# Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: Role of NADPH oxidase

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## Abstract

**Objective:** Chronic administration of moderate amounts of red wine has been associated with a protective effect on the cardiovascular system. This study examined whether red wine polyphenols prevent the angiotensin II (Ang II)-induced hypertension and endothelial dysfunction in rats, and, if so, to elucidate the underlying mechanism.
**Methods:** Hypertensive rats were obtained by a 14-day infusion of Ang II. Red wine polyphenols were administered in the drinking water one week before and during the Ang II infusion. Arterial pressure was measured in conscious rats. *Ex vivo* vascular relaxation was assessed in organ chambers, vascular superoxide anion production by dihydroethidine and vascular NADPH oxidase expression by immunohistochemistry.
**Results:** Ang II-induced hypertension was associated with decreased relaxation to acetylcholine but not to red wine polyphenols. The Ang II treatment also increased vascular superoxide anion production and expression of nox1 and p22phox NADPH oxidase subunits. Intake of red wine polyphenols prevented the Ang II-induced hypertension and endothelial dysfunction and normalized vascular superoxide anion production and NADPH oxidase subunit expression. Red wine polyphenol treatment alone did not affect blood pressure.
**Conclusion:** Intake of red wine polyphenols prevents Ang II-induced hypertension and endothelial dysfunction. Prevention of vascular NADPH oxidase induction and preservation of arterial nitric oxide availability during Ang II administration likely contribute to this effect.
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*Keywords:* Angiotensin II; Endothelial function; Hypertension; NADPH oxidase; Redox signaling

## 1. Introduction

Epidemiological studies have indicated that regular intake of moderate amounts of wine, in particular red wine, is associated with a reduced risk in overall mortality [1,2]. This effect has been attributable to a reduction in death from cardiovascular diseases and cancer [1,2].

Although the alcohol component may contribute to the protective effect by increasing the concentration of high density lipoproteins, and by decreasing the fibrinogen level and the reactivity of platelets [3–5], several recent studies suggest also a key role of the polyphenolic component (for review see: [6,7]). Indeed, intake of red wine polyphenols reduced the development of atherosclerosis in several experimental models of atherosclerosis but was without effect on mature atherosclerosis [8–10]. Red wine polyphenols and a grape skin extract also reduced blood pressure in $N^G$-nitro-L-arginine methyl ester (L-NAME)- and desoxycorticosterone acetate (DOCA) salt-induced hypertensive rats [11,12].

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Cardiovascular diseases such as atherosclerosis and hypertension are characterized by an impaired endothelium-dependent relaxation in both animals and humans [13]. Such an endothelial dysfunction is observed early in the development of the pathology and is due, at least in part, to an excessive vascular formation of reactive oxygen species in particular superoxide anions, which reduce the bioavailability of nitric oxide (NO) [14–17]. Although red wine polyphenols have antihypertensive properties, the possibility that they prevent the oxidative stress-induced endothelial dysfunction remains to be determined. Amongst experimental models of hypertension, that induced by angiotensin II (Ang II) has been well characterized and is of potential clinical importance. Indeed, a variety of pathologic states, including certain forms of hypertension, congestive heart failure, and nitrate tolerance, are associated with elevated plasma renin activity and circulating levels of Ang II [18,19]. In transgenic hypertensive rats overexpressing the Ren2 gene and in Ang II-induced hypertensive rats, a marked upregulation of the vascular expression of NADPH oxidase is observed and this effect is associated with an excessive formation of superoxide anions, which, in turn, decreases the bioavailability of endothelium-derived NO [15,20]. Therefore, the aim of the present study was to examine whether chronic intake of red wine polyphenols prevents the Ang II-induced hypertension and endothelial dysfunction, and, if so, to determine the underlying mechanism.

## 2. Methods

The study conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by the local ethics committee.

### 2.1. Preparation of red wine polyphenolic extract

Red wine phenolic extract dry powder was obtained from French red wine (Corbières A.O.C.) and provided by Dr. M. Moutounet (Institut National de la Recherche Agronomique, Montpellier, France) and analyzed by Dr. P.-L. Teissedre (Département d'Oenologie, Bordeaux, France). The extract was prepared as previously described [21]: briefly, phenolic compounds were adsorbed on a preparative column, then alcohol desorbed; the alcoholic eluent was gently evaporated; the concentrated residue was lyophilized and finely sprayed to obtain the phenolic extract dry powder. One liter of red wine produced 2.9 g of phenolic extract, which contained 471 mg/g of total phenolic compounds expressed as gallic acid. Phenolic levels in phenolic extract were measured by HPLC. The extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g, B2: 8.0 mg/g, B3: 20.7 mg/g and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g, peonidin-3-glucoside: 0.66 mg/g, and cyanidin-3-glucoside: 0.06 mg/g)

and phenolic acids (gallic acid: 5.0 mg/g, caffeic acid: 2.5 mg/g, and caftaric acid: 12.5 mg/g).

