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Original Contribution

Polyphenols restore endothelial function in DOCA-salt hypertension: Role of endothelin-1 and NADPH oxidase

Rosario Jiménez [a], Rocío López-Sepúlveda [a], María Kadmiri [a], Miguel Romero [a], Rocío Vera [a], Manuel Sánchez [a], Félix Vargas [b], Francisco O'Valle [c], Antonio Zarzuelo [a], Montserrat Dueñas [d], Celestino Santos-Buelga [d], Juan Duarte [a,*]

[a] *Department of Pharmacology, School of Pharmacy, Spain*
[b] *Department of Physiology, School of Medicine, 18071 Granada, Spain*
[c] *Department of Pathological Anatomy, School of Medicine, 18071 Granada, Spain*
[d] *Laboratory of Nutrition and Bromatology, School of Pharmacy, University of Salamanca, 37007 Salamanca, Spain*

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Abstract

Red wine polyphenols (RWPs) have been reported to exert beneficial effects in preventing cardiovascular diseases, such as hypertension. We studied the effects of chronic treatment with RWPs and apocynin, an inhibitor of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, on blood pressure, endothelial function, and oxidative status in deoxycorticosterone acetate (DOCA)-salt-induced hypertension. Rats were administered RWPs (40 mg/kg) or apocynin (33 μg/kg) daily by gavage for 5 weeks. Plasma catechin levels were detected only after RWP treatment. RWPs and apocynin prevented both the increase in systolic blood pressure and the proteinuria induced by DOCA-salt. Plasma malonyldialdehyde levels, urinary iso-prostaglandin $F_{2\alpha}$ excretion, aortic superoxide production, and aortic NADPH oxidase activity were found to be increased in animals of the DOCA group. RWP and apocynin treatments reduced these parameters in DOCA-salt rats, having no effect on control rats. However, only RWPs reduced the increase in plasma endothelin-1 (ET-1) levels and aortic $p22^{phox}$ gene overexpression found in DOCA-salt animals. RWPs and apocynin also improved the blunted endothelium-dependent relaxation response to acetylcholine in noradrenaline-precontracted aortic rings. All these results suggest that chronic treatment with RWPs prevents hypertension and vascular dysfunction. RWPs prevent vascular oxidative stress by inhibiting NADPH oxidase activity and/or by reducing ET-1 release.
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Keywords: Red wine polyphenols; DOCA-salt hypertension; Endothelial dysfunction; NADPH oxidase; Endothelin-1; Free radicals

Moderate consumption of red wine is associated with a lower incidence of cardiovascular disease [1,2]. However, the source of the cardioprotective effect of wine is still uncertain. 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 for the polyphenolic component (for reviews see [6,7]). Indeed, intake of red wine polyphenols (RWPs) reduced the development of atherosclerosis in several experimental models of atherosclerosis but was without effect on mature atherosclerosis [8–11].

Hypertension is a well-established risk factor for the development and acceleration of atherosclerosis. RWPs and a grape skin extract also reduced blood pressure in several models of experimental hypertension, such as N^G-nitro-L-arginine methyl ester (L-NAME)- [12,13], angiotensin II (Ang II)-

Abbreviations: Ang II, angiotensin II; DAPI, 4,6-diamidin-2-phenylindol dichlorohydrate; DHE, dihydroethidium; DOCA, deoxycorticosterone acetate; DPI, diphenylene iodonium; ET-1, endothelin-1; iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; L-NAME, N^G-nitro-L-arginine methyl ester; MDA, malonyldialdehyde; MS, mass spectrometer; NADPH, nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet -}$, superoxide anion; PAD, photodiode array detector; RLU, relative luminescence units; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; RWP, red wine polyphenol; SBP, systolic blood pressure; SEM, standard error of mean.

* Corresponding author. Fax: +34 958248264.
E-mail address: jmduarte@ugr.es (J. Duarte).

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[14], and deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rats [15], which was related to a combination of vasodilator and antioxidant actions. Oxidative stress and the inactivation of nitric oxide (NO) by vascular superoxide anion ($O_2^{\bullet -}$) play a critical role in the pathogenesis of vascular disease, including hypertension [16,17]. Arterial $O_2^{\bullet -}$ is elevated in Ang II-induced hypertension [18], attributable to a large extent to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation by Ang II [19,20]. However, an excess of vascular $O_2^{\bullet -}$ production has also been found in DOCA-salt hypertension [21–23], a model with a markedly depressed plasma renin activity because of sodium retention [24]. Endothelin-1 (ET-1) has been shown to contribute to the pathogenesis of salt-sensitive hypertension in animals and humans [25] secondary to a low-renin state [26,27]. ET-1 may be one of the most potent vasoconstrictors produced in the blood vessel wall known to date [28] and also augments vascular $O_2^{\bullet -}$ production, at least in part, via the ET_A/NADPH oxidase pathway [29], leading to endothelial dysfunction and hypertension. Because red wine extract [30] and polyphenol constituents [31] decreased ET-1 synthesis in cultured bovine aortic endothelial cells by suppressing transcription of the ET-1 gene, we hypothesized that in vivo RWPs would affect the development of DOCA-salt hypertension and its vascular features by interfering with ET-1 production. The involvement of the antioxidant properties of RWPs in their protective effect in this model of low-renin hypertension was also tested by comparison with the NADPH oxidase inhibitor apocynin.

