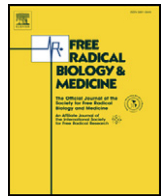




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Original Contribution

Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension

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ABSTRACT

Flavanol-rich diets have been reported to exert beneficial effects in preventing cardiovascular diseases, such as hypertension. We studied the effects of chronic treatment with epicatechin on blood pressure, endothelial function, and oxidative status in deoxycorticosterone acetate (DOCA)-salt-induced hypertension. Rats were treated for 5 weeks with (–)-epicatechin at 2 or 10 mg kg⁻¹ day⁻¹. The high dose of epicatechin prevented both the increase in systolic blood pressure and the proteinuria induced by DOCA-salt. Plasma endothelin-1 and malondialdehyde levels and urinary iso-prostaglandin F_{2α} excretion were increased in animals of the DOCA-salt group and reduced by the epicatechin 10 mg kg⁻¹ treatment. Aortic superoxide levels were enhanced in the DOCA-salt group and abolished by both doses of epicatechin. However, only epicatechin at 10 mg kg⁻¹ reduced the rise in aortic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and p47^{phox} and p22^{phox} gene overexpression found in DOCA-salt animals. Epicatechin increased the transcription of nuclear factor-E2-related factor-2 (Nrf2) and Nrf2 target genes in aortas from control rats. Epicatechin also improved the impaired endothelium-dependent relaxation response to acetylcholine and increased the phosphorylation of both Akt and eNOS in aortic rings. In conclusion, epicatechin prevents hypertension, proteinuria, and vascular dysfunction. Epicatechin also induced a reduction in ET-1 release, systemic and vascular oxidative stress, and inhibition of NADPH oxidase activity.

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Flavanols, such epicatechin and catechin, and their oligomers, the procyanidins, represent a major class of flavonoids commonly present in higher plants. They are found at high concentrations in certain food plants, such as *Vitis vinifera* (wine grape), *Camellia sinensis* (tea), and *Theobroma cacao* (cocoa). Several epidemiological investigations and dietary interventions in humans using flavanol-containing foods

indicate an inverse relationship between flavanol intake and the risk of cardiovascular disease [1,2]. A very wide range of biological actions of flavanol-rich foods supports these potential cardiovascular protective effects including the improvement of vasodilation and endothelial function [3–9], blood pressure [10–13], and insulin resistance and glucose tolerance [14]; the attenuation of platelet reactivity [15]; and the improvement of immune responses and antioxidant defense system [16,17]. In contrast, some studies have shown that black tea does not modify blood pressure, platelet aggregation, or several markers of oxidative stress or inflammation in coronary artery disease patients [4,18]. However, little is known about the molecular mechanisms of flavanol-mediated bioactivities in both humans and animals. The reasons for these shortcomings are, at least in part, based on the fact that food matrices contain a multitude of phytochemical constituents, of which an unknown number may exert physiological effects. However, the effect of high-flavanol cocoa was mimicked by oral intake of pure epicatechin isolated from cocoa, and the maximum effect on endothelial function coincided with the

Abbreviations: Ang II, angiotensin II; DAPI, 4,6-diamidino-2-phenylindole dichlorohydrate; DHE, dihydroethidium; DOCA, deoxycorticosterone acetate; DPI, diphenylene iodonium; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; GCLC, γ -glutamylcysteine synthetase; HMOX1, heme oxygenase-1; iso-PGF_{2α}, 8-iso-prostaglandin F_{2α}; Keap-1, Kelch-like erythroid cell-derived protein-1; L-NAME, N^G-nitro-L-arginine methyl ester; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NQO1, NADPH:quinone oxidoreductase 1; Nrf2/ARE, nuclear factor-E2-related factor-2/antioxidant-response element; O₂⁻, superoxide anion; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; SBP, systolic blood pressure.

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peak of the plasma level of epicatechin metabolites [8]. Therefore, epicatechin seems to be a major bioactive constituent of cocoa and other flavanol-rich foods and beverages. Epicatechin controls vascular tone, inducing endothelium-independent and endothelium-dependent nitric oxide (NO)-mediated vasodilation in resistance arteries [19,20] and improving endothelial dysfunction found in pathological conditions such as diabetes and hypertension [21]. Epicatechin induced endothelial NO synthase (eNOS) activation through several mechanisms: (i) eNOS phosphorylation under normal calcium conditions through the participation of the phosphatidylinositol 3-kinase pathway [22], (ii) activation mediated via the Ca^{2+} /calmodulin-dependent kinase II pathway [22], and (iii) eNOS phosphorylation under calcium-depleted conditions [23].

Hypertension is a well-established risk factor for the development and acceleration of atherosclerosis. Oxidative stress and the inactivation of NO by vascular superoxide anion (O_2^-) play critical roles in the pathogenesis of vascular disease, including hypertension [24]. Arterial O_2^- is elevated in angiotensin II (Ang II)-induced hypertension, attributable to a large extent to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation by Ang II [25,26]. However, an excess of vascular O_2^- production has also been found in deoxycorticosterone acetate (DOCA) salt hypertension [27–29], a model with a markedly depressed plasma renin activity because of sodium retention [30]. Patients with low renin (i.e., salt-sensitive hypertension) represent approximately 30% of the essential hypertensives and show a poor therapeutic response to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. Endothelin-1 (ET-1) has been shown to contribute to the pathogenesis of salt-sensitive hypertension in animals and humans, secondary to a low-renin state [31,32]. ET-1 is the most potent vasoconstrictor produced in the blood vessel wall and also augments vascular O_2^- production, at least in part, via the ET_A /NADPH oxidase pathway [33], leading to endothelial dysfunction and hypertension. Because flavanols [34,35] decreased ET-1 synthesis in cultured bovine and human aortic endothelial cells by suppressing transcription of the ET-1 gene, we hypothesized that *in vivo* epicatechin would affect the development of DOCA-salt hypertension and its vascular features by interfering with ET-1 production. Therefore, the aim of this study was to examine whether chronic intake of epicatechin 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.