### 2.2. In vivo treatment of rats

Male Wistar rats (12 to 15 weeks-old) were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). A 1-cm incision was made in the midscapular region, and an osmotic minipump (Alzet model 2004) was implanted. Pumps contained Ang II, which was dissolved in 0.15 M NaCl containing 0.01 N acetic acid, and the infusion rate was 0.4 mg/kg/day. Sham-operated rats underwent an identical surgical procedure without pump implantation. After 14 days of
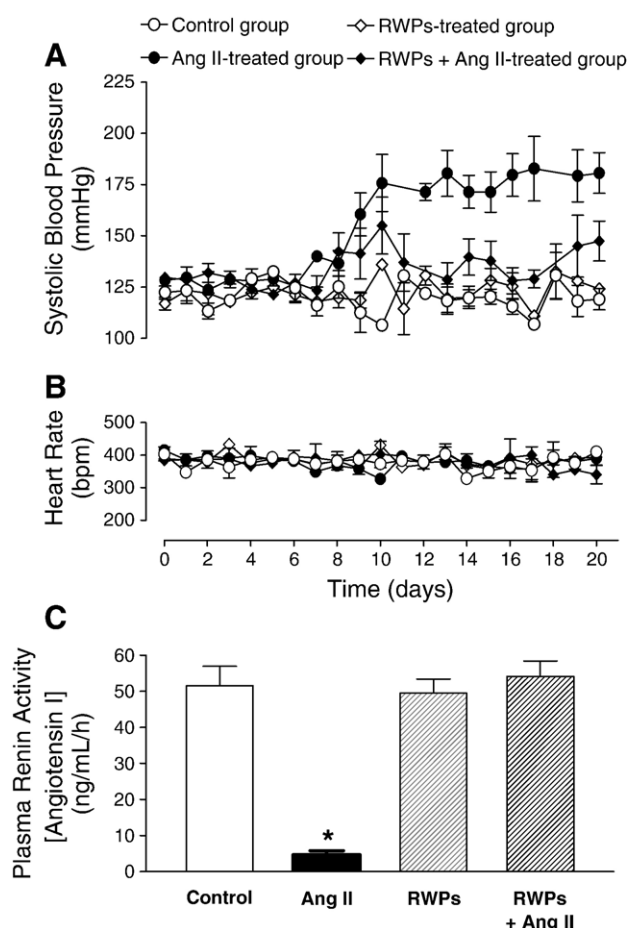


Fig. 1. Red wine polyphenols prevent the Ang II-induced hypertension. The control group of rats received the solvent (10% ethanol, pH 3.3) for 21 days in the drinking water; the red wine polyphenols (RWPs) group received 150 mg/kg/day RWPs in the drinking water for 21 days; the Ang II group received the solvent in the drinking water and the sub-cutaneous infusion of Ang II (0.4 mg/kg/day) using osmotic minipumps from day 7 through day 21; the RWPs plus Ang II group received RWPs in the drinking water for 21 days and the subcutaneous infusion of Ang II from day 7 through day 21. Changes in systolic blood pressure are shown in (A), those in heart rat in (B) and those of the plasma renin activity determined at day 21 in (C). Results are shown as mean±S.E.M. of 9 (Control), 10 (Ang II), 9 (RWPs+Ang II) and 8 (RWPs) different rats. * indicates a significant inhibitory effect versus control.
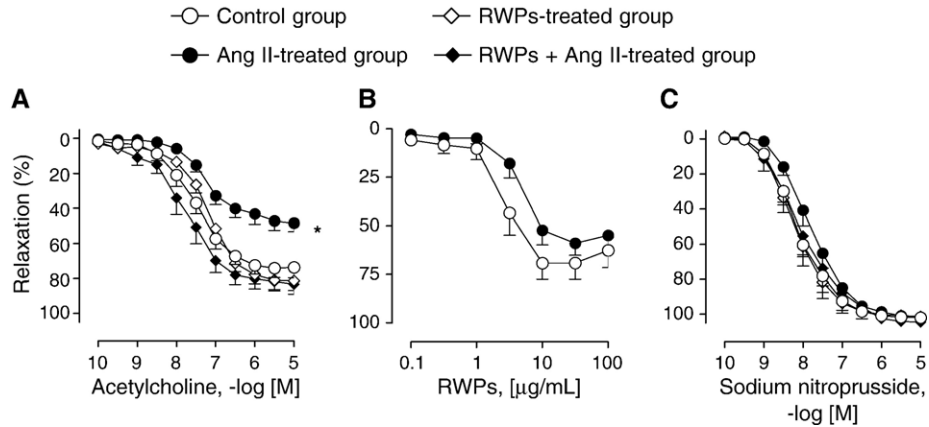
Fig. 2. Red wine polyphenols prevent the Ang II-induced endothelial dysfunction. Concentration–relaxation curves to acetylcholine (A) and red wine polyphenols (B) in aortic rings with endothelium from the indicated groups of rats. (C) Relaxations to sodium nitroprusside in aortic rings without endothelium are also shown. Results are shown as mean±S.E.M. of (A, C) 9 (Control), 8 (RWPs), 7 (Ang II), 7 (RWPs plus Ang II) and (B) 9 (Control), 9 (Ang II) different rats. * indicates a significant inhibitory effect versus control group.

