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

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Short title: RWPs in ET-1-induced endothelial dysfunction

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Abstract

Red wine polyphenols (RWPs) exert antihypertensive effects and improve endothelial function by reducing the plasma levels of endothelin-1 (ET-1) and the subsequent vascular production of superoxide ($O_2^{\cdot-}$). Our present study was designed to evaluate if RWPs act directly in the vascular wall improving endothelial dysfunction and $O_2^{\cdot-}$ production induced by ET-1 and to analyze 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} g/L to 10^{-2} g/L) or catechin (0.2 μ M), epicatechin (10 μ M) and resveratrol (0.1 μ M). ET-1 reduced the relaxant responses to acetylcholine, increased intracellular $O_2^{\cdot-}$ production, NADPH oxidase activity, and protein expression of NADPH oxidase subunit p47^{phox}. All these changes were prevented by RWPs. The preventive effects of RWPs were unaffected by co-incubation with either ICI-182780, an estrogen receptor (ER) antagonist, or GW9662, a peroxisome proliferator-activated receptor γ (PPAR γ) antagonist. RWPs inhibited the phosphorylation of the mitogen-activated protein kinase, extracellular signal-regulated kinases (ERK1/2), a key regulator of p47^{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 $O_2^{\cdot-}$ production by reducing overexpression of p47^{phox} and the subsequent increased NADPH oxidase activity, leading to improvement of endothelial function. The effects of RWPs seem to be independent of ER and PPAR γ activation and related to ERK1/2 inhibition.

Introduction

Endothelin-1 (ET-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 deoxycorticosterone acetate (DOCA)-salt or 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 endothelial 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 ET_A/ET_B receptor activation, increases reactive oxygen species (ROS) production in the vasculature (e.g. superoxide anion, O₂⁻), resulting in endothelial dysfunction [6-8].

The major source of intracellular ROS in vascular cells is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multi-subunit enzymatic complex which comprises two membrane-bound subunits, Nox (Nox-1, Nox-2 or gp91^{phox}, Nox-4 or Nox-5) and p22^{phox} which are regulated by cytoplasmic subunits such as p47^{phox}, p67^{phox} and a low-molecular-weight G protein (rac 1 or rac 2) [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 uncoupling leading to further ROS generation which ultimately leads to endothelial dysfunction [8,10,11]. Moreover, we recently found that ET-1 induces NADPH oxidase-driven O₂⁻ generation through increase of p47^{phox} protein expression [11]. The signaling pathway for this effect involves sequential activation of PKC, c-Src and ERK1/2 [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 which, in addition to ethanol, that 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 coronary heart disease (CHD), which is reduced by 50% compared with other European countries and the USA, despite similar intakes of saturated fats [17,18]. However, the molecular basis of this phenomenon, commonly known as the 'French paradox', remains unknown.

Red wine polyphenols (RWPs) reduced cardiovascular risk factors such as hypertension and endothelial dysfunction. In fact, chronic administration of RWPs 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, RWPs prevent vascular oxidative stress and inhibit NADPH oxidase activity [20]. Similarly, in Zucker rats RWPs reduced $O_2^{\cdot-}$ release via decreased expression of NADPH oxidase membrane sub-unit Nox-1 [21]. However, it is not clear if these protective effects of RWPs are a result of decreased ET-1 synthesis [20] and/or interference with ET-1 signaling pathways to lead oxidative stress in the vascular wall. Furthermore, the main compounds of RWPs 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 tree main monomeric polyphenols (catechin, epicatechin and resveratrol) included in this RWPs extract.

Methods

All of the procedures conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication no. 85-23, revised 1996) and 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 (Barcelona, Spain) weighing 200-250 g. The descending thoracic aortae were dissected and cut into rings. Rings were incubated in Krebs solution (composition in mM: NaCl 118, KCl 4.75, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 2, KH₂PO₄ 1.2 and glucose 11) 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 (dimethylsulfoxide, DMSO 0.1%), RWPs (10⁻⁴, 10⁻³ and 10⁻² g/L), apocynin (10⁻⁴M), catechin (0.2 μM), epicatechin (10 μM), or resveratrol (0.1 μM). The concentration of RWPs used in the present study has been found to produce the maximal endothelium-dependent relaxation in rat vascular tissues [22] and was comparable to the concentration of polyphenols present in the plasma with a dose of 20 mg/kg of RWPs when used in vivo experiments [21]. Aortae were immediately used for

O_2^- production or contractile tension recording or frozen in liquid nitrogen and stored at -80°C for Western blotting or included in optimum cutting temperature (OCT) 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 UF1, Cibertec, Madrid) and connected to a computer via an A/D interface. Contractile tension was recorded by a PowerLab 800 (AD Instruments, Cibertec, Madrid), 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 ($1\ \mu\text{M}$ or $0.1\ \mu\text{M}$, 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 acetylcholine (ACh). In some experiments, endothelium-independent responses to sodium nitroprusside were also performed in the dark in rings pre-contracted with $1\ \mu\text{M}$ phenylephrine. To evaluate the participation of endothelial NO on endothelial dysfunction induced by ET-1, several aortic rings were incubated with or without eNOS inhibitor L-NAME ($100\ \mu\text{M}$) in organ chambers for 30 min before the addition of phenylephrine (10^{-9}M - 10^{-4}M).

In situ detection of vascular O_2^- production

Dihydroethidium (DHE) 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, previously incubated as mentioned above, were cryopreserved by incubation with phosphate buffered solution (0.1M) containing 30% sucrose for 1-2 h, included in OCT, frozen, and 10 μ m cross sections were obtained in a cryostat (Microm international Model HM500 OM). Sections were incubated in a humidified chamber for 30 min in Hepes buffered solution (in mM: NaCl 130, KCl 5, MgCl₂ 1.2, glucose 10, and HEPES 10, pH 7.3 with NaOH) at 37°C. Then, the sections were further incubated for 30 min in Hepes solution containing DHE (10 μ M) in the dark. Preparations were counterstained with the nuclear stain 4',6-diamidino,2-phenylindol dichlorohydrate (DAPI, 0.3 μ M) 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 μ M) before DHE. Four sections of each preparation were examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany) using a 40x objective with the following pairs of excitation/emission filters: 340-380 nm/ 425 nm long pass to visualize DAPI blue fluorescence, BP450-490 nm/ 515 nm long pass to visualize the green autofluorescence of elastin and 545-30 nm/ 610-75 nm long pass to visualize ethidium red fluorescence. Sections were photographed with a Leica DC300F color 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 1.32j, 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 minutes at 37 °C in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, MgSO₄ 1, Na₂HPO₄ 0.15, KH₂PO₄ 0.4, NaHCO₃ 1, CaCl₂ 1.2 and glucose 5.5. 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 a higher concentrations. NADPH oxidase activity were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 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 relative luminescence units (RLU)/min/mg 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 previously described [11].