Materials 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 (150–180 g) were obtained from the Laboratory Animal Service of the University of Granada (Spain). All rats were kept at five per cage at a constant temperature ($24 \pm 1^\circ\text{C}$), 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 five groups: control (1 ml of 1% methylcellulose once daily, $n = 10$), (–)-epicatechin (EPI10; 10 mg kg^{-1} po by gavage, mixed in 1 ml of 1% methylcellulose, once daily, $n = 10$), DOCA-salt (1 ml of 1% methylcellulose once daily, $n = 9$), DOCA-salt–EPI2 (2 mg kg^{-1} po by gavage, mixed in 1 ml of 1% methylcellulose, once daily, $n = 10$), and DOCA-salt–EPI10 (10 mg kg^{-1} po by gavage, mixed in 1 ml of 1% methylcellulose, once daily, $n = 9$). DOCA-salt hypertension was created as previously described [34]. Briefly, DOCA-salt animals were anesthetized with 2.5 ml/kg equitensin (500 ml contains 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 rest of two groups received a sham operation. During the experimental period, DOCA-salt-treated rats were allowed free access to water containing 1% NaCl. Epicatechin treatment was stopped 2 days before the end of the experiments, to study its 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, to measure urine output for 24 h. After 5 weeks of treatment, the rats were anesthetized with 2.5 ml/kg equitensin (ip) and blood was collected from the abdominal aorta.

Blood pressure measurements

SBP and heart rate were 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. The 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, aliquotted, and frozen until analysis. Plasma malondialdehyde (MDA) content was evaluated as described by Esterbauer and Cheeseman [36]. 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 using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the instructions of the manufacturer.

For total 8-iso-prostaglandin (iso-PG) $\text{F}_{2\alpha}$ determination, 50 μl of urine was used for assay. 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, using bovine serum albumin as standard, and the results were expressed as milligrams of protein excreted per 100 g of rat during 24 h.

Vascular O_2^- levels

Vascular O_2^- was assayed with oxidative dihydroethidium (DHE) fluorescence as previously described [37,38]. Unfixed thoracic aortic rings were cryopreserved (phosphate buffer solution 0.1 M, plus 30% sucrose for 1–2 h), placed in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), and frozen (-80°C), and 10- μm 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, MgCl_2 1.2, glucose 10, and Hepes 10, pH 7.3 with NaOH) at 37°C , further incubated for 30 min in Hepes solution containing DHE (10^{-5} M) in the dark, counterstained with the nuclear stain 4,6-diamidino-2-phenylindole 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.gov/ij/>). $O_2^{\cdot-}$ content was estimated from the ratio of ethidium/DAPI fluorescence. In preliminary experiments, DHE fluorescence was almost abolished by the $O_2^{\cdot-}$ scavenger tiron, indicating the specificity of this reaction.

Vascular NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described [29]. 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 $O_2^{\cdot-}$ was stimulated by addition of NADPH (100 μ M). 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 μ M to avoid known artifacts when used a 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/min/mg dry aortic tissue.

Western blot analysis

Aortic homogenates were run on a sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Proteins were transferred to polyvinylidene difluoride membranes and incubated with primary monoclonal mouse anti-eNOS antibody (Transduction Laboratories, San Diego, CA, USA), anti-phospho-eNOS (Ser-1177) (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-Akt (Ser-473), rabbit anti-Akt (Cell Signaling Technology), polyclonal goat anti-p22^{phox} (Santa Cruz Biotechnology, Santa Cruz, C, USA), or polyclonal rabbit anti-p47^{phox} (Upstate Cell Signaling) overnight, all at 1:1000 dilution, and with the corresponding 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>). Samples were reprobated for expression of smooth muscle α -actin. Films were scanned and densitometric analysis was performed as previously described.

Nrf-2 nuclear protein expression was measured in the nuclear extract of aortic homogenates. Aortae were lysed with buffer A (10 mM Hepes-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on ice for 20 min and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fraction. The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM Hepes, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14,000 g for 15 min at 4 °C. The nuclear

fraction was subjected to immunoblot analysis using rabbit anti-Nrf2 polyclonal (1:1000 dilution; Santa Cruz Biotechnology) and goat anti-lamin B polyclonal (1:2000 dilution; Santa Cruz Biotechnology).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

A quantitative real-time RT-PCR technique was used to analyze mRNA expression of the nuclear factor-E2-related factor-2 (Nrf2)/antioxidant-response element (ARE) target genes NADPH:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HMOX1), and γ -glutamylcysteine synthetase (GCLC) in aortic segments. In brief, total RNA was isolated with a Mini RNA isolation kit (Zymo Research, Orange, CA, USA) and was reverse transcribed using Superscript III RT (Invitrogen). A real-time RT-PCR technique was used to analyze mRNA expression using the Stratagen MX3000. Amplification efficiencies were determined using a dilution series of a standard vascular sample. Quantification was performed using the efficiency-corrected $\Delta\Delta C_t$ method. The relative quantities of the reference gene β -actin were determined and a normalization factor was calculated based on their geometric mean for internal normalization. Oligonucleotides used for quantitative real-time RT-PCR are listed in Table 1. The fidelity of the PCR was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Vascular functional studies

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 kept 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 [39]. 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 for acetylcholine (10^{-9} – 10^{-4} M) were performed in intact rings precontracted by 10^{-6} M phenylephrine. 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 phenylephrine. Relaxant responses to acetylcholine and sodium nitroprusside were expressed as a percentage of precontraction induced by phenylephrine. In some rings with and without endothelium, a concentration-response curve for phenylephrine (10^{-9} M– 10^{-5} M) was carried out by cumulative addition of the drugs.