Therefore, the aim of the present study was to examine whether chronic intake of RWPs prevents DOCA-salt-induced hypertension and endothelial dysfunction and, if so, to determine the underlying mechanism, focusing on the involvement of ET-1 and oxidative stress.

Material and methods

DOCA-salt-hypertensive rats and in vivo pharmacological intervention

The experimental protocol followed the European Union guidelines for animal care and protection. Twelve-week-old male Wistar rats (250–285 g) were obtained from the Laboratory Animal Service of the University of Granada (Spain). All rats were maintained at five per cage at a constant temperature (24±1°C), with a 12-h dark/light cycle and on standard rat chow. An adaptation period of 2 weeks for vehicle administration and systolic blood pressure (SBP) measurements was allowed before the initiation of experiments.

Rats were randomly divided into six groups: control, RWPs, apocynin, DOCA-salt, DOCA-salt–RWPs, and DOCA-salt–apocynin. DOCA-salt hypertension was created as previously described [32]. Briefly, DOCA-salt animals were anesthetized with 2.5 ml/kg equitensin (500 ml containing 43% w/v chloral hydrate in 81 ml ethanol, 4.86 mg Nembutal, 198 ml propylene glycol, 10.63 g $MgSO_4$, distilled water) (ip) and uninephrectomized. On the following day, they were administered DOCA subcutaneously at a dose of 12.5 mg/rat per week for 5 weeks. The remaining three groups received a sham operation. During the experimental period, DOCA-salt-treated rats were allowed free access to water containing 1% NaCl. RWPs and apocynin were given by intragastric gavage at doses of 40 mg/kg and 33 μg/kg per day, respectively. This dose of RWPs has been previously shown to prevent cardiovascular alterations in L-NAME-induced hypertension [13]. On average, the absorption of RWPs in the digestive tract was estimated to be about 5–10% [33].Thus, the dose used in the present study may lead to a concentration of polyphenols present in the plasma about 0.01 g/L, the concentration that has been found to produce the maximal endothelium-dependent relaxation in rat vascular tissues [34,35]. The dose of apocynin used has been previously used to prevent the development of DOCA-salt hypertension and to abolish the increase in $O_2^{\bullet -}$ generation [23,36]. RWP or apocynin treatment was stopped 2 days before the end of the experiments, in order to study their long-term effects without the involvement of acute administration effects. All rats of each group were then housed in metabolic cages with free access to food and their respective drinking fluids, in order to measure 24-h urine output.

In order to study the plasma levels of the main components of this mixture we prepared another four groups of rats: control, RWPs, DOCA-salt, and DOCA-salt–RWPs. After 5 weeks of treatment, the rats were anesthetized with 2.5 ml/kg equitensin (ip) 2 h after the last RWP administration and blood was collected from the abdominal aorta.

Blood pressure measurements

SBP was determined once a week, in the morning, 18–20 h after administration of the drugs in conscious, prewarmed, restrained rats by tail-cuff plethysmography (digital pressure meter, LE 5000; Letica S.A., Barcelona, Spain). At least seven determinations were made in every session and the mean of the lowest three values within 5 mm Hg was taken as the SBP level.

Cardiac and renal weight indices

At the end of the experimental period, animals were anesthetized with 2.5 ml/kg equitensin (ip) and blood was collected from the abdominal aorta. Animals were sacrificed and kidneys and hearts excised, cleaned, and weighed. The atria and the right ventricle were then removed and the remaining left ventricle was weighed. The cardiac, left ventricular, and renal weight indices were calculated by dividing the heart, left ventricle, and kidney weight by the body weight.