Western blotting analysis

Aortic homogenates were run on a sodium dodecyl sulphate (SDS)-polyacrilamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (PVDF), incubated with primary polyclonal rabbit anti-p47phox (SantaCruz Biotechnology, Santa Cruz, California, USA), anti-ET_A receptor (SantaCruz Biotechnology, Santa Cruz, California, USA), anti-phospho-eNOS (Ser-1177) (Cell Signalling Technology, MA, USA), mouse anti-eNOS (Cell Signalling Technology, MA, USA) or phospho-ERK1/2 (Thr183 and Tyr185) (1:1000 dilution, Sigma-Aldrich) monoclonal antibody overnight and with the secondary peroxidase conjugated antibody. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK) 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 ET_A protein abundance/ α -actin ratio or phospho-ERK1/2/ERK1/2 and phospho-eNOS/eNOS were calculated and data is expressed as a percentage of the values in control aorta from the same gel.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

For Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis, total RNA was extracted from aorta by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). Initial denaturation was done at 95°C for 3 min and followed by 30 to 40 (30 for caveolin-1, p22phox and eNOS, and 40 for p47phox) 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 base pairs) sense, 5'-CCCAGCGACAGATTAGAAGC -3' and antisense, 5'-TGGATTGTCCTTTGAGTCAGG -3'; p22phox (220 base pairs) sense, 5'-GCGGTGTGGACAGAAGTACC-3' and antisense, 5'-CTTGGGTTTAGGCTCAATGG-3'. The sequences for primers for caveolin-1, and eNOS were as follows: caveolin-1 (304 base pairs) sense, 5'-TCTACAAGCCCAACAACAAGG-3' and antisense, 5'-AGGAAAGAGAGGATGGCAAAG-3'; eNOS (164 base pairs) sense 5'-CGAGATATCTTCAGTCCCAAGC-3' and antisense 5'-GTGGATTTGCTGCTCTCTAGG-3'. GAPDH 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 dry powder): 5.3 catechin, 222.0 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, except DAPI from Calbiochem. RWPs, apocynin, catechin, epicatechin and resveratrol were initially dissolved in DMSO and all other drugs in distilled water.

Statistical analysis

Results are expressed as means \pm standard error of mean and n reflects the number of animals. Statistically significant differences between groups were calculated by Students' t test for unpaired observations or for multiple comparisons by an analysis of variance (ANOVA) followed by a Newman Keuls test. $P < 0.05$ was considered statistically significant. Concentration-response curves were fitted to the logistic equation: $E = E_{\max} / (1 + 10^{-k * ([\text{drug}] - \text{pD}_2)})$, where E_{\max} is the maximal effect, k is a factor which represents the slope of the curve, and pD_2 is the drug concentration exhibiting 50% of the E_{\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 (data 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 NO synthase inhibition with L-NAME, in both control and ET-1 treated aortic rings (data not shown). The concentration-contractile response induced by phenylephrine in intact aortic rings was significantly reduced in aortae incubated with ET-1 as 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 analyzed in phenylephrine pre-contracted rings. Under these more physiological experimental conditions, ET-1 incubation also impaired the vasodilatation induced by ACh as compared with control rings (Maximal effects (%): 35.2 ± 4.6 , $n = 6$; 69.2 ± 5.1 , $n = 6$, respectively, $P < 0.01$).

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 $O_2^{\cdot -}$ production

In order to characterize $O_2^{\cdot-}$ production and localize it within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE which is converted into ethidium by $O_2^{\cdot-}$ -induced oxidation. The nuclear staining was almost suppressed by the $O_2^{\cdot-}$ intracellular scavenger tiron. Positive red nuclei could be observed in adventitial, medial and endothelial cells (Figure 3A). Red fluorescence was quantified and the data was normalized by the blue fluorescence of the nuclear stain DAPI (Figure 3B). At 4 h, ET-1 induced an increase in $O_2^{\cdot-}$ production which was evident in all layers of the vessel. RWPs (10^{-3} g/L) prevented ET-1-induced increase in DHE staining (Figure 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 previous incubation for 30 min with the flavoprotein inhibitor DPI ($10\ \mu\text{M}$) (not shown). NADPH oxidase activity was increased in aortic rings incubated with ET-1 as compared to control rings. Apocynin ($100\ \mu\text{M}$), a specific intracellular inhibitor of NADPH oxidase which prevents the translocation of $p47^{\text{phox}}$ and $p67^{\text{phox}}$ 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 raise in NADPH oxidase activity induced by ET-1 (Figure 4A).

ET-1 also increased aortic $O_2^{\cdot-}$ generation, measured by lucigenin-enhanced chemiluminescence, in rings stimulated with the calcium ionophore A23187, as a result of uncoupled eNOS activation. This increased $O_2^{\cdot-}$ production was suppressed by L-

NAME (100 μ M), endothelial denudation (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, as compared to control rings (Figure 5A). In aortic segments, incubation with ET-1 for 4 h increased both gene and protein expression of p47^{phox} (Figure 5C, 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 estrogen receptor, peroxisome proliferator activated receptor and extracellular signal-regulated kinases in the effects of RWPs

The protective effects of RWPs in endothelial function have been attributed to estrogen receptor (ER)- α activation [26]. To test the involvement of ER- α on 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 μ M) [27]. This agent did not modify the effect of RWPs (10^{-2} g/L) in ACh-induced relaxation (Figure 7A).

In order to determine whether PPAR γ activation is involved in the protective effects of RWPs in endothelial dysfunction induced by ET-1, aortic rings were coincubated with the PPAR γ antagonist GW9662 (1 μ M) [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 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).

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 μ M prevented the increased O_2^- production (Figure 8B), NADPH oxidase activity (Figure 8C), p47^{phox} expression (Figure 8D) and ERK1/2 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 $O_2^{\cdot-}$ 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 α and PPAR γ 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 1) the DOCA-salt hypertensive rats, a low renin-angiotensin model of hypertension [7], 2) streptozotocin-induced diabetic rats [30], 3) ET-1-infused rats [31], and 4) 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 evidenced by the reduced relaxant response after eNOS activation induced by ACh, and by the increased vasoconstrictor response to phenylephrine. Chronic RWPs reduces blood pressure and improves endothelial function in DOCA-salt rats [20]. These protective effects have been related to its 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 $O_2^{\cdot-}$ generating systems, such as NADPH oxidase subunits. Moreover, the increased p47^{phox} protein expression induced by ET-1 was inhibited by the ET_A-receptor antagonist BQ123, but not by the ET_B-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 ET_A receptor 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 RWPs-induced increase of endothelial-derived NO responses, such as changes in the activity and/or expression of eNOS, changes in the vascular levels of $O_2^{\cdot-}$ and, thus, $O_2^{\cdot-}$ -driven NO inactivation and, changes in the sensitivity to 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 to 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 heat shock protein 90 (hsp90) and caveolin or suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin (BH4). 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 presence of L-NAME, was reduced by ET-1 and restored by RWPs. These data are consistent to the improvement of eNOS uncoupling induced by RWPs.