To evaluate the formation of basal NO, the contraction induced by 10^{-6} M phenylephrine was obtained in rings incubated for 30 min with the NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME, 10^{-4} M).

Drugs

All drugs used were obtained from Sigma (Alcobendas, Madrid, Spain). All drugs and chemicals were dissolved in distilled deionized

Table 1
Oligonucleotides for real-time RT-PCR.

mRNA target	Description	Sense	Antisense
<i>Actb</i>	β -Actin	AATCTGCGTGACATCAAAG	ATGCCACAGGATTCATACC
<i>Nrf2</i>	Nuclear respiratory factor-2	GTTGAGAGCTCAGTCTTCAC	CAGAGAGCTATCGAGTGACT
<i>HMOX1</i>	Heme oxygenase-1	GCACAGGGTGACAGAAGAGG	ATGGCATAAATCCCACTGC
<i>GCLC</i>	Glutamate-cysteine ligase, catalytic subunit	CCTCATTCCTACTGCCAAGG	GGATGAGTCCAGGAAACCG
<i>NQO1</i>	NAD(P)H dehydrogenase:quinone 1	GGGATATGAATCAGGGAGAGG	TGCCCTAAACCACAGAGAGG

water, except for DOCA, which was mixed with ethanol/sesame oil (1/5, v/v).

Statistical analysis

Results are expressed as means \pm standard error of the 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 value of $P < 0.05$.

Results

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

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 47 mm Hg at the end of the treatment. Chronic treatment with 2 mg kg^{-1} epicatechin did not modify this rise in SBP. However, epicatechin at 10 mg kg^{-1} prevented ($P < 0.01$) the increase in SBP (about 70%) in DOCA-salt-treated rats from the second week, being without effect in control animals. No change in heart rate was observed among groups.

Values of body weight and cardiac and renal indices and proteinuria are shown in Table 2. At the end of the experimental period, animals of the DOCA-salt group showed significantly increased cardiac (~38%), left-ventricular (~49%), and renal (~93%) 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 epicatechin. However, at the higher dose, epicatechin treatment reduced the marked proteinuria detected in the DOCA-salt rats.

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 epicatechin 10 mg kg^{-1} reduced ET-1 levels significantly, being without effect at 2 mg kg^{-1} (Fig. 2).

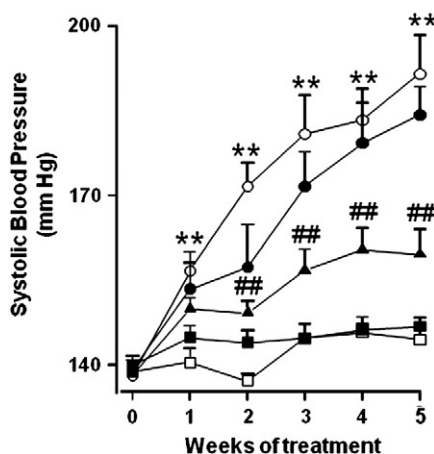


Fig. 1. Effects of chronic epicatechin treatment on the time course of systolic blood pressure as measured by tail-cuff plethysmography. Experimental groups: control (□; $n = 10$), DOCA (○; $n = 9$), epicatechin 10 mg kg^{-1} (■; $n = 10$), DOCA-epicatechin 2 mg kg^{-1} (●; $n = 10$), and DOCA-epicatechin 10 mg kg^{-1} (▲; $n = 9$). Values are expressed as means \pm SEM. ** $P < 0.01$ compared to the control group. ## $P < 0.01$ compared to the DOCA group.

Systemic reactive oxygen species (ROS), vascular $\text{O}_2^{\cdot-}$ level, NADPH oxidase activity, and gene and protein expression

The plasma levels of MDA, a marker of lipid peroxidation induced by ROS, were increased in DOCA-salt-treated animals compared to the control group. In the DOCA-salt-EPI10 group, MDA concentrations were similar to those found in control rats (Fig. 3A). The 24-h urinary iso-PGF_{2α} excretion, a more specific marker for lipid peroxidation, was also increased in the DOCA-salt group and, again, this increase was prevented by epicatechin 10 mg kg^{-1} (Fig. 3B). In the DOCA-salt-EPI2 group neither plasma MDA nor iso-PGF_{2α} excretion were altered compared to the DOCA-salt group.

To characterize and localize ROS content within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE. It is known that DHE is oxidized by $\text{O}_2^{\cdot-}$ to yield ethidium, which stains DNA. Positive red nuclei could be observed in adventitial, medial, and endothelial cells (Fig. 4A). Nuclear red ethidium fluorescence, indicative of $\text{O}_2^{\cdot-}$ 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 almost suppressed when aortic sections were incubated for 30 min with tiron (10 mM), an intracellular $\text{O}_2^{\cdot-}$ scavenger (data not shown). Rings from DOCA-salt rats showed marked increased staining in adventitial, medial, and endothelial cells compared to control rats, which was prevented by epicatechin at either 2 or 10 mg kg^{-1} .

NADPH increased lucigenin luminescence in normal aortic rings, which was strongly inhibited (85 \pm 4%) by previous incubation for 30 min with the flavoprotein inhibitor DPI (10 μM), indicating 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 treatments with epicatechin 10 mg kg^{-1} reduced significantly this activity only in DOCA salt-treated rats, being without effects in control animals (Fig. 5A).

Significant p47^{phox} (Fig. 5B) and p22^{phox} (Fig. 5C) protein overexpression was observed in aortic tissue from DOCA-salt compared with control rats. Epicatechin 10 mg kg^{-1} treatment prevented the upregulation of both proteins in DOCA-salt animals, being without effects in control animals. However, epicatechin 2 mg kg^{-1} treatment was unable to significantly alter the upregulation of both subunits in hypertensive rats.