Plasma and urinary determinations

Plasma was obtained by blood centrifugation at 2000*g* for 15 min, aliquoted, and frozen until analysis. Plasma malonyldialdehyde (MDA) content was evaluated as described by Esterbauer and Cheeseman [37]. One hundred microliters of plasma was reacted with a chromogenic reagent, 1-methyl-2-

phenylindole (10.3 mM) in acetonitrile and 37% aqueous HCl (10.4 M). After incubation of the reaction mixture for 40 min in a 45°C water bath, the absorbance was measured at 586 nm in a GBC 920 spectrophotometer. Plasma ET-1 levels were determined with the use of a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Analysis of phenolic compounds in plasma was performed as described by Pignatelli et al. [38]. Plasma samples were incubated with a hydrolyzing solution to obtain free phenolic compounds. Briefly, 0.8 ml of plasma was mixed with 1.1 ml of 100 mM acetate buffer (pH 5.0) containing 1000 U of glucuronidase and 100 U of sulfatase. The mixture was incubated for 90 min at 37°C and then extracted three times with ethyl acetate. Three milliliters of ethyl acetate was added each time and the mixture was vortexed for 4 min. After the mixture was centrifuged for 15 min at 3500*g* the top layer was removed. The extracts were combined and dried under nitrogen and the residue was dissolved in 300 μl aqueous 0.1% trifluoroacetic acid. Samples were stored at −30°C until analysis. HPLC–mass spectrometric (MS) analysis was performed using Hewlett–Packard 1100 equipment provided with a quaternary pump, automatic injector, diode array detector, and data treatment station. The column was a Phenomenex (Torrance, CA, USA) ODS-2 C8, 3 μm (4.6×150 mm). Solvents were (A) 2.5% acetic acid, (B) acetic acid/acetonitrile (10/90, v/v), and (C) gradient grade acetonitrile. The following gradient was established: 0–5 min, 100% A–100% B; 5–30 min, 100% B–85% B and 15% C; 30–35 min, 85% B and 15% C–50% B and 50% C at a flow rate of 0.5 ml/min. Detection was carried out in a photodiode array detector (PAD) and in a MS connected to the HPLC system via the DAD cell outlet. LC–MS analyses were performed with a Finningan LCQ MS detector (Thermoquest, San Jose, CA, USA) equipped with an API source, using an electrospray ionization interface. Both the sheath gas and the auxiliary gas were nitrogen at flow rates of 1.2 and 6 L/min, respectively. The capillary and source voltage were 4 V and 4.5 kV, respectively, and the capillary temperature was 195°C. Spectra were recorded in positive ion mode. The spectrometer was programmed to record the MS^2 of the ion at *m/z* $[M+H]^+$ 291. The positive presence of catechins was established from the detection of the characteristic fragment ions at *m/z* 123, 139, 151, 165. Catechins detected were quantified from the areas of their peaks recorded in the mass chromatogram by comparison with an external calibration curve prepared with (+)-catechin. The limit of quantification of the method was 0.01 μM.

For total 8-iso-prostaglandin $F_{2\alpha}$ (iso-$PGF_{2\alpha}$) determination, 50 μl of urine was assayed. The total iso-$PGF_{2\alpha}$ concentration was measured using a competitive enzyme immunoassay kit (Cayman Chemical), and the results were expressed as nanograms excreted during 24 h.

Proteinuria was determined according to Bradford [39], using bovine serum albumin as standard, and the results were expressed as milligrams of protein excreted per 100 g of rat body weight during 24 h.

Vascular $O_2^{\bullet-}$ levels

Vascular $O_2^{\bullet-}$ was assayed with oxidative dihydroethidium (DHE) fluorescence as previously described [40]. Unfixed thoracic aortic rings were cryopreserved (phosphate buffer solution 0.1 M, plus 30% sucrose for 1–2 h), covered in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), and frozen (−80°C), and 10-μm cross sections were made with 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, $MgCl_2$ 1.2, glucose 10, and Hepes 10, pH 7.3 with NaOH) at 37°C, further incubated in the dark for 30 min in Hepes solution containing DHE (10^{-5} M), counterstained with the nuclear stain 4,6-diamidin-2-phenylindol dichlorohydrate (DAPI; 3×10^{-7} M), and examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Four sections of each preparation were photographed, and ethidium and DAPI fluorescence was quantified using ImageJ (version 1.32j; NIH, http://rsb.info.nih/ij/). $O_2^{\bullet-}$ production was estimated from the ratio of ethidium/DAPI fluorescence. In preliminary experiments, DHE fluorescence was almost abolished by the $O_2^{\bullet-}$ scavenger Tiron, indicating the specificity of this reaction.

NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described [40]. Aortic rings from all experimental groups were incubated for 30 min 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, $MgSO_4$ 1, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 1, $CaCl_2$ 1.2, and glucose 5.5. Aortic production of $O_2^{\bullet-}$ was stimulated by the 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, Germany) 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 relative luminescence units (RLU)/min/mg dry aortic tissue.