Ang II infusion, rats were anesthetized with pentobarbital (60 mg/kg i.p.) and thereafter blood was taken from the abdominal aorta into EDTA-containing tubes and their aortas were harvested. Blood was immediately centrifuged at 4 C for 10 min at 2000×g. Plasma samples were frozen in liquid nitrogen and stored at −80 °C until assayed. Red wine polyphenolic extract (150 mg/kg/day, 10% ethanol, pH 3.3) or solvent (10% ethanol, pH 3.3) was given in the drinking water beginning 7 days before Ang II infusion.
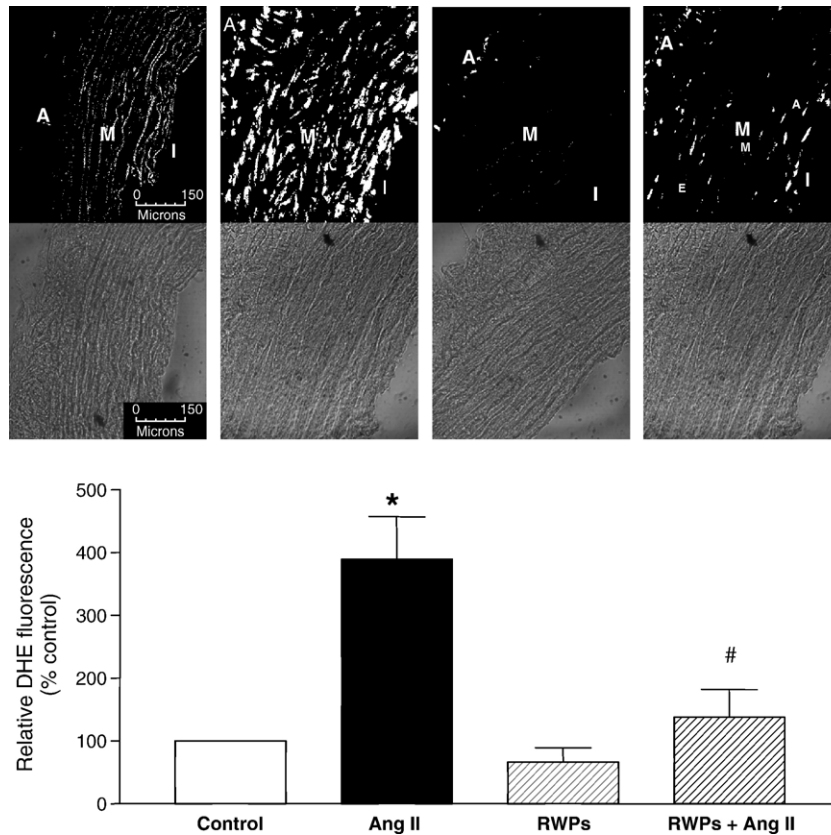


Fig. 3. Red wine polyphenols prevent the Ang II-induced vascular formation of reactive oxygen species. Aortic sections were exposed to the redox-sensitive fluorescent dye dihydroethidine (DHE) for 30 min at 37 °C. Thereafter, ethidium fluorescence was determined by confocal microscopy. Upper panel represents ethidium bromide staining and corresponding phase contrast; lower panel represents corresponding cumulative data. Results are shown as mean±S.E.M. of 3 different rats. * indicates a significant stimulatory effect and # a significant inhibitory effect. A: adventitia; M: media; I: intima.