Nrf2 and Nrf2/ARE-driven genes

In DOCA-salt rats we found an increased expression of Nrf2 mRNA (Fig. 6A), Nrf2 protein in nuclear fraction (Fig. 6B), and the Nrf2 target genes NQO1 (Fig. 6C), GCLC (Fig. 6D), and HMOX1 (Fig. 6E), compared to the control group. Chronic treatment with epicatechin 10 mg kg^{-1} in control animals increased both Nrf2 and Nrf2/ARE-driven genes compared to the control untreated group. In DOCA-salt plus epicatechin 2 mg kg^{-1} -treated rats the Nrf2 pathway was upregulated compared with vehicle-treated-rats. However, in the DOCA-salt plus EPI10 group the expression of Nrf2 genes was similar to that found in the DOCA-salt group.

In vitro endothelial function

Aortic rings from DOCA-salt-treated animals showed reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by phenylephrine compared to control aortic rings (Fig. 7). Both doses of epicatechin prevented the DOCA-salt-induced impairment of endothelium-dependent vasodilatation. No significant effect was observed in the rings from control rats treated with epicatechin 10 mg kg^{-1} . No differences were observed among groups in the endothelium-independent vasodilator response to the NO donor sodium nitroprusside in vessels precontracted with phenylephrine (Fig. 7B).

Table 2

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)	281 ± 8	760 ± 15	539 ± 13	785 ± 26	2.71 ± 0.04	1.92 ± 0.04	2.80 ± 0.08	44.1 ± 11.6
EPI10 (n = 10)	280 ± 9	732 ± 19	521 ± 13	731 ± 13	2.62 ± 0.05	1.87 ± 0.04	2.62 ± 0.07	55.3 ± 6.8
DOCA (n = 9)	260 ± 6*	972 ± 34**	744 ± 31**	1404 ± 46**	3.74 ± 0.10**	2.86 ± 0.099**	5.40 ± 0.15**	103.3 ± 21.9*
DOCA-EPI2 (n = 10)	260 ± 6*	916 ± 24**	683 ± 17**	1396 ± 33**	3.54 ± 0.15**	2.64 ± 0.10**	5.39 ± 0.20**	97.7 ± 21.1*
DOCA-EPI10 (n = 9)	253 ± 8**	917 ± 11**	676 ± 15**	1291 ± 56**	3.65 ± 0.12**	2.69 ± 0.13**	5.12 ± 0.20**	41.1 ± 12.4#

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

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

** $P < 0.01$ compared to the control group.

$P < 0.05$ compared to the DOCA group.

No differences were found among all experimental groups in the concentration–contractile response induced by phenylephrine in aortic rings without endothelium (Fig. 8A). However, in intact aortic rings the vasoconstrictor response to phenylephrine was increased in the DOCA-salt group compared to the control group (Fig. 8B). Chronic treatment with both doses of epicatechin significantly reduced the increased contractile response to phenylephrine in aortas with endothelium. However, vasoconstriction induced by phenylephrine 10^{-6} M was significantly reduced in aortae from the DOCA-salt group compared with control animals (Fig. 8C) when the rings were incubated previously with the NO synthase inhibitor L-NAME, indicating a reduced basal NO formation in DOCA-salt rats. In aortae from DOCA-salt rats treated with epicatechin this contractile response was not significantly reduced, suggesting a higher NO formation in these vessels compared with those from control DOCA-salt rats.

Effects on vascular Akt pathway

Aortas from DOCA-salt rats exhibited decreased levels of Akt (Ser-473) (Fig. 9A) and eNOS (Ser-1177) (Fig. 9B) phosphorylation compared to control rats, without change in total Akt or eNOS levels. Control rats chronically treated with epicatechin showed increased levels of both Akt and eNOS phosphorylation compared to control rats. The phospho-Akt and phospho-eNOS protein expression was also restored by both doses of epicatechin treatment in DOCA-salt rats.

Discussion

The main novel finding of this study is that chronic treatment with epicatechin prevents the progressive increase in SBP, the proteinuria, and the endothelial dysfunction in uninephrectomized rats subjected

to chronic administration of DOCA-salt. The effects on aortic endothelial function were associated with an attenuation of vascular O_2^- content and increased phosphorylation of Akt and eNOS. The reductions in blood pressure and proteinuria were achieved only by the high dose employed and were accompanied by reduced systemic markers of oxidative stress and endothelin-1.

According to previous studies [29,30], we found that both systemic and vascular oxidative stress is present in DOCA-salt-induced hypertension. Renal injury, characteristic of mineralocorticoid-induced hypertension, has been shown to be partly independent of blood pressure [40,41]. Long-term administration of epicatechin at 10 mg kg^{-1} to DOCA-salt rats significantly decreased the systemic markers of oxidative stress (plasma MDA and urinary iso-PGF $_{2\alpha}$) and this was associated with reduced blood pressure and proteinuria. However, epicatechin at the lower dose, which was unable to reduce systemic oxidative stress markers, was also unable to prevent the development of hypertension or reduce protein excretion.

Sustained high blood pressure is a powerful determinant of cardiac and renal hypertrophy development [42]. Accordingly, DOCA-salt hypertensive rats showed increased cardiac, left-ventricular, and kidney weight indices compared to normotensive control rats. However, epicatechin did not reduce significantly any of these indices, despite its preventive effect on the development of hypertension. Thus, we found a dissociation between high blood pressure and cardiac hypertrophy in this model. This suggests the involvement of other trophic stimuli that are unaffected by drugs with antioxidant properties, such as epicatechin (present results), red wine polyphenols, or apocynin [29], or by drugs without antioxidant properties, such as verapamil [43]. In fact, our group reported that rats receiving the same doses of DOCA used in the present study, and drinking water not containing salt, were able to develop renal and cardiac hypertrophy without increasing blood pressure [44].