In order to analyze the in vitro effects of RWPs on NADPH oxidase-derived $O_2^{\bullet-}$, NADPH-stimulated lucigenin luminescence was analyzed in homogenates from cultured aortic smooth muscle cells, as previously described [40]. Cell culture was obtained from aortic explants. Briefly, aortas from control rats were cleared of connective tissue, cut into pieces, and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids. At subconfluence, cells (passages 5–10) were placed in serum-free medium overnight, washed in ice-cold phosphate buffer solution, and

incubated in lysis buffer (20 mM monobasic potassium phosphate, 1 mM EGTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) for 10–20 min at 4°C. Protein content was measured by using the bicinchoninic acid method. NADPH (100 μM) was added to the buffer containing the aortic homogenate suspension (30 μg protein in 500 μl) and lucigenin at 5 μM was injected automatically. RWPs (0.1–10 μg/ml) or the NADPH oxidase inhibitor diphenylene iodonium (DPI; 10^{-5} M) was added 30 min before NADPH addition.

NADPH oxidase subunit gene and protein expression

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 25 to 32 cycles of amplification. Each cycle consisted of 1 min of denaturation at 94°C, 45 s of annealing at 60°C for $p47^{phox}$ or 55°C for $p22^{phox}$, 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 $p47^{phox}$ and $p22^{phox}$ were selected according to the published sequences in GenBank and were as follows: $p47^{phox}$ (191 bp) sense, 5′-CCCAGCGACAGATTAGAAGC-3′, and antisense, 5′-TGGATTGTCCTTTGAGTCAGG-3′; $p22^{phox}$ (220 bp) sense, 5′-GCGGTGTGGACAGAAGTACC-3′, and antisense, 5′-CTTGGGTTTAGGCTCAATGG-3′. β-Actin, a constitutively expressed gene, was chosen as a control. The sequence for β-actin (200 bp) was sense, 5′-AATCGTGCGTGACATCAAAG-3′, and antisense, 5′-ATGCCACAGGATTCCATACC-3′.

For Western blotting experiments, aortas were homogenized and centrifuged. Western blottings were performed with 30 μg of protein from the supernatant per lane. Sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis was performed in a minigel system (Bio-Rad Laboratories). The proteins were transferred to nitrocellulose membranes for 1 h and incubated with rabbit anti-$p47^{phox}$ polyclonal antibodies (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. The membranes were then washed five times for 10 min in Tris-buffered saline–0.1% Tween 20 and then incubated with secondary peroxidase-conjugated goat anti-rabbit antibodies (1:2000; Santa Cruz Biotechnology). All incubations were performed at room temperature for 2 h. After washing the membranes, antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Films were scanned and densitometric analysis was performed as previously described. Samples were reprobed for expression of α-actin. Data are expressed as $p47^{phox}$ protein abundance/α-actin ratio.

Isolated vascular ring experiments

Three-millimeter ring segments of the descending thoracic aorta were dissected and mounted in individual organ chambers filled with Krebs buffer (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2, glucose 11). The solution was continuously gassed with a 95% O_2 and 5% CO_2 mixture and maintained at 37°C. Rings were stretched to 2 g of resting tension by means of two L-shaped stainless steel wires, which were inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (Letigraph Model 2000; Letica S.A.), as previously described [32]. Rings were equilibrated for 60 to 90 min, and during this period, tissues were restretched and washed every 30 min with warm Krebs solution. The concentration–relaxation response curves to acetylcholine (10^{-9}–10^{-4} M) were performed in intact rings precontracted by 10^{-6} M noradrenaline. The relaxant responses to sodium nitroprusside (10^{-9}–10^{-5} M) were studied in the dark in endothelium-denuded vessels precontracted by 10^{-6} M noradrenaline. Relaxant responses to acetylcholine and sodium nitroprusside were expressed as a percentage of precontraction induced by noradrenaline.

Drugs

RWPs from red wine in dry powder form were provided by Mr. D. Ageron (Société Francaise de Distillerie, Vallont Pont d'Arc, France). The content of polyphenols in RWPs was 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 antocyanins, 4.7 gallic acid, 15.1 *p*-coumaric acid, and 1.5 resveratrol. DOCA was obtained from ICN Biomedicals (Solon, OH, USA). All other drugs used were obtained from Sigma (Alcobendas, Madrid, Spain). All drugs and chemicals were dissolved in distilled deionized water, except for DOCA, which was mixed with ethanol/sesame oil (1/5, v/v).

Statistical analysis

Results are expressed as means ± standard error of mean (SEM) of measurements. For statistical analysis, we used one-way analysis of variance followed by Bonferroni's multiple comparisons test. For all comparisons, differences were considered significant at a $p < 0.05$.