## 2.3. Blood pressure measurement

Systolic blood pressure and heart rate were measured in conscious rats by tail-cuff plethysmography connected to a computerized system (LE 5002®, BIOSEB). Before the administration of red wine polyphenols, the rats were trained in the blood pressure device to accustom them to the procedure for 1 week before the minipump implantation. On each day of blood pressure determination, 10 measurements were obtained and averaged for each rat.

## 2.4. Vascular reactivity studies

Aortas were cleaned of connective tissue and cut into rings (3–4 mm in length). As indicated the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% $O_2$; 5% $CO_2$) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37 °C) for the determination of changes in isometric tension. Following equilibration for 90 min under a resting tension of 2 g, rings were contracted with phenylephrine (1 μM) to about 80% of the maximal contraction reached by increasing concentrations of phenylephrine. After washout and a 30-min equilibration period, rings were contracted again with phenylephrine (1 μM) and the relaxation to acetylcholine (1 μM) was determined. After washout and a 30-minute equilibration period, rings were again contracted with phenylephrine (1 μM) before a concentration–relaxation curve to either red wine polyphenols, acetylcholine or sodium nitroprusside was constructed.

## 2.5. Determination of plasma renin activity

Plasma renin activity was measured by determining the level of Ang I generated during a 30-min incubation of plasma at 37 °C in the presence of 8-hydroxyquinoline (5 mM). Ang I was measured by radioimmunoassay as previously described [22].

## 2.6. Determination of vascular reactive oxygen species formation

The oxidative fluorescent dye dihydroethidine was used to evaluate *in situ* formation of reactive oxygen species by use of a method described by Miller et al. [16]. Aortic rings
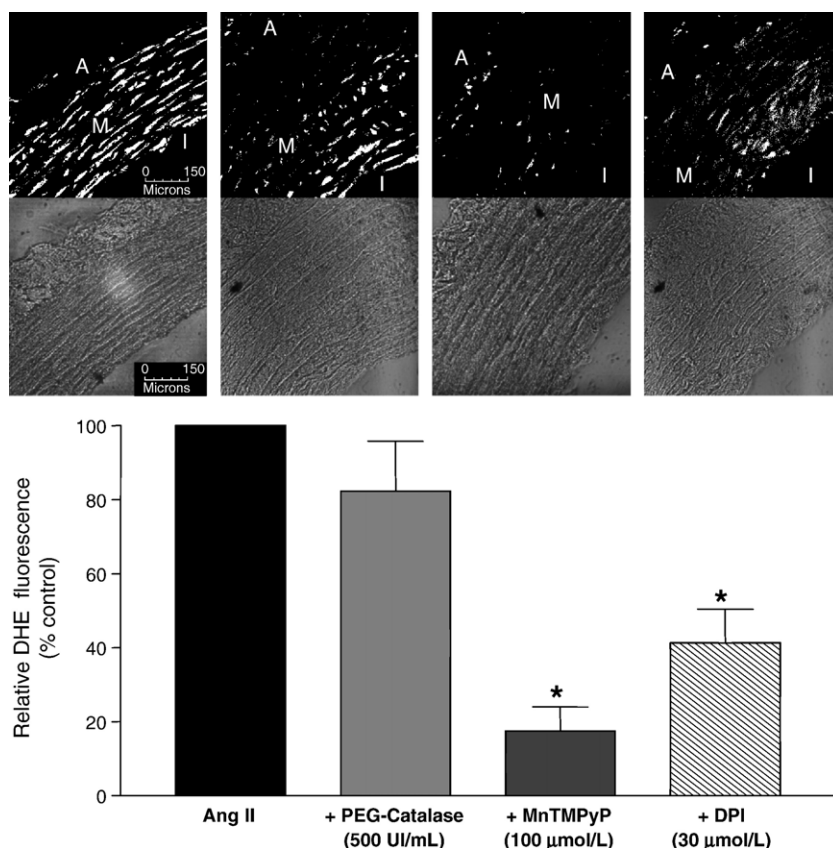


Fig. 4. Characterization of the pro-oxidant response induced by the Ang II treatment in the rat aorta. Aortic sections were exposed to the redox-sensitive fluorescent dye dihydroethidine (DHE) for 30 min at 37 °C. Thereafter, ethidium fluorescence was determined by confocal microscopy. Aortic rings were exposed to a pharmacological modulator for 30 min prior to being processed for dihydroethidine staining. Upper panel represents ethidium bromide staining and corresponding phase contrast; lower panel represents corresponding cumulative data. Results are shown as mean±S.E.M. of 3 different rats. * indicates a significant inhibitory effect.