ET-1 is involved in the development of oxidative stress and hypertension in DOCA-salt rats, because ET $_A$ receptor blockade reduced arterial O_2^- levels with a concomitant decrease in SBP [33]. Recently, it has been reported that epicatechin reduced plasma ET-1 levels in healthy men [45] and in the apolipoprotein E (ApoE $^{-/-}$) gene knockout mouse [46]. In agreement with these results, we found that epicatechin 10 mg kg^{-1} treatment is able to prevent hypertension, reducing plasma ET-1 levels in this low-renin model of hypertension. This inhibitory effect might be mediated via Akt regulation of the ET-1 promoter, as previously suggested in in vitro experiments [35]. In the present study, both doses of epicatechin abolished the increased aortic intracellular O_2^- content measured by ethidium fluorescence but only the higher dose reduced plasma ET-1 levels in DOCA-salt rats, suggesting that other mechanisms could be involved in the protective effects of low doses of epicatechin in vascular oxidative stress, such as (i) interfering the signaling pathway of ET-1-driven O_2^- generation and/or (ii) increasing vascular antioxidant systems.

It is well established that ET-1 activates NADPH oxidase to produce vascular O_2^- generation in DOCA-salt hypertensive rats via upregulation of the NADPH oxidase subunits p22 $^{\text{phox}}$ and p47 $^{\text{phox}}$

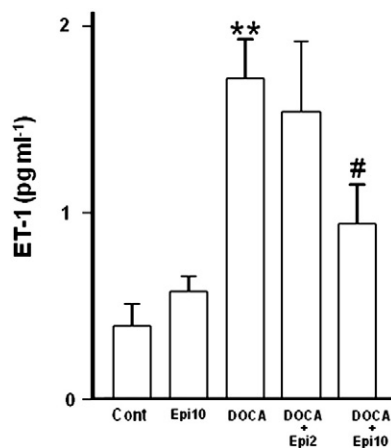


Fig. 2. Plasma ET-1. Values are expressed as means ± SEM (n = 9 or 10). Experimental groups: control (Cont; n = 10), (–)–epicatechin (Epi10; 10 mg kg^{-1} , n = 10), DOCA-salt (n = 9), DOCA-salt + Epi2 (2 mg kg^{-1} , n = 10), and DOCA-salt + Epi10 (10 mg kg^{-1} , n = 9). ** $P < 0.01$ compared to the control group. # $P < 0.05$ compared to the DOCA group.

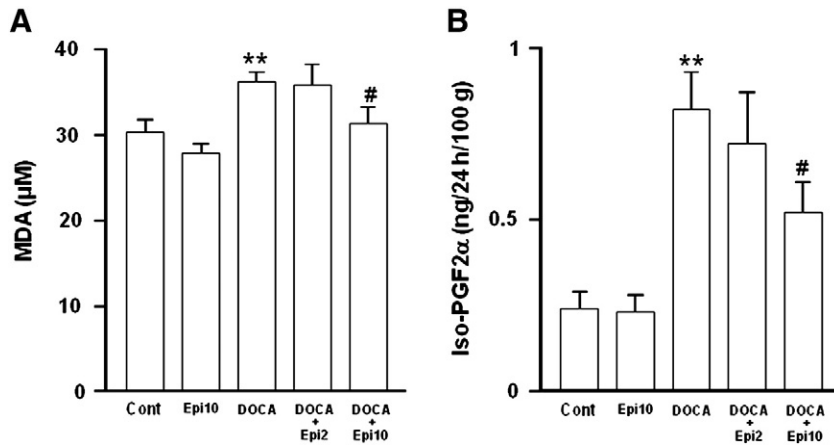


Fig. 3. (A) Plasma MDA levels and (B) 24-h urinary iso-PGF_{2α} excretion. Experimental groups: control (Cont; $n = 10$), (–)-epicatechin (Epi10; 10 mg kg^{-1} , $n = 10$), DOCA-salt ($n = 9$), DOCA-salt + Epi2 (2 mg kg^{-1} , $n = 10$), and DOCA-salt + Epi10 (10 mg kg^{-1} , $n = 9$). Values are expressed as means \pm SEM ($n = 9$ or 10). ** $P < 0.01$ compared to the control group. # $P < 0.05$ compared to the DOCA group.

[28,29,33,47]. ET-1 has also been reported to increase xanthine oxidase and mitochondrial-derived ROS in this model of hypertension [47,48]. We also found that NADPH oxidase activity and the NADPH oxidase subunits, p22^{phox} and p47^{phox}, are increased in the aorta of DOCA-salt hypertensive rats and that this increase is associated with elevated vascular O₂^{•-} production. In vitro, epicatechin reduced NADPH oxidase-driven O₂^{•-} production stimulated by ET-1 in aortic rings through ERK1/2 inhibition [38]. Likewise, in the present study, the high dose of epicatechin prevented the upregulation of p22^{phox} and p47^{phox}, the increased NADPH oxidase activity, and the vascular O₂^{•-} content in DOCA-salt rats. The low dose, however, had no significant effect on NADPH oxidase activity or expression but still prevented the increased vascular O₂^{•-} content, suggesting

that it might not reduce the increased vascular O₂^{•-} production but rather increase the vascular O₂^{•-} metabolism via enhanced antioxidant defenses.