Results

Blood pressure, cardiac and renal hypertrophy, urinary protein excretion, and plasma polyphenols

Rats receiving DOCA-salt showed a progressive increase in SBP compared to animals of the control group (Fig. 1). This increase was significant ($p < 0.05$) from the first week, reaching a difference of approximately 40 mm Hg at the end of the treatment. Chronic treatment with RWPs or apocynin prevented ($p < 0.05$) SBP increase (about 68 and 78%, respectively) in DOCA-salt-treated rats from the first week. A small decrease in

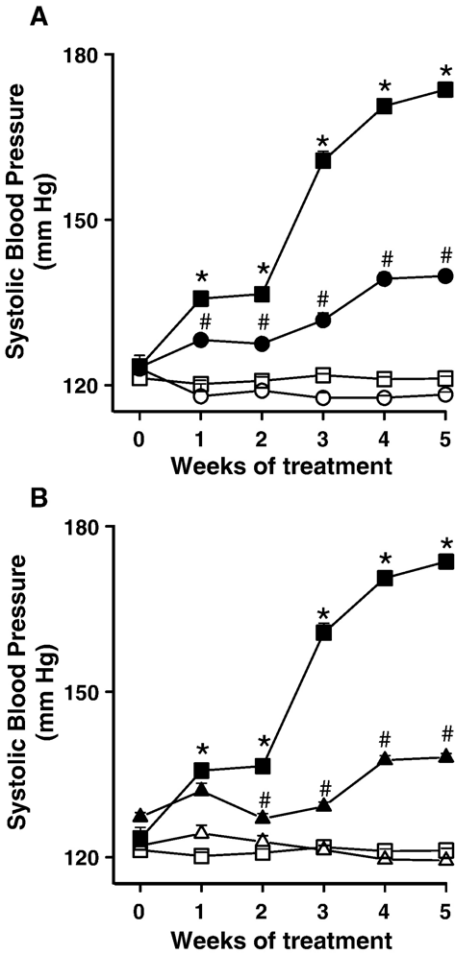

Fig. 1. Effects of (A) chronic RWPs and (B) apocynin treatment on the time course of the systolic blood pressure as measured by tail-cuff plethysmography. Experimental groups: control (□) ($n=10$), DOCA (■) ($n=10$), RWPs (○) ($n=10$), DOCA–RWPs (●) ($n=7$), apocynin (△) ($n=10$), and DOCA–apocynin (▲) ($n=9$). Values are expressed as means±SEM. *$p<0.05$ compared to the control group. #$p<0.05$ compared to the DOCA group.

SBP was observed in control animals treated with RWPs after 3 weeks of treatment.

Values of body weight and cardiac and renal indices and proteinuria are shown in Table 1. At the end of the experimental period, animals of the DOCA-salt group showed significantly increased cardiac (~25%), left ventricular (~24%), and renal (~83%) weight indices, compared with the animals of the control group. No significant modifications of these parameters were observed in the rats receiving DOCA-salt plus RWPs or apocynin. However, both RWPs and apocynin treatment reduced the marked proteinuria detected in the DOCA-salt rats.

LC–MS analyses revealed the presence of catechins (catechin and epicatechin) in the plasma samples of the rats (control or DOCA-salt) treated with RWPs, whereas no catechins were detected in any of the samples corresponding to the untreated animals. The amounts of catechins detected were always below (although around) the limit of quantification of the employed method, meaning that their plasma concentrations were less than 0.01 μM. No catechin dimers or higher oligomers were detected in any of the plasma samples, suggesting that in the model assayed only monomeric catechins were bioavailable. However, we have not found any change corresponding to catechins in PAD chromatograms.

Plasma ET-1 levels

ET-1 levels in plasma were significantly higher in DOCA-salt rats than in sham-control rats. Chronic treatment of DOCA-salt rats with RWPs reduced ET-1 levels to values similar to those found in the control group. However, apocynin was unable to prevent plasma ET-1 accumulation in DOCA-salt animals (Fig. 2).

Systemic reactive oxygen species (ROS), vascular $O_2^{\bullet -}$ production, and NADPH oxidase activity and gene and protein expression

Plasma MDA levels, a marker of lipid peroxidation induced by ROS, of DOCA-salt-treated animals were increased compared to the control group. In rats treated with DOCA-salt plus RWP or apocynin, MDA concentration was reduced to values similar to those found in control rats (Fig. 3A). The 24-h urinary iso-$PGF_{2\alpha}$ excretion, a more specific marker of $O_2^{\bullet -}$ production, was also increased in the DOCA-salt group. In both RWP- and apocynin-treated DOCA-salt rats, iso-$PGF_{2\alpha}$ excretion showed values similar to those of control rats (Fig. 3B).