Excess of O_2^- generation is critically involved in the breakdown of NO associated to 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 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 polyethylene-glycol SOD, a cell-permeant O_2^- scavenger, completely prevented the impairment in endothelium dependent relaxation 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 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 coincubation with apocynin. Coincubation of RWPs with ET-1 also prevented both the increased vascular O_2^- production and the increased NADPH oxidase activity. These experiments were carried 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 by 4 hours 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 O₂⁻ 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 O₂⁻ production stimulated by ET-1 by downregulating the expression of the p47^{phox} subunit of vascular NADPH oxidase. In recent studies in SHR [38], in DOCA-salt [20], and Angiotensin II-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 as compared to normotensive rats. Our present results suggest that reduced NADPH oxidase derived O₂⁻ 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 O₂⁻ production in aortic tissue. The calcium ionophore A23187, which induces calcium-dependent eNOS activation, increased O₂⁻ 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 O₂⁻ generation is required for eNOS uncoupling. In fact RWPs, that inhibit NADPH oxidase, also inhibited A23187-induced O₂⁻ 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 BH₄ is likely to be involved in eNOS uncoupling.

ER α 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 α are not involved in this beneficial effect. Some isolated RWPs have been suggested to show agonistic effects on PPAR γ [41]. Because the PPAR γ ligands reduce O $_2^{\cdot-}$ 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 γ antagonist GW9662 did not affect the effects induced by RWPs, suggesting that these protective effects are unrelated to PPAR γ activation.

A key signalling event evoked by ET-1 in vascular wall is the activation of the mitogen-activated protein kinases (MAPKs) cascade [43-45]. Of the major MAPKs, extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK) are the best characterized [46]. Recently, Romero et al., [12], using pharmacological inhibition of these major MAPKs found that only ERK1/2 inhibition are able to prevent the increased of both O $_2^{\cdot-}$ production and p47^{phox} protein expression induced by ET-1, being without effect p38MAPK and JNK inhibition. 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 $_2^{\cdot-}$ production induced by these compounds in aortic rings stimulated by ET-1.

The major monomeric polyphenols in this RWPs 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 10^{-2} g/L RWPs, would be responsible of 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 wall. These results suggest that epicatechin content of RWPs mixture would be the main polyphenol involved in the improvement of endothelial dysfunction induced by RWPs. Moreover, the data highlight that RWPs components such as resveratrol may exert interesting protective effects but its concentration in wine is too low as to be effective.

Taken together these results indicate that RWPs prevent ET-1-induced vascular O_2^- production by reducing overexpression of p47^{phox} and the subsequent increased NADPH oxidase activity, leading to improvement of endothelial function. The effects of RWPs seem to be independent of ER α and PPAR γ activation and related to ERK1/2 inhibition. The epicatechin content of RWPs plays a key role in these protective effects.

Author contribution

R.L.-S. and M.G.-G. are equally contributed as first authors. F.P.-V., J.D. and R.J. designed research; R.L.-S., M. G.-G., M.J.Z., M.R., M.S., A.M.Q. and P.G. performed research; M.S. and F.O. contributed new reagents/analytic tools; M.S., F.O., J.T. F.P.-V., J.D. and R.J. analyzed data; and J.T. F.P.-V., J.D. and R.J. wrote the paper.

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Figure legends

Figure 1. *Effects of RWPs on ET-1-induced endothelial dysfunction.* Endothelium-intact aortic rings were incubated for 4 h with vehicle (dimethylsulfoxide, 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 acetylcholine (Ach, 0.001-10 μ M) was carried out in a cumulative fashion. Concentration-response curves to phenylephrine obtained in aortic rings in the absence (C) or in the presence (D) of L-NAME (100 μ M), added to the organ chamber 30 min before the addition of phenylephrine. Results are means \pm SEM, n = 5-9, * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, vs control rings, # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, vs ET-1-treated rings.

Figure 2. *Effects of RWPs on the endothelium-independent relaxant response in aortic ET-1-treated rings.* Endothelium-independent vasodilator response induced by sodium nitroprusside (SNP) in endothelium-denuded aortae precontracted by 10^{-6} M phenylephrine from rings incubated with vehicle (DMSO 0.1%) or RWPs (10^{-2} g/L) in the presence or absence of ET-1 (10 nM). Results are means \pm SEM, n = 5-6.

Figure 3. *Effects of RWPs on ET-1-induced vascular O_2^- production measured by dihydroethidium (DHE) fluorescence.* A) Upper pictures show 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 \pm SEM of 4 sections obtained from each ring ($n = 4$). ****P** < 0.01, *vs* control rings, **#P** < 0.05, *vs* 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 (DMSO, 0.1%) or RWPs (10^{-4} , 10^{-3} and 10^{-2} g/L) or apocynin (100 μ M) in the presence or in the absence of ET-1 (10 nM). (A) NADPH oxidase activity stimulated by NADPH (100 μ M) and (B) O_2^- production stimulated by the calcium ionophore A23187 (10 μ M). Results are means \pm SEM, $n = 5-12$, * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, *vs* control rings, # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, *vs* ET-1-treated rings.

Figure 5. *Effects of RWPs on expression of ET_A receptor, p22^{phox}, and p47^{phox}.* Gene expression of p22^{phox} (B) and p47^{phox} (C) was measured by RT-PCR and protein expression of ET_A (A) and p47^{phox} (D) by Western blot in aortic rings incubated for 4 h either with vehicle (DMSO, 0.1%) or RWPs (10^{-3} g/L) in the presence or in the absence of ET-1 (10 nM). Results are means \pm S.E.M. ($n = 4-6$) of densitometric values normalized to the corresponding RT-PCR products of GAPDH (B, C) or to the corresponding α -actin (A, D) and expressed as a percentage of control. * and ** $P < 0.05$ and $P < 0.01$, respectively, *vs* control rings, **#P** < 0.05, *vs* ET-1-treated rings.