Nrf2 is a transcription factor that regulates the expression of numerous ROS-detoxifying and antioxidant genes. Under basal nonactivated conditions, Nrf2 interacts with Kelch-like erythroid cell-derived protein 1 (Keap-1), a cytosolic repressor protein. Upon activation, the Keap-1–Nrf2 complex is dissociated and Nrf2 translocates to the nucleus, where it binds to the ARE, triggering the transcription of phase II and antioxidant defense enzymes, including NQO1, HMOX1, and GCLC, the rate-limiting enzyme for glutathione synthesis. Nrf2 is activated by oxidative stress, which seems to be a countervailing mechanism to protect tissue from oxidative injury [49]. In agreement with previous reports of

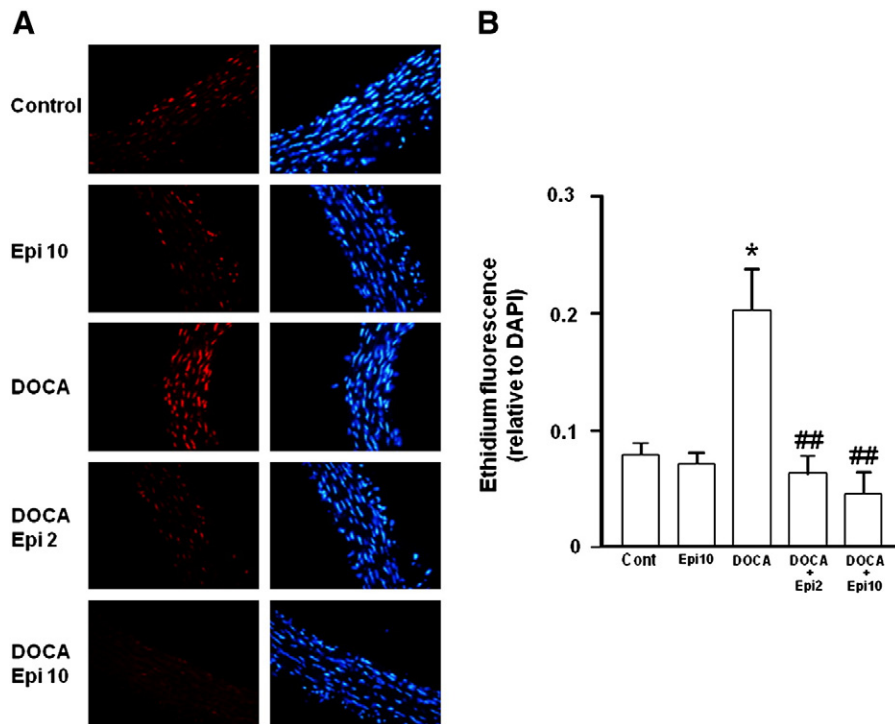


Fig. 4. In situ localization of O₂^{•-} production in aortic rings. (A) Left column shows arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by O₂^{•-}. Right column shows blue fluorescence of the nuclear stain DAPI ($\times 400$ original magnification). (B) Averaged values, means \pm SEM ($n = 5$ or 6), of ethidium red fluorescence normalized to blue DAPI. Experimental groups: control (Cont; $n = 10$), (–)-epicatechin (Epi10; 10 mg kg^{-1} , $n = 10$), DOCA-salt ($n = 9$), DOCA-salt + Epi2 (2 mg kg^{-1} , $n = 10$), and DOCA-salt + Epi10 (10 mg kg^{-1} , $n = 9$). * $P < 0.05$ compared to the control group. ## $P < 0.01$ compared to the DOCA group.

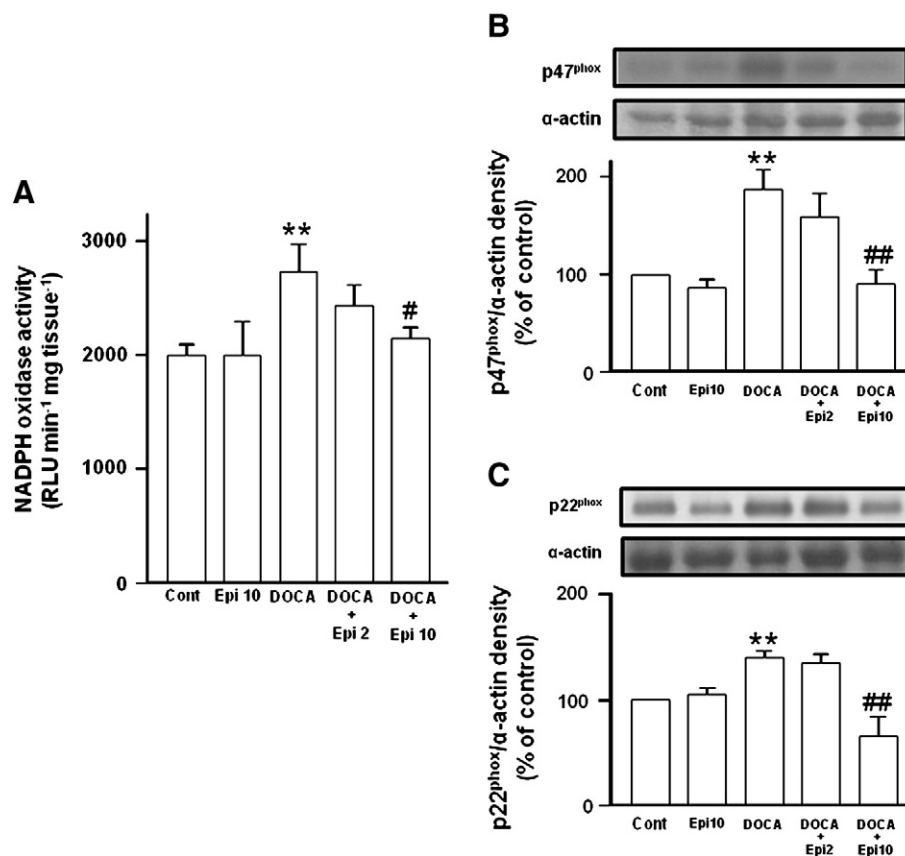


Fig. 5. NADPH oxidase pathway. (A) NADPH oxidase activity measured by lucigenin luminescence and expression of NADPH oxidase subunit proteins (B) p47^{phox} and (C) p22^{phox} in aortic rings from all experimental groups. Histograms represent densitometric values normalized to the corresponding α -actin. Values are expressed as means \pm SEM ($n = 9$ or 10). Experimental groups: control (Cont; $n = 10$), (-)-epicatechin (Epi10; 10 mg kg^{-1} , $n = 10$), DOCA-salt ($n = 9$), DOCA-salt + Epi2 (2 mg kg^{-1} , $n = 10$), and DOCA-salt + Epi10 (10 mg kg^{-1} , $n = 9$). ** $P < 0.01$ compared to the control group. # $P < 0.05$ and ## $P < 0.01$ compared to the DOCA group.