To characterize and localize ROS production within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE. It is known that DHE is oxidized by $O_2^{\bullet -}$ to yield ethidium, which stains DNA. Positive red nuclei could be observed in adventitial, medial, and endothelial cells

Table 1
Body and organ weights and cardiac and renal indices

Group	BW (g)	HW (mg)	LVW (mg)	KW (mg)	HW/BW	LVW/BW	KW/BW	Proteinuria
Control ($n=10$)	352±8	835±29	593±17	883±27	2.37±0.06	1.69±0.04	2.51±0.06	8.1±2.2
RWPs ($n=10$)	333±6	780±22	543±15	828±21	2.35±0.06	1.63±0.04	2.49±0.06	13.7±2.1
Apocynin ($n=10$)	335±5	798±15	551±9**	837±21	2.39±0.05	1.65±0.02	2.50±0.07	14.5±2.7
DOCA ($n=10$)	299±10**	875±26	621±22	1371±46**	2.95±0.13**	2.10±0.12**	4.60±0.14**	27.5±7.2**
DOCA–RWPs ($n=7$)	287±8**	822±28	578±16	1223±40*,**	2.87±0.13**	2.02±0.07**	4.27±0.16**	12.2±2.1*
DOCA–apocynin ($n=9$)	298±7**	893±34	620±16	1302±75**	3.01±0.11**	2.09±0.06**	4.36±0.20**	8.2±1.6*

BW, body weight; HW, heart weight; LVW, left ventricle weight; KW, kidney weight. Proteinuria was expressed as milligrams of protein excreted per 100 g of rat BW during 24 h. Values are expressed as means±SEM.

* $p<0.05$ compared to the DOCA group.

** $p<0.05$ compared to the control group.

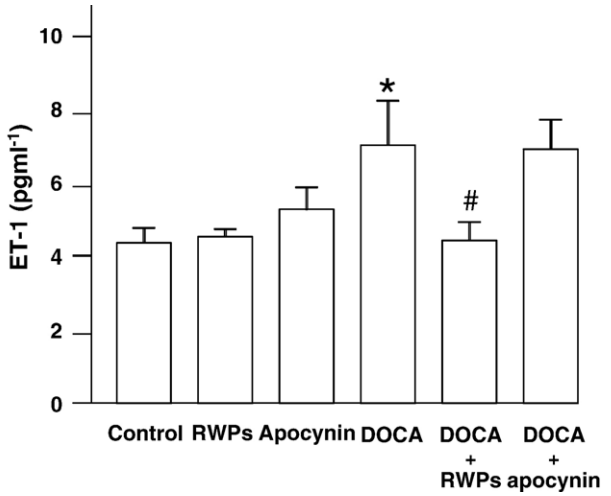

Fig. 2. Plasma ET-1 levels from all experimental groups. Values are expressed as means±SEM (n=7–10). *p<0.05 compared to the control group. #p<0.05 compared to the DOCA group.

(Fig. 4A). Nuclear red ethidium fluorescence, indicative of $O_2^{\bullet -}$ production, was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections (Fig. 4B). In fact, red staining was suppressed when aortic sections were incubated for 30 min with Tiron (10 mM), an intracellular $O_2^{\bullet -}$ scavenger, (data not shown). Rings from DOCA-salt rats showed markedly increased staining in adventitial, medial, and endothelial cells compared to control rats, which was restored after RWP or apocynin treatment.

NADPH increases lucigenin luminescence in normal aortic rings, which was almost abolished by previous incubation for 30 min with the flavoprotein inhibitor DPI (10 μM) or with the selective NADPH oxidase inhibitor apocynin (100 μM) (not shown), showing that external NADPH increased NADPH oxidase activity in vascular tissue. NADPH oxidase activity was increased in aortic rings from DOCA-salt rats compared to control rats. Chronic treatment with RWPs or apocynin reduced this activity significantly only in DOCA-salt-treated rats, being without effect in control animals (Fig. 5A). In another set of experiments, NADPH oxidase activity in homogenates from vascular smooth muscle cells in culture obtained from control rats was $1197 \pm 361 \times 10^3$ RLU/min/mg of protein. RWPs reduced this NADPH oxidase activity in a concentration-dependent manner (Fig. 5B).

Significant $p22^{phox}$ gene overexpression without change in $p47^{phox}$ gene expression was observed in aortic tissue of DOCA-salt compared with control rats (Fig. 6A). Total $p47^{phox}$ protein content was also similar in aortas from DOCA-salt and control rats (Fig. 6B). RWP but not apocynin treatment was able to inhibit the $p22^{phox}$ gene overexpression in DOCA-salt animals.

In vitro endothelial function

Aortic rings from DOCA-salt-treated animals showed strongly reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by noradrenaline compared to the control aortic rings (Fig. 7). The aortic rings obtained from DOCA-salt-hypertensive animals treated with both RWPs (Fig. 7A) and apocynin (Fig. 7B) showed a significant increase in vasodilatation induced by acetylcholine compared to the animals from the DOCA-salt group. No significant effect was observed in the rings from control rats treated with RWPs and apocynin. No differences were observed among groups in the endothelium-independent vasodilator response to the NO donor sodium nitroprusside in vessels precontracted with noradrenaline (Figs. 7C and 7D).