Figure 5. *Effects of RWPs on expression of eNOS, caveolin, and eNOS phosphorylation.* Aortic eNOS (A), and caveolin-1 (B) gene expression measured by RT-PCR and protein expression of Ser-1177-phospho-eNOS (C) by Western blot in aortic rings incubated for 4 h either with vehicle (DMSO, 0.1%) or RWPs (10^{-3} g/L) in

the presence or in the absence of ET-1 (10 nM). Results are means \pm S.E.M. ($n = 4-6$) of densitometric values normalized to the corresponding RT-PCR products of GAPDH (A, B) or to the corresponding eNOS (C) and expressed as a percentage of control. * and ** $P < 0.05$ and $P < 0.01$, respectively, vs control rings, # $P < 0.05$, vs ET-1-treated rings.

Figure 7. *Role of estrogen receptor, peroxisome proliferator activated receptor and extracellular signal-regulated kinase.* Effects of RWPs (10^{-2} g/L) coincubated with (A) a non selective ER antagonist, ICI-182780 (1 μ M), or (B) a PPAR γ antagonist, GW9662 (1 μ M), in presence or absence of ET-1 (10 nM). (C) Effect of RWPs (10^{-3} g/L) on vascular protein expression of phosphorylation of ERK1/2 in ET-1-treated rings. Results are quantitative evaluation of phospho-ERK1/2/ERK1/2 protein levels expressed as a percentage of control. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, vs control rings, # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, vs ET-1-treated rings.

Figure 8. *Epicatechin displays similar protective effects to RWPs.* (A) Endothelium-intact aortic rings were incubated for 4 h with vehicle (dimethylsulfoxide, DMSO 0.1%) or ET-1(10 nM) or ET-1(10 nM) plus catechin (0.2 μ M), or epicatechin (10 μ M), or resveratrol (0.1 μ M), then stimulated with phenylephrine and a concentration-response curve to acetylcholine (Ach, 0.01-10 μ M) was carried out in a cumulative fashion. Effects of epicatechin (epi, 10 μ M) in the increase of O_2^- production measured by dihydroethidium fluorescence (B), NADPH oxidase activity measured by lucigenin-enhanced chemiluminiscence (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 \pm SEM, n = 5-7, * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, vs control rings, # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, vs ET-1-treated rings.

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Table 1. Contractile responses to phenylephrine and pD₂ and E_{max} values for Ach-induced relaxation in aortic rings incubated with ET-1 (10 nM) for 4 h in the presence of vehicle (DMSO 0.1%, control) or RWPs (10⁻⁴, 10⁻³ and 10⁻² g/L) or apocynin (10⁻⁴M) calculated from data in Figure 1.

Treatment	n	Phenylephrine contraction (g)	Ach relaxation	
			pD ₂	E _{max} (%)
Control	5	2.40 ± 0.31	6.99 ± 0.11	57.0 ± 7.6
ET-1	9	2.39 ± 0.33	7.16 ± 0.45	23.4 ± 6.5**
RWPs (10 ⁻² g/L)	6	2.50 ± 0.14	6.98 ± 0.12	49.4 ± 3.0
ET-1+ RWPs (10 ⁻⁴ g/L)	6	2.38 ± 0.12	7.03 ± 0.27	40.5 ± 4.1#
ET-1+ RWPs (10 ⁻³ g/L)	5	2.10 ± 0.32	6.98 ± 0.13	42.1 ± 4.3#
ET-1+ RWPs (10 ⁻² g/L)	9	2.32 ± 0.22	6.97 ± 0.05	57.2 ± 5.3##
Apocynin	5	2.16 ± 0.32	6.85 ± 0.13	60.9 ± 9.9
ET-1+ apocynin	6	2.14 ± 0.28	7.08 ± 0.13	57.2 ± 11.8#

Values are means ± SEM. ** indicate P < 0.01, vs control rings; # and ## indicate P < 0.05 and P < 0.01, respectively, vs ET-1-treated rings.

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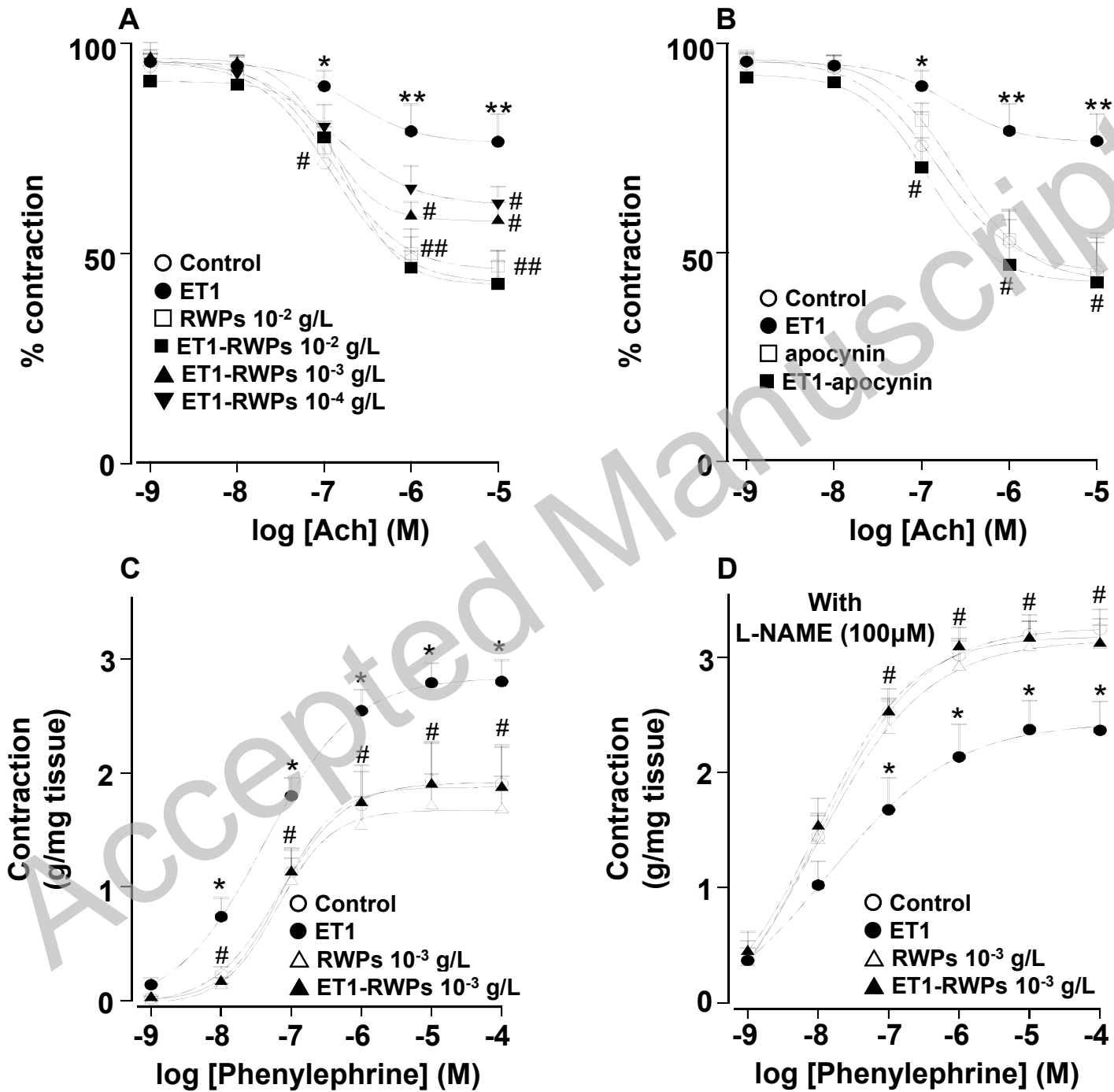