(3 to 4 mm length) from the control group, the Ang II group, the red wine polyphenols group and the red wine polyphenols plus Ang II group were embedded in OCT compound (Tissue-Tek), and frozen in a pentane/liquid nitrogen bath for cryostat sections. In addition, rings from the Ang II group were also incubated in minimum essential medium containing 0.1% of bovine serum albumin in the absence or presence of an inhibitor for 60 min at 37 °C before being embedded. These unfixed frozen aortic rings were cut into sections 30 µm thick and placed on polylysine-coated-plus glass slides. Dihydroethidine (2.5 µM) was applied to all tissue sections on the slide, which were then incubated in a light-protected humidified chamber at 37 °C for 30 min before being sealed with coverslips. The sections were examined under a confocal microscope (1024 MRC; Bio-Rad, Hercules, CA) with a 60× epifluorescence objective (Nikon, Tokyo, Japan). After excitation at 488 nm with a Krypton/Argon laser, emission signal was recorded with a Zeiss 565–610 nm filter. Z-series were collected in 3-µm steps from position 0.00 (Z-Start) to 30.00 (Z-Stop). Final images were obtained after stacking. All images were corrected for unspecific fluorescence. Images were analysed by the Confocal Assistant™ (CAS 40 version 4.02, 1024×728 Pixels, 32-Bits per Pixels). After a 3-D projection and average of each pixel value, the intensity value of fluorescence was recorded considering an intensity level threshold (Minimum, 0; Maximum, 255).

### 2.7. Immunohistochemical determination of NADPH oxidase subunit expression

Aortas were removed, embedded in OCT compound, and snap-frozen. Frozen aortas were cryosectioned at 14 µm. Sections were air-dried for 1 h and stored at −80 °C until use. The slices were first treated with 10% normal serum in PBS containing 0.1% BSA and 0.05% Tween-20 for 45 min to block any nonspecific binding. After rinsing, the sections were then incubated overnight at 4 °C with control nonimmune serum or an antibody directed against either nox1 or p22phox. The antibodies used were a purified goat polyclonal nox1 (*sc-5821*, 1:100 dilution) and a purified goat polyclonal p22phox (*sc-11712*, 1:100 dilution). Sections were washed with PBS, incubated with the secondary antibody (donkey anti-goat alexia fluor 567-conjugated, 1:200) diluted in the same buffer for 2 h at room temperature in the dark, and washed before being mounted and
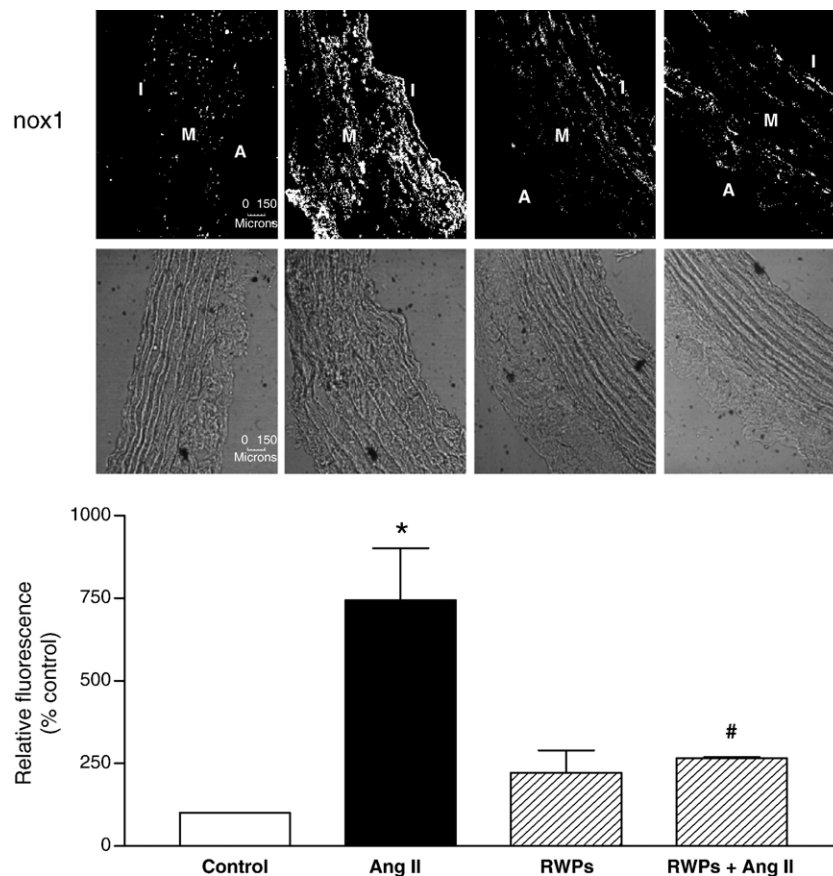
Fig. 5. Red wine polyphenols prevent the Ang II-induced expression of nox1 NADPH oxidase subunit in the rat aorta. The expression level of nox1 NADPH oxidase subunit was determined using a purified polyclonal antibody and a fluorescence-tagged secondary antibody by confocal microscopy. Upper panel shows representative immunofluorescent staining and corresponding phase contrast; lower panel represents corresponding cumulative data. Results are shown as mean ±S.E.M. of 3 different rats. * indicates a significant stimulatory effect and # indicates a significant inhibitory effect.