Discussion

The major new findings of this study are that chronic treatment with RWPs prevents the progressive increase in SBP, the proteinuria, and the endothelial dysfunction in uninephrectomized rats subjected to chronic administration of DOCA-salt and that this effect seems to be related to attenuation of vascular $O_2^{\bullet -}$ production mediated by NADPH oxidase inhibition. In

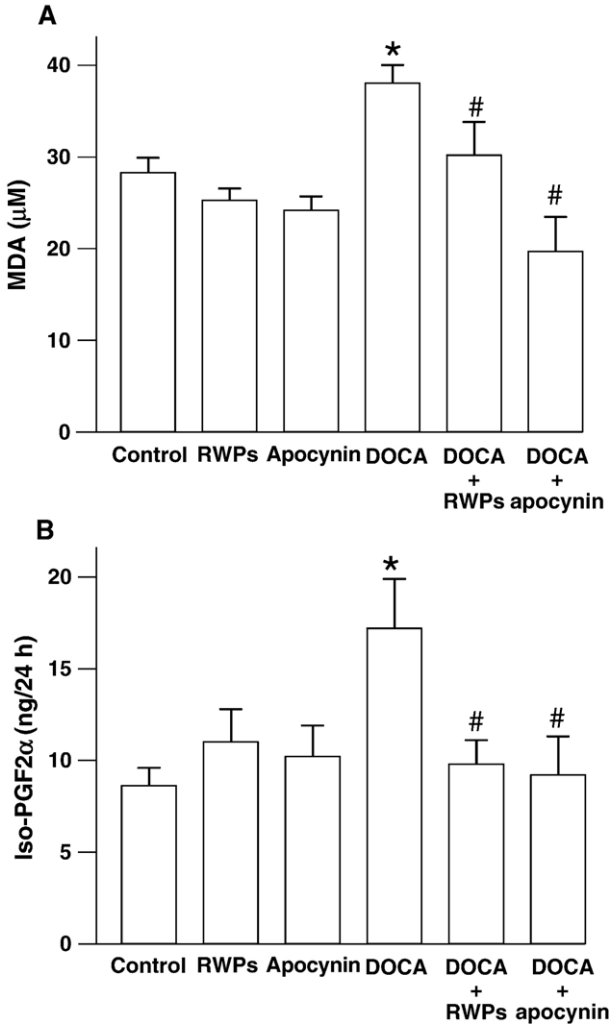

Fig. 3. (A) Plasma MDA levels and (B) 24-h urinary iso-$PGF_{2\alpha}$ excretion from all experimental groups. Values are expressed as means±SEM (n=7–10). *p<0.05 compared to the control group. #p<0.05 compared to the DOCA group.