Figure 1

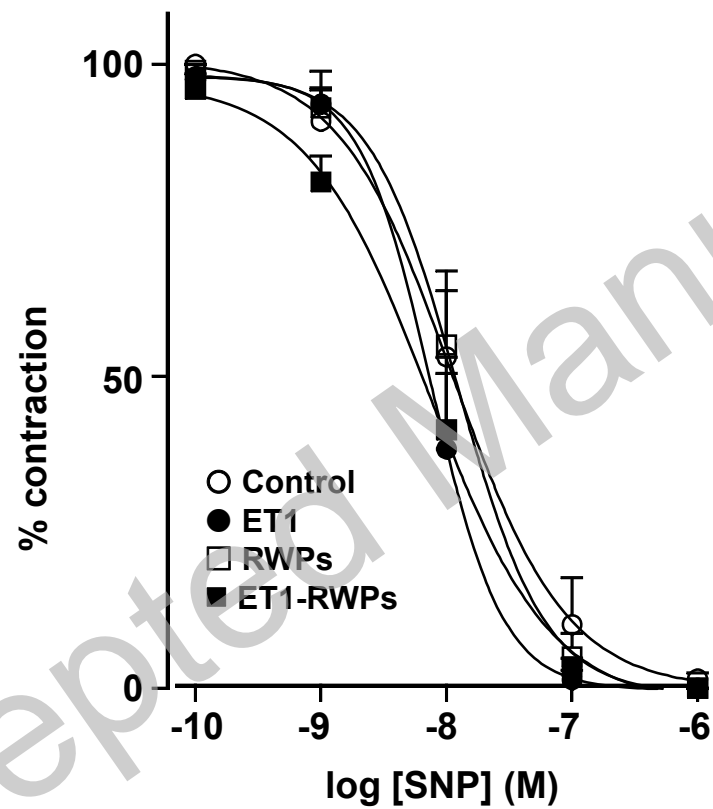


Figure 2

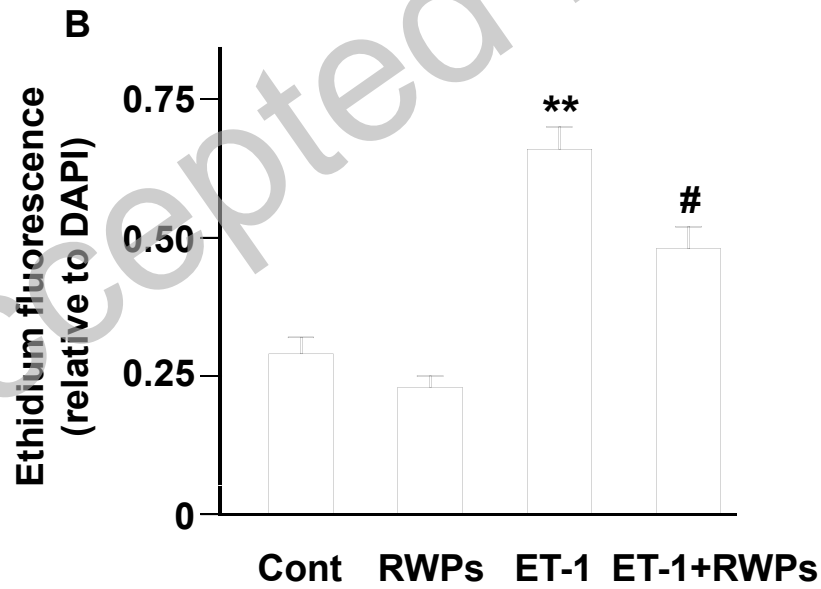
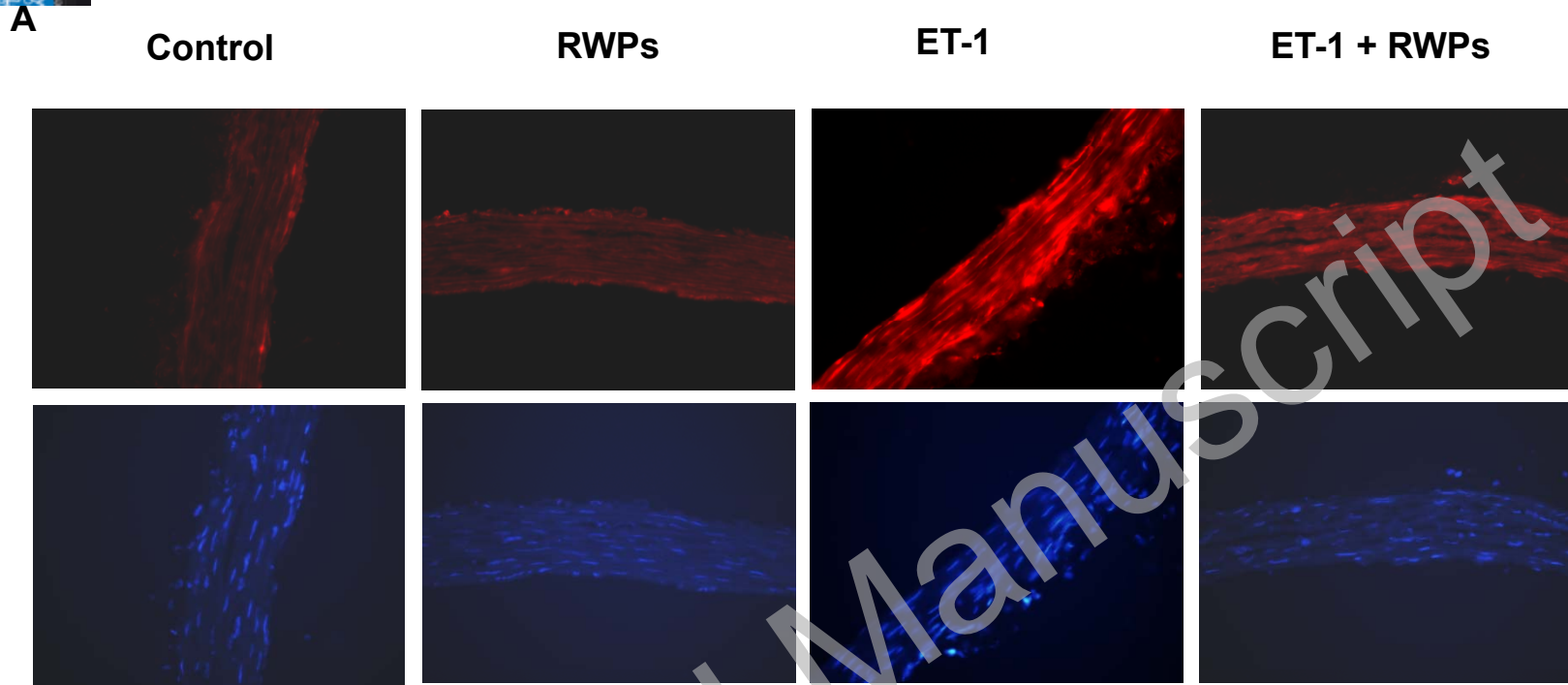