coverslipped. Negative controls included omission of primary antibodies.

## 2.8. Materials

All chemicals were obtained from Sigma Chemical Co unless specified. The superoxide dismutase mimetic [Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin, MnTMPyP] was obtained from Alexis Chemicals and Alzet osmotic mini-pumps from Charles Rivers Laboratories. Antibodies directed against either nox-1 or p22phox NADPH oxidase proteins were obtained from Santa Cruz.

## 2.9. Statistical analysis

Values are expressed as mean ± S.E.M. Statistical analyses were performed with Student's *t*-test for unpaired data or by ANOVA followed by the Bonferroni test to compare 2 treatments where appropriate. A value of $P<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Red wine polyphenols prevent the Ang II-induced hypertension

Infusion of Ang II at a rate of 0.4 mg/kg/day to rats caused a significant increase in systolic blood pressure,

which reached a steady state plateau 3 days after the start of Ang II infusion and, thereafter, remained elevated throughout the study (Fig. 1A). In contrast to systolic blood pressure, heart rate was unaffected by the Ang II treatment (Fig. 1B). The Ang II-induced elevation in systolic blood pressure was associated with a pronounced reduction of the plasma renin activity (Fig. 1C). Intake of 150 mg/kg/day red wine polyphenols in the drinking water 7 days before the infusion of Ang II significantly reduced the increase in systolic blood pressure (Fig. 1A, $F=7.7$, $P<0.001$ Ang II treatment versus RWPs plus Ang II treatment) and the decrease in plasma renin activity induced by Ang II (Fig. 1C). The red wine polyphenols treatment alone affected neither systolic blood pressure nor the plasma renin activity (Fig. 1A and C).

### 3.2. Red wine polyphenols prevent the Ang II-induced endothelial dysfunction

Acetylcholine caused concentration-dependent relaxations in aortic rings with endothelium, which were markedly reduced in the Ang II-treated group compared to the control group (Fig. 2A). In contrast, relaxations to red wine polyphenols in intact aortic rings were only slightly shifted to the right and the maximal relaxation was not impaired in the Ang II-treated group compared to the control group [$EC_{50}$s (µg/ml) were 3.4±0.9 and 7.8±2.0, respectively, $P=0.06$; Fig. 2B]. In addition, RWPs did not cause
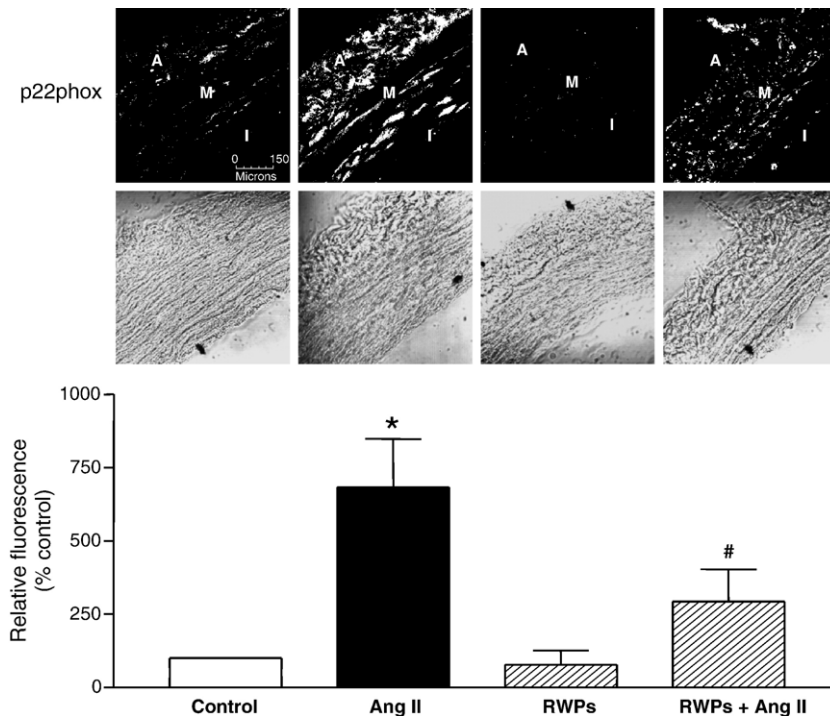