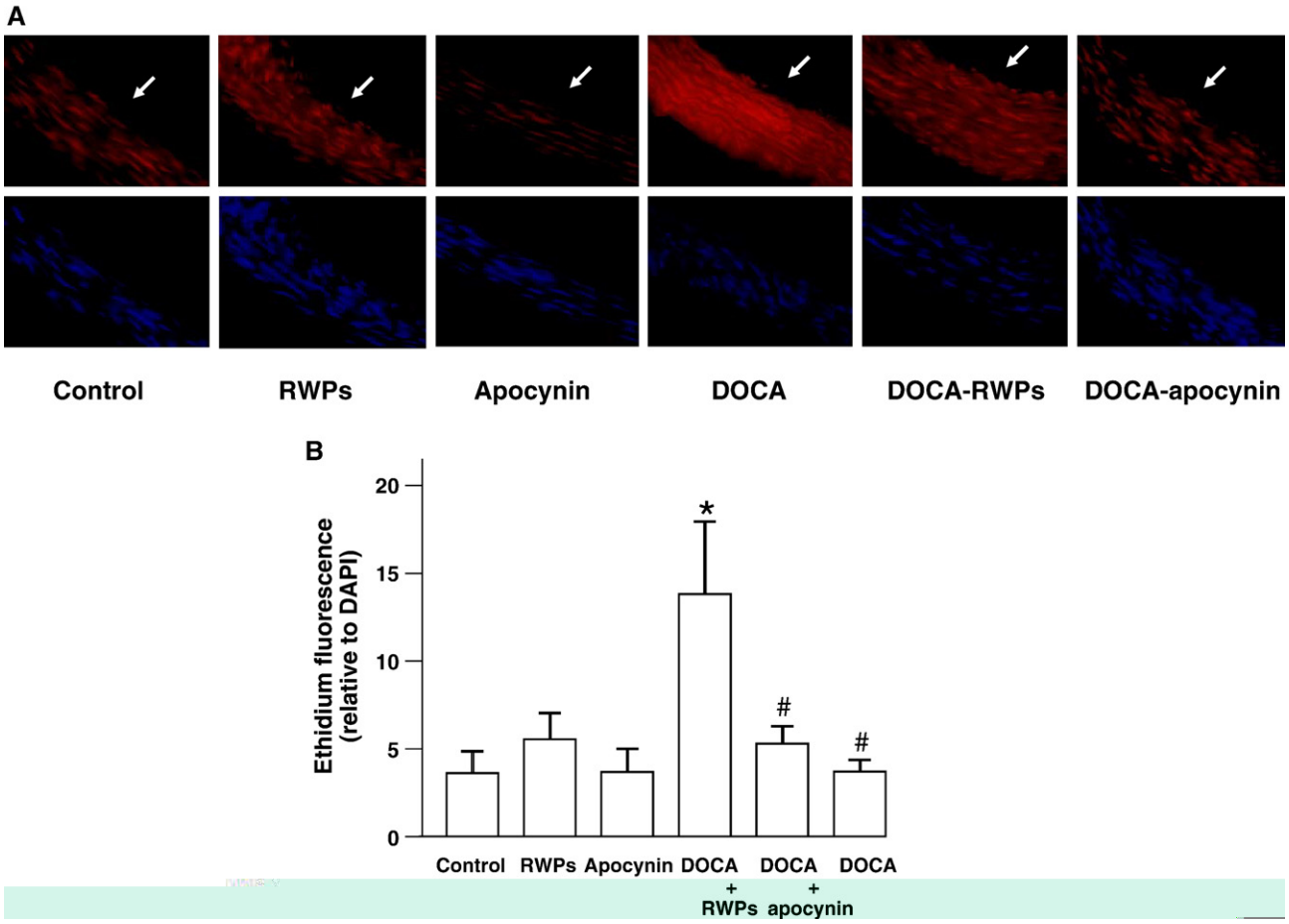

Fig. 4. In situ localization of $O_2^{\bullet -}$ production in aortic rings from all experimental groups. (A) Upper pictures show arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by $O_2^{\bullet -}$. Lower pictures show blue fluorescence of the nuclear stain DAPI (×400 magnification). The lumen is indicated by the arrow. (B) Averaged values, means ± SEM ($n = 3–4$), of red ethidium fluorescence normalized to blue DAPI. *$p < 0.05$ compared to the control group. #$p < 0.05$ compared to the DOCA group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition, we show for the first time that RWPs reduce ET-1 production in vivo, which would collaborate with their beneficial cardiovascular effects.

This study confirms and extends previous evidence about antihypertensive effects and the improvement in endothelial function of RWPs in several animal models of hypertension [12–15]. In rats with chronic inhibition of NO and in Ang II-infused rats, two experimental models in which vascular oxidative stress mediated by the renin–angiotensin system play a major role in the development of hypertension [41,42], RWPs prevent hypertension, at least in part, by reducing vascular $O_2^{\bullet -}$ production. Moreover, a grape-derived polyphenol extract [15] and RWPs (present results) also prevented the rise in blood pressure induced by DOCA-salt-treated rats, an Ang II-independent model of hypertension because AT_1 receptor blockers failed to reduce blood pressure [43–46]. In agreement with previous studies [23,36,47] we also found that oxidative stress is involved in DOCA-salt-induced hypertension, because long-term administration of apocynin to DOCA-salt rats significantly decreased the urinary iso-$PGF_{2\alpha}$, a prostaglandin-like compound produced in a nonenzymatic reaction of arachidonic acid and $O_2^{\bullet -}$; the plasma MDA levels, a reliable marker for lipid peroxidation and oxidative stress; and the SBP compared with that of rats treated with DOCA-salt alone. Similarly, chronic treatment with RWPs also normalized urinary iso-$PGF_{2\alpha}$ and plasma MDA and prevented the increase in SBP in DOCA-salt rats. Moreover, renal injury characteristic of mineralocorticoid hypertension is associated with oxidative stress and is partly independent of blood pressure [46,48]. In our study, proteinuria, a marker for renal damage, was increased in DOCA-salt rats and both RWP and apocynin treatments reduced protein excretion to values similar to those found in control rats.

Analysis of plasma polyphenols revealed the presence of low concentrations (<0.01 μM) of catechins (the main components of RWPs) in rats treated with RWPs. Nevertheless, because no quantification of their circulating levels was possible, it cannot be concluded if the vascular effects observed in the treated rats could be directly related to the plasmatic levels of catechins, although the low detected levels would not support such an assumption. On the other hand, the low rate of catechin absorption may be due, at least in part, to an extensive degradation of the gastrointestinal microflora (present in relevant counts in the small gut of the rat compared to humans).