Figure 3

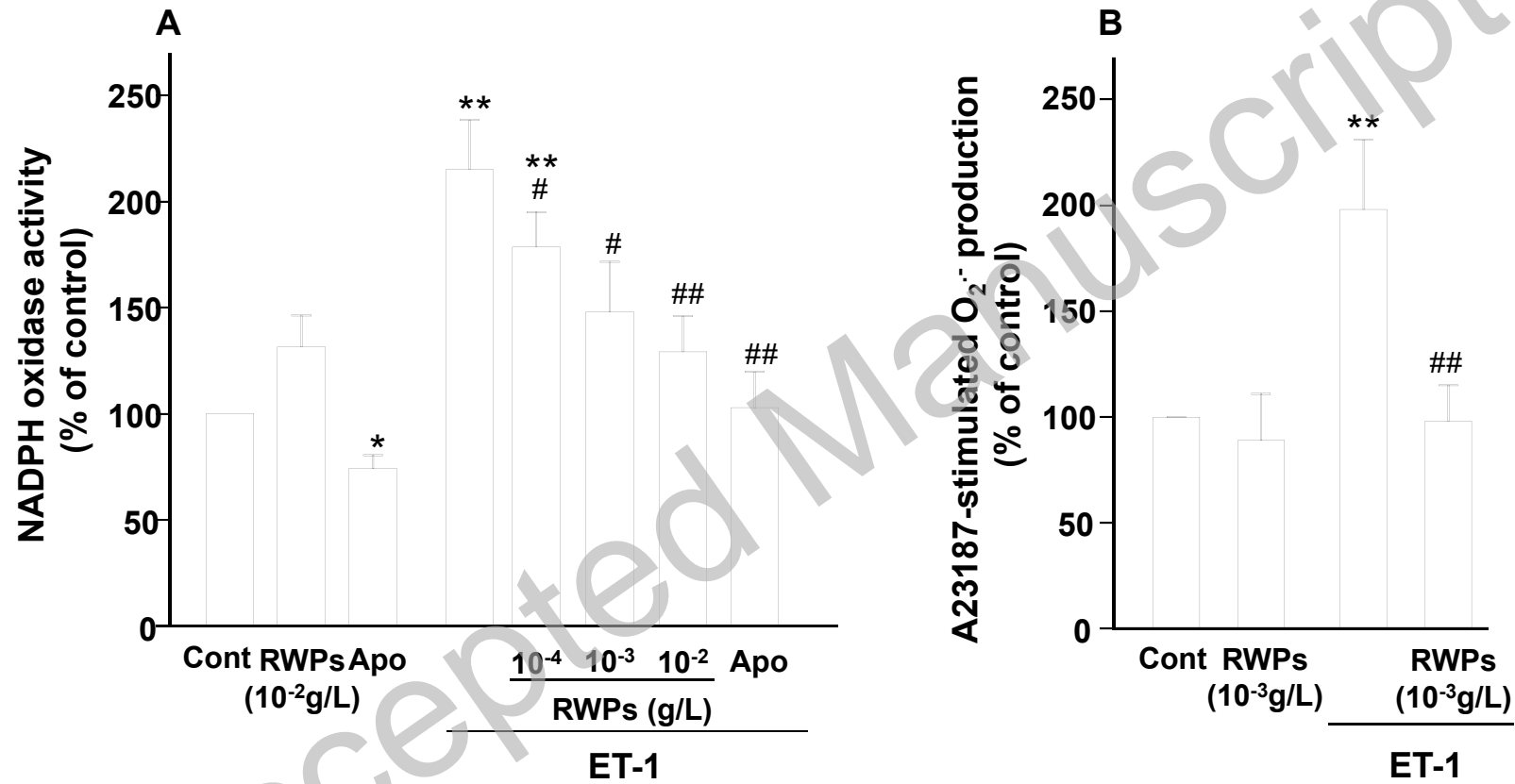


Figure 4

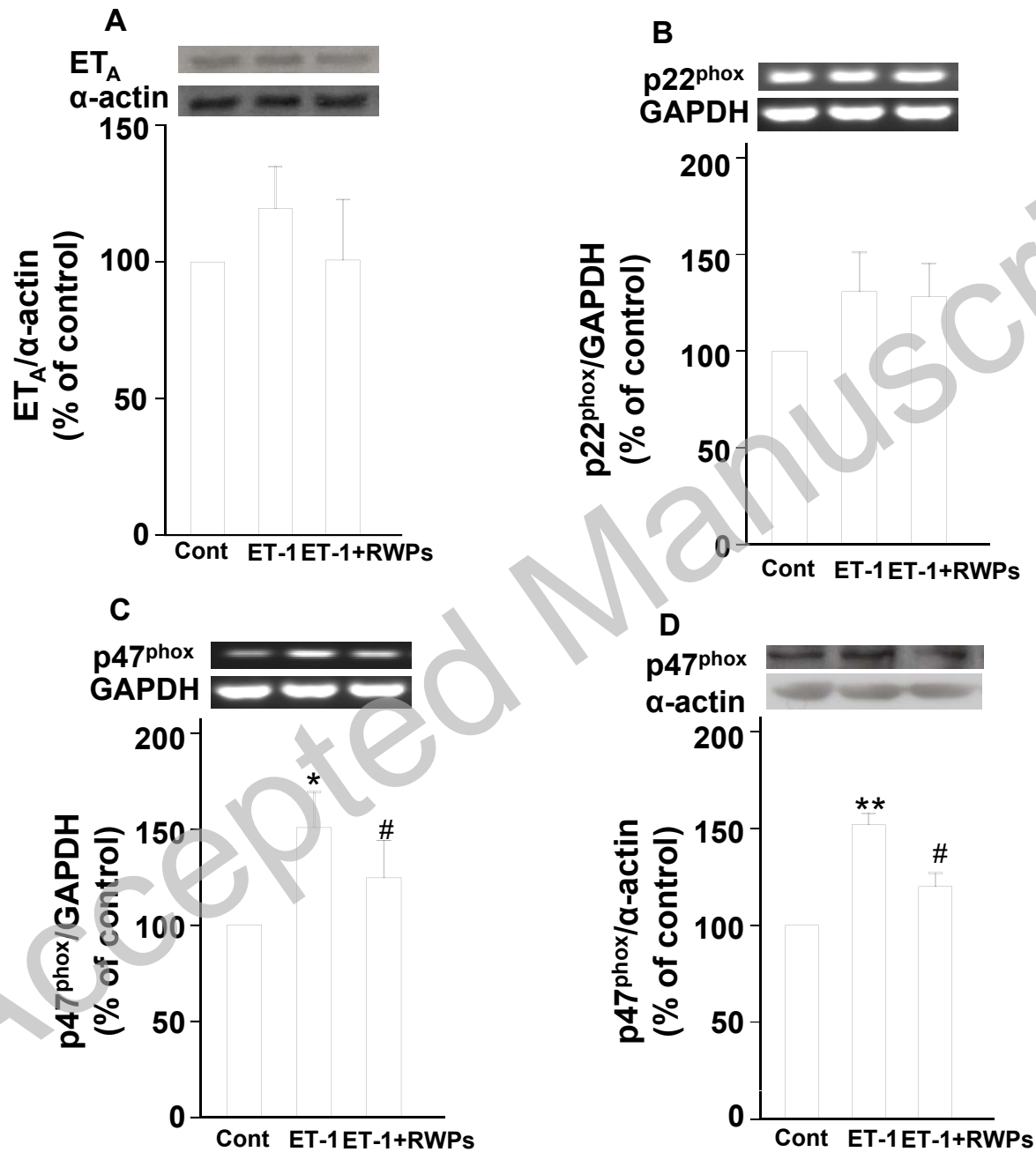