HMOX overexpression in the DOCA-salt rat model [50], we found upregulation of Nrf2, increased Nrf2 protein in the nucleus, and increased mRNA for NQO1, HMOX1, and GCLC. This increase in the Nrf2 pathway is consistent with increased oxidative stress in this model. Recent studies suggest that the Nrf2/ARE pathway can also be activated both pharmacologically and by dietary means [51]. Thus, epicatechin ($10 \mu\text{M}$) increased nuclear translocation of Nrf2 and nuclear content of phosphorylated Nrf2 in HepG2 cells [52]. In this paper we show for the first time that chronic epicatechin increased the expression of Nrf2 and Nrf2 target genes (NQO1, GCLC, and HMOX) in the vascular wall. In aorta from control rats treated with epicatechin at 10 mg kg^{-1} , the increased Nrf2/ARE pathway seems to be unrelated to changes in ROS production. Additionally, it should be mentioned that increased phosphatidylinositol-3-kinase/Akt activity has been linked to the activation of Nrf2 induced by epicatechin in astrocytes [53] and HepG2 cells [52]. We also found that chronic epicatechin increased Akt phosphorylation in the aorta, which might be involved in the Nrf2 upregulation. In our experiments, epicatechin at a dose of 2 mg kg^{-1} , which was unable to reduce vascular NADPH oxidase-derived $\text{O}_2^{\cdot-}$ production, increased the Nrf2 pathway in DOCA-salt rats, leading to reduced $\text{O}_2^{\cdot-}$ content in vessel. These results suggest that epicatechin is a more potent stimulator of the Nrf2/ARE pathway than inhibitor of NADPH oxidase-driven $\text{O}_2^{\cdot-}$ production. However, the high-dose epicatechin in DOCA-salt rats had no effect on the Nrf2 pathway. The increase in Nrf2 activity evoked by the high-dose epicatechin in control rats might be counterbalanced by its inhibitory effects on $\text{O}_2^{\cdot-}$ production in DOCA-salt rats, despite increased Akt phosphorylation found in these vessels.

Endothelial dysfunction is usually associated with hypertension and it is a risk factor (independent of blood pressure) for major cardiovascular events. Our findings are compatible with previous studies showing

that endothelium-dependent vasodilator response induced by acetylcholine is altered in conductance vessels in DOCA-salt hypertension [29,43,50]. Endothelial dysfunction was unrelated with changes in the guanylate cyclase/cGMP pathway or with changes in eNOS expression, because responses to the NO donor sodium nitroprusside and eNOS protein were unchanged. In agreement with previous findings in this model [29,43,54], endothelial dysfunction was associated with an increased vascular $\text{O}_2^{\cdot-}$ content and decreased phosphorylated eNOS levels in aortic rings. Chronic epicatechin at the two doses analyzed restored the impaired endothelial function and this can be attributed to increase eNOS phosphorylation at the activation site (Ser-1177) and also by a reduction in $\text{O}_2^{\cdot-}$ content at the vascular wall (as shown by DHE fluorescence), and thereby protection of NO from inactivation. It should be noted that improvement of vascular oxidative stress and aortic endothelial function is not necessarily accompanied by reduced blood pressure (e.g., NADPH oxidase inhibition attenuates vascular oxidative stress but not hypertension produced by chronic ET-1 [55]). Accordingly, the low-dose epicatechin affected the former parameters but not the latter.

In conclusion, these results demonstrate that chronic treatment with epicatechin reduces hypertension, proteinuria, and endothelial dysfunction in this model of DOCA-salt hypertension. This study supports epidemiological evidence and intervention studies on antihypertensive effects and the improvement in endothelial function by flavanol-enriched food.