Fig. 6. Red wine polyphenols prevent the Ang II-induced expression of p22phox NADPH oxidase subunit in the rat aorta. The expression level of p22phox NADPH oxidase subunit was determined using a purified polyclonal antibody and fluorescence-tagged secondary antibody by confocal microscopy. Upper panel shows representative immunofluorescent staining and corresponding phase contrast; lower panel represents corresponding cumulative data. Results are shown as mean±S.E.M. of 3 different rats. * indicates a significant stimulatory effect and # indicates a significant inhibitory effect.

significant relaxations in rings without endothelium from the control group ($3.3\pm2.6\%$ at 100 µg/ml RWPs, $n=4$) and those in rings with endothelium were abolished by $N^G$-nitro-L-arginine (30 µM; from $68.2\pm6.7$ to $5.9\pm3.2\%$ at 100 µg/ml RWPs, $n=4$). The inhibitory effect of the Ang II treatment on acetylcholine-induced relaxations was prevented by the intake of red wine polyphenols before the infusion of Ang II to rats (Fig. 2A; $F=14.2$, $P<0.0001$ Ang II treatment versus RWPs plus Ang II treatment). Intake of red wine polyphenols alone did not affect relaxations to acetylcholine (Fig. 2A). Neither the Ang II treatment nor the red wine polyphenols treatment affected relaxations to sodium nitroprusside in aortic rings without endothelium (Fig. 2C).

### 3.3. Red wine polyphenols prevent the Ang II-induced vascular formation of reactive oxygen species and expression of NADPH oxidase

Previous studies have indicated that the Ang II-induced endothelial dysfunction is associated with an excessive NADPH oxidase-dependent vascular formation of reactive oxygen species, which scavenge NO [15]. Therefore, experiments were performed to test whether the protective effect of red wine polyphenols on the Ang II-induced endothelial dysfunction is associated with a reduced vascular pro-oxidant response as assessed using the redox-sensitive fluorescent probe dihydroethidine. The Ang II treatment caused a marked increase of the low basal fluorescence signal throughout the entire aortic wall (Fig. 3). The stimulatory effect was markedly reduced by the membrane permeant mimetic of superoxide dismutase MnTMPyP and the inhibitor of flavin-dependent enzymes including the NADPH oxidase, diphenylene iodonium whereas it was not affected by the membrane permeant analogue of catalase, polyethyleneglycol catalase (Fig. 4). In contrast to the Ang II treatment, no such increase in fluorescence was observed in intact aortic rings from the red wine polyphenols and Ang II group (Fig. 3). Administration of red wine polyphenols alone did not affect the low basal fluorescence signal (Fig. 3).

The *in vivo* Ang II-induced vascular formation of reactive oxygen species has been associated with an upregulation of several NADPH oxidase subunits including p22phox, gp91phox, and nox1 in rats [23]. Moreover, Ang II increased the expression of p22phox, nox1, p40phox, p47phox and p67phox in vascular smooth muscle cells [24,25]. Therefore, the possibility that red wine polyphenols prevent the Ang II-induced vascular expression of NADPH oxidase subunits was assessed by immunohistochemistry. In the control group, a low fluorescent signal for nox1 and p22phox subunits was detected throughout the aortic wall (Figs. 5 and 6). Both of these signals were markedly increased in aortic rings from Ang II-treated rats but not in those from rats receiving either red wine polyphenols before the infusion of Ang II or only red wine polyphenols (Figs. 5 and 6).

## 4. Discussion

The present findings demonstrate that administration of red wine polyphenols in the drinking water effectively prevents the Ang II-induced hypertension in rats. An antihypertensive effect has also been observed after intake of red wine polyphenols in insulin-resistant fructose-fed rats [26] and of a grape skin extract in both L-NAME- and DOCA salt-induced hypertension [11]. Moreover, a grape-derived polyphenol extract accelerated the decrease of systolic blood pressure after cessation of oral intake of L-NAME [11,12]. In addition, polyphenols from cocoa reduced blood pressure in elderly humans with mild isolated hypertension [27] and those from black and green tea attenuated blood pressure in stroke-prone spontaneously hypertensive rats [28]. Altogether, these studies indicate that several polyphenol-rich extracts are able not only to retard effectively the development of hypertension in several experimental models of hypertension but also to normalize blood pressure once hypertension is established in both humans and animals. Although the antihypertensive effect of polyphenols is well established, the mechanism underlying the protective effect still remains to be determined.