Figure 5

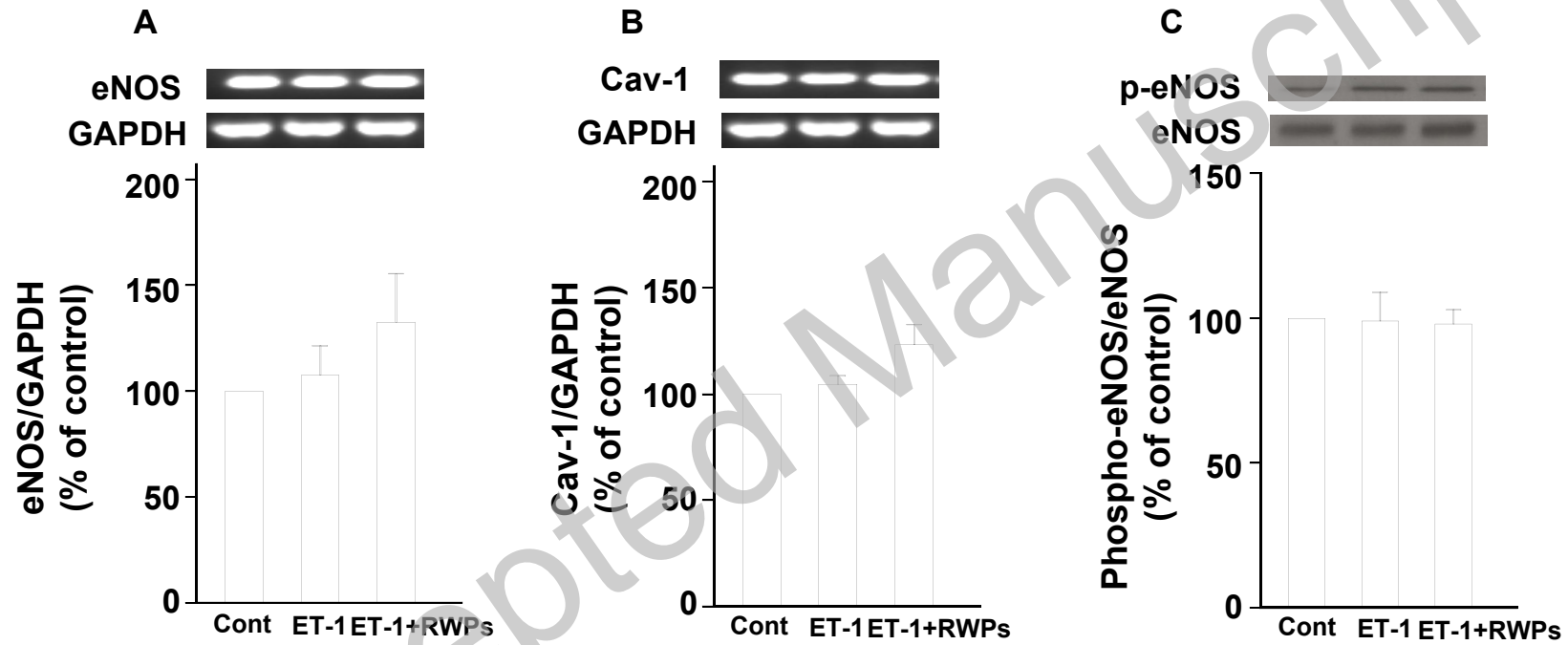


Figure 6