Acknowledgments

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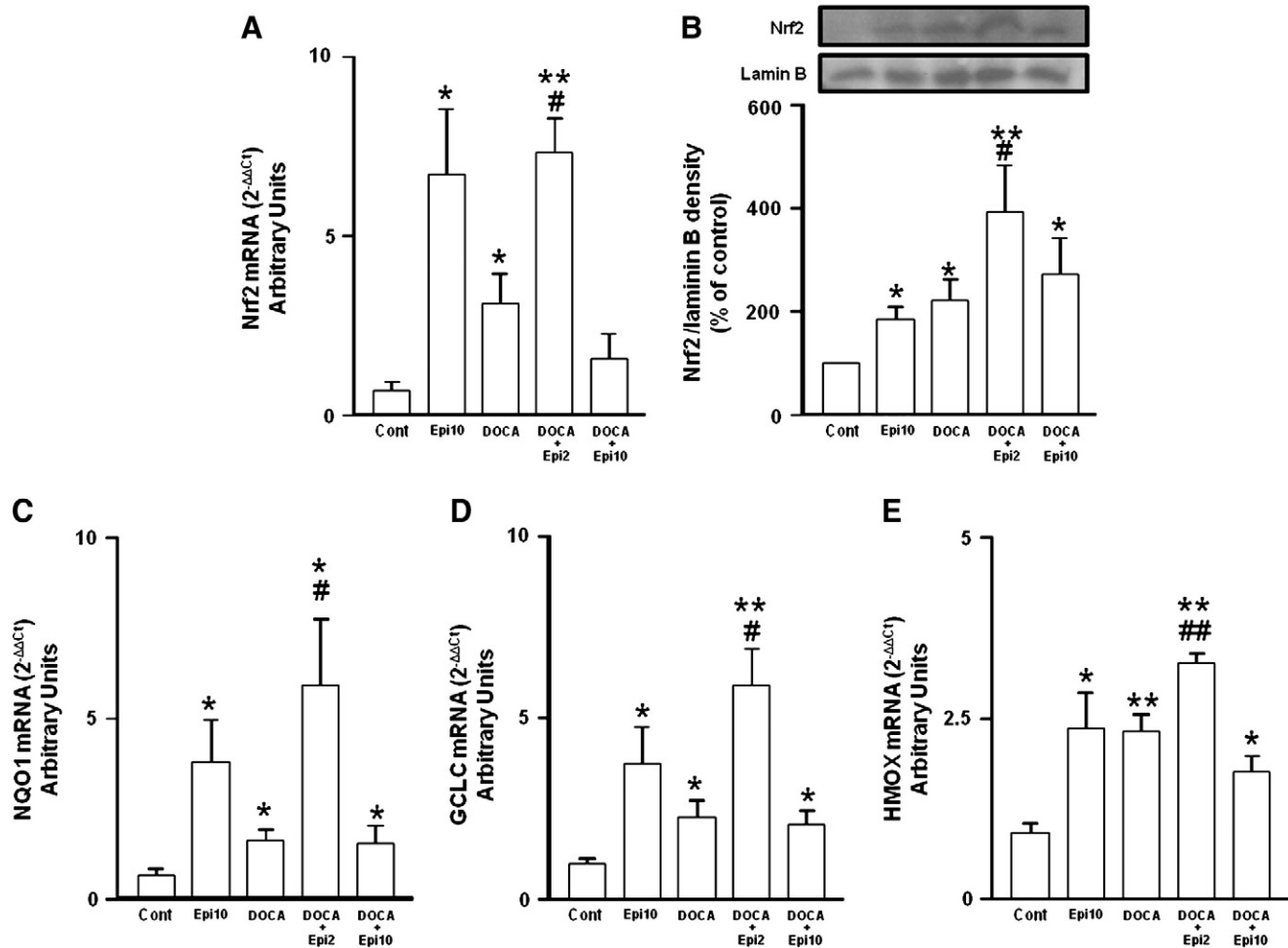


Fig. 6. Nrf2/ARE pathway. mRNA expression of (A) Nrf2, (C) NQO1, (D) GCLC, and (E) HMOX1 and (B) Nrf2 protein expression in the nuclear fraction in aortic rings from all experimental groups. Data are presented as the ratio of arbitrary units of mRNA (2^{-ΔΔCT}). Values are expressed as means ± SEM (n = 9 or 10). Experimental groups: control (Cont; n = 10), (-)-epicatechin (Epi10; 10 mg kg⁻¹, n = 10), DOCA-salt (n = 9), DOCA-salt + Epi2 (2 mg kg⁻¹, n = 10), and DOCA-salt + Epi10 (10 mg kg⁻¹, n = 9). (B) shows representative bands, and the histograms represent densitometric values (n = 5) of Nrf2 relative to nuclear protein laminin B. *P < 0.05 and **P < 0.01 compared to the control group. #P < 0.05 and ##P < 0.01 compared to the DOCA group.

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(Red HERACLES RD06/0009), Spain. María José Zarzuelo, Pilar Galindo, Miguel Romero, Rocío López-Sepulveda, and Ana María Quintela are holders of studentships from the Spanish Ministry of

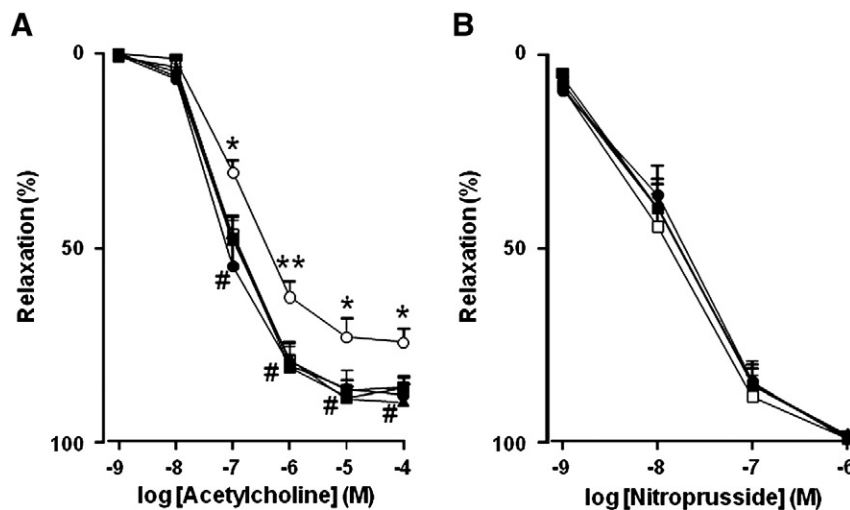


Fig. 7. Effects on relaxant responses. Vasorelaxation induced by (A) acetylcholine and (B) sodium nitroprusside in aortae with or without endothelium, respectively, in aortic rings from all experimental groups. Relaxant responses were analyzed in arteries previously contracted by 10⁻⁶ M phenylephrine. Values are expressed as means ± SEM (n = 9 or 10). Experimental groups: control (□; n = 10), DOCA (○; n = 9), epicatechin 10 mg kg⁻¹ (■; n = 10), DOCA-epicatechin 2 mg kg⁻¹ (●; n = 10), and DOCA-epicatechin 10 mg kg⁻¹ (▲; n = 9). *P < 0.05 and **P < 0.01 compared to the control group. #P < 0.05 compared to the DOCA group.