Ang II-induced hypertension is associated with an endothelial dysfunction as indicated by the reduced endothelium-dependent relaxation to agonists such as acetylcholine, and an increased vascular formation of reactive oxygen species [15]. An impaired endothelium-dependent relaxation is also observed in patients with essential hypertension [14]. Several lines of evidence support the concept that the vascular pro-oxidant response accounts for the endothelial dysfunction most likely by inactivating NO. Indeed, administration of antioxidants including liposome encapsulated superoxide dismutase, the permeant superoxide dismutase mimetic tempol, vitamin C and vitamin E improved endothelium-dependent relaxation, vascular oxidative stress and hypertension in several experimental models of hypertension and in humans [15,29,30]. Interestingly, the present findings indicate that although the Ang II treatment caused a pronounced impairment of endothelium-dependent relaxations to acetylcholine, those induced by the direct application of red wine polyphenols were maintained. Since relaxations to red wine polyphenols are exclusively mediated by NO in the rat aorta ([31], present findings), these findings strongly suggest that the red wine polyphenols-induced endothelial formation of NO persists in pathologic arteries presenting an oxidative stress. Such an effect may be due to the fact that red wine polyphenols have antioxidant properties thereby preventing the degradation of NO by reactive oxygen species. Indeed, polyphenols can directly interact with superoxide anions and other reactive oxygen species such as hydroxy and peroxy radicals [32–34]. Alternatively, it may also be due to the fact that distinct signaling pathways mediate the stimulatory effect of receptor coupled to G protein activation and polyphenols to endothelial NO synthase activation.

Activation of receptors coupled to protein G induce the endothelial formation of NO via the calcium/calmodulin signaling pathway whereas that induced by polyphenols involves the phosphatidylinositol 3-kinase/Akt pathway with the subsequent phosphorylation of Ser1177 of endothelial NO synthase [35,36].

The present study also indicates that intake of red wine polyphenols before the infusion of Ang II prevents the development of an endothelial dysfunction to acetylcholine and the vascular pro-oxidant response. Although the protective effect may be explained, at least in part, by the direct antioxidant properties of polyphenols, it may also be due to changes in the expression pattern of endogenous pro-oxidant and antioxidant enzymes in the arterial wall. Previous studies have indicated a key role of the NADPH oxidase in the endothelial dysfunction, vascular oxidant stress and development of hypertension to Ang II. Indeed, infusion of Ang II to rats increased membrane-bound NADH/NADPH oxidase activity and upregulated the expression of several NADPH oxidase subunits including nox1, nox4 and p22phox in the arterial wall [15,37,38]. Moreover, the hypertensive response to Ang II is markedly blunted in mice deficient in p47phox, a cytosolic subunit of the NADPH oxidase [39]. Consistent with these previous findings are the present ones indicating that the Ang II-treatment caused an upregulation of both p22phox and nox1 proteins in the arterial wall, which was associated with an increased diphenylene iodonium-sensitive formation of superoxide anions. They further indicate that administration of red wine polyphenols to rats prior to the infusion of Ang II prevents vascular oxidative stress and expression of p22phox and nox1. Therefore, the beneficial effect of red wine polyphenols on Ang II-induced hypertension and endothelial dysfunction appears also due to the prevention of the expression of several NADPH oxidase subunits and, hence, the excessive vascular formation of superoxide anions. Previous studies have also shown that tea polyphenols down-regulate the expression of NADPH oxidase subunits p22phox and p67phox in vascular cells, and up-regulate the expression of catalase [28,40]. Although polyphenols prevent oxidative stress in the vascular smooth muscle [41], they are also able to induce a modest pro-oxidant response in endothelial cells, which initiates activation of the phosphatidylinositol 3-kinase/Akt pathway leading to an enhanced formation of NO and endothelium-derived hyperpolarizing factor [35,42–44]. Thus, the dual ability of polyphenols to cause a redox-sensitive formation of vasoprotective endothelial factors and, on the other hand, to prevent excessive vascular oxidative stress via their antioxidant properties, might help to explain their beneficial effect on the cardiovascular system.

In conclusion, the present findings indicate that intake of red wine polyphenols effectively blunts hypertension and endothelial dysfunction induced by the infusion of Ang II to rats. The protective effect is most likely due to the ability of red wine polyphenols to prevent vascular oxidative stress by inhibiting NADPH oxidase expression and to induce an unaltered endothelial formation of NO in pathologic arteries.

## Acknowledgements

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