed the critical role of NADPH oxidase activity in modulating vascular tone. However, caution might be taken with this result, since apocynin in vitro is not a specific inhibitor of NADPH oxidase [37].

We found that ET-1 induced an increase in DHE staining in medial and adventitial layers of the vessel and an increase in NADPH oxidase activity in aortic rings, an effect which was prevented by co-incubation with apocynin. Co-incubation of RWPs with ET-1 also prevented both the increased vascular \( O_2^{*−} \) production and the increased NADPH oxidase activity. These experiments were carried out in rings that had been treated previously with ET-1 and RWPs for 4 h, but the actual measurements were done in the absence of ET-1 and RWPs, which suggested a possible interference with the expression rather than the activity of NADPH oxidase subunits. In fact, aortic rings incubated for 4 h with ET-1 showed increased expression of p47\(_{phox}\) but not of p22\(_{phox}\). Thus this increased p47\(_{phox}\) protein expression is consistent with the increased \( O_2^{*−} \) production found in aortae stimulated by ET-1. Co-incubation with RWPs decreased the levels of this protein in ET-1-treated aortae. These results suggest that RWPs reduced \( O_2^{*−} \) production stimulated by ET-1 by down-regulating the expression of the p47\(_{phox}\) subunit of vascular NADPH oxidase. In recent studies in SHR (spontaneously hypertensive rats) [38], in DOCA-salt rats [20] and in AngII-infused rats [39], it has been demonstrated that the improvement of endothelial function by chronic oral administration of RWPs is associated with a reduction in the NADPH oxidase activity, which is abnormally high in these animals compared with normotensive rats. Our present results suggest that reduced NADPH oxidase-derived \( O_2^{*−} \) and, thus, reduced NO inactivation may be an important mechanism contributing to the prevention of ET-1-mediated endothelial dysfunction induced by chronic RWPs.

Uncoupled eNOS is also a source of \( O_2^{*−} \) production in aortic tissue. The calcium ionophore A23187, which induces calcium-dependent eNOS activation, increased \( O_2^{*−} \) production in aortic rings exposed to ET-1. As expected, this effect was reduced by eNOS inhibition with \( l \)-NAME. Previous studies [8,11] suggest that NADPH oxidase-derived \( O_2^{*−} \) generation is required for eNOS uncoupling. In fact, RWPs that inhibit NADPH oxidase, also inhibited A23187-induced \( O_2^{*−} \) generation, i.e. they prevented ET-1-induced eNOS uncoupling. Because ET-1-induced eNOS uncoupling was secondary to NADPH oxidase and prevented by sepiapterin [11], oxidation of BH\(_4\) is likely to be involved in eNOS uncoupling.

ER\(\alpha\) is a key target of RWPs extract action on the endothelium [27]. Activation of ER by 17\(β\)-estradiol inhibits NADPH oxidase activity through the regulation of p47\(_{phox}\) mRNA and protein expression [40]. However, in our experiments, the ER antagonist ICI 182780 was unable to reduce the protective effects of RWPs in endothelial dysfunction induced by ET-1, which suggests that ER\(\alpha\) are not involved in this beneficial effect. Some isolated RWPs have been suggested to show agonistic effects on PPAR\(\gamma\) [41]. Because the PPAR\(\gamma\) ligands reduce \( O_2^{*−} \) generation stimulated by AngII in human coronary artery endothelial cells [42], we hypothesized that RWPs might also prevent endothelial dysfunction via activation of these receptors. However, the PPAR\(\gamma\) antagonist GW9662 did not affect the effects induced by RWPs, suggesting that these protective effects are unrelated to PPAR\(\gamma\) activation.

A key signalling event evoked by ET-1 in vascular wall is the activation of the MAPK (mitogen-activated protein kinase) cascade [43–45]. Of the major MAPKs, ERK1/2, p38 MAPK and SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase) are the best characterized [46]. Recently, Romero et al. [12], using pharmacological inhibition of these major MAPKs, found that only ERK1/2 inhibition is able to prevent the increase in both \( O_2^{*−} \) production and p47\(_{phox}\) protein expression induced by ET-1, with p38 MAPK and JNK inhibition being without effect. Red wines inhibit ERK1/2 phosphorylation induced by platelet-derived growth factor in rat and human vascular smooth muscle cells. This inhibitory effect was related to the accumulation of flavonoids in wine [47]. In our experimental conditions, ET-1 stimulates ERK1/2 phosphorylation, and this effect was inhibited by RWPs, which suggests that this inhibitory effect might be responsible, at least in part, to the reduced NADPH-oxidase-driven \( O_2^{*−} \) production induced by these compounds in aortic rings stimulated by ET-1.

The major monomeric polyphenols in this RWP mixture are catechin, epicatechin and resveratrol. Epicatechin is, by far, the most abundant in this wine, being about 50- and 100-fold more concentrated than the other two, respectively. In order to test if these compounds, at the concentrations found in RWPs at \( 10^{-2} \) g/l, would be responsible for the protective effects of RWPs, we studied the endothelium-dependent relaxant response to ACh in ET-1-incubated rings. Only epicatechin restored the impaired relaxant response to ACh. Moreover, epicatechin also prevented all biochemical changes induced by ET-1 in the vascular
The overexpression of p47phox and the subsequent increase in NADPH oxidase activity, leading to improvement in endothelial function. The effects of RWPs appear to be independent of ERα and PPARγ activation and related to ERK1/2 inhibition. The epicatechin content of RWPs plays a key role in these protective effects.

**AUTHOR CONTRIBUTION**

Francisco Pérez-Vizcaíno, Juan Duarte and Rosario Jiménez designed the research; Rocío López-Sepúlveda, Manuel Gómez-Guzmán, María José Zarzuelo, Miguel Romero, Manuel Sánchez, Ana María Quintela and Pilar Galindo performed the research; Manuel Sánchez and Francisco O’Valle contributed new reagents/analytical tools; Manuel Sánchez, Francisco O’Valle, Juan Tamargo, Francisco Pérez-Vizcaíno, Juan Duarte and Rosario Jiménez analysed the data; Juan Tamargo, Francisco Pérez-Vizcaíno, Juan Duarte and Rosario Jiménez wrote the paper.

**FUNDING**

This work was supported by the Comisión Interministerial de Ciencia y Tecnología [grant number AGL2007–66108/ALI, SAF2007–62731, SAF2008–03948, SAF2010–22066–C02–01], the Junta de Andalucía, Proyecto de Excelencia [grant number P06-CTS-01555] and the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, Spain [grant number Red HERACLES RD06/0009]. R.L.-S., M.G.-G., M.J.Z., A.M.Q., P.G. and M.R. are supported by studentships from the Spanish Ministry of Science and Education.

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