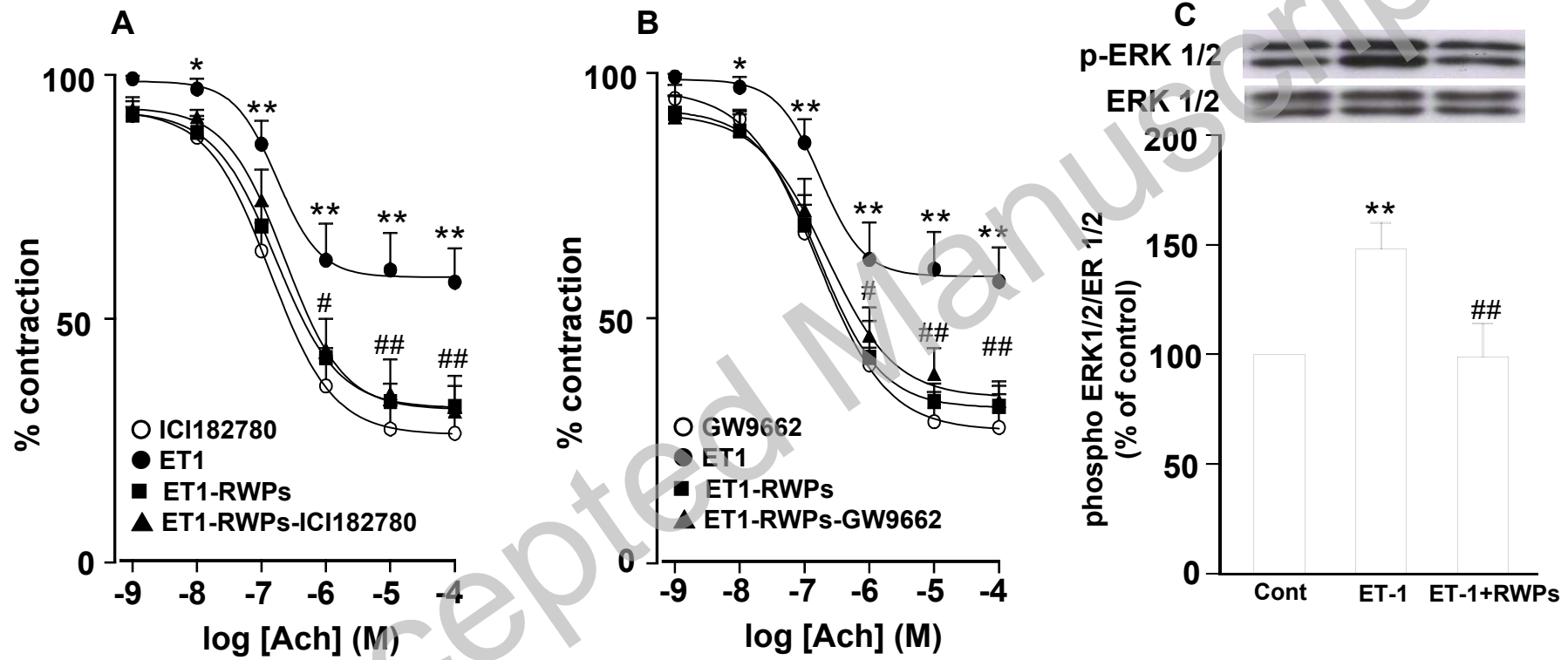


Figure 7

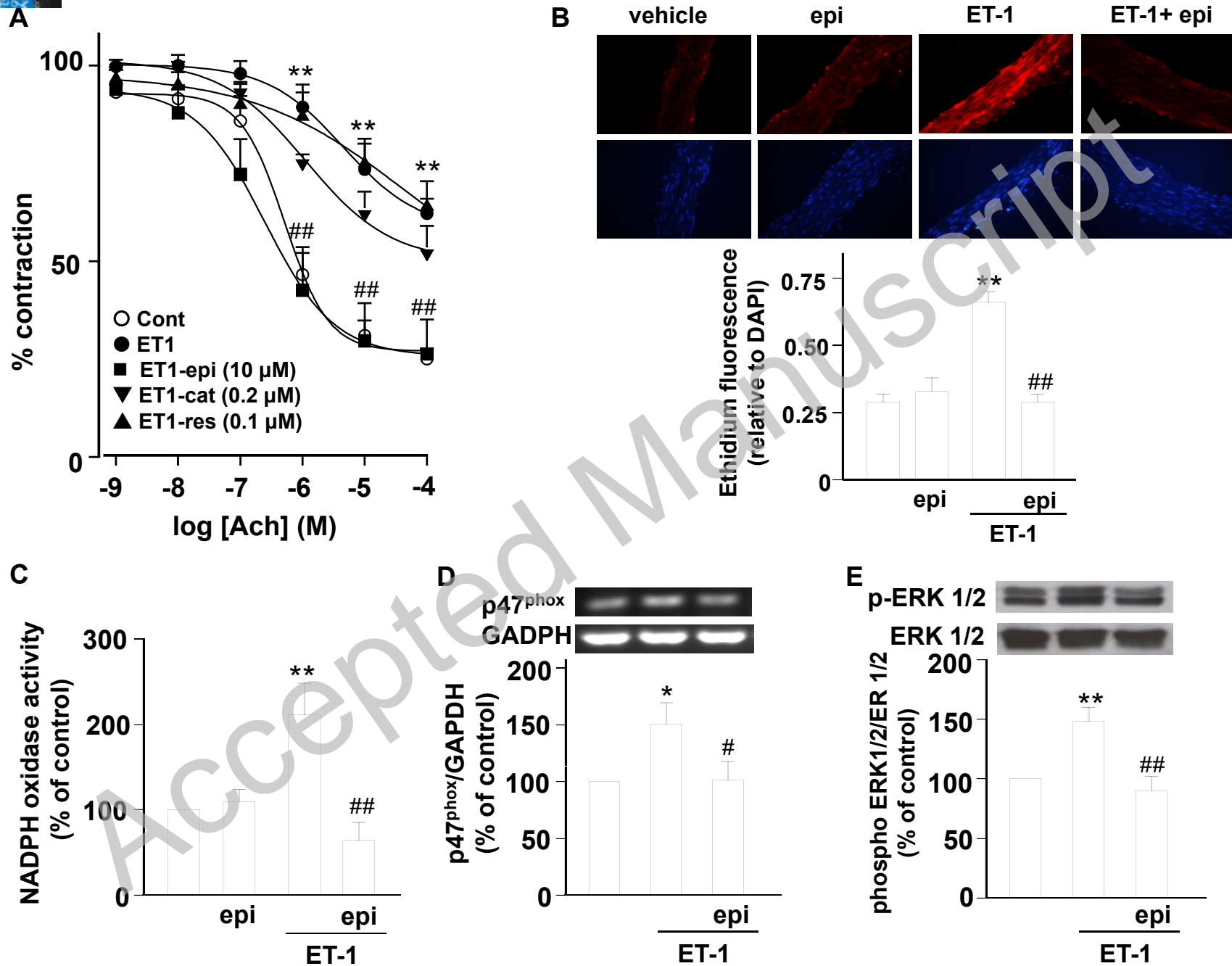


Figure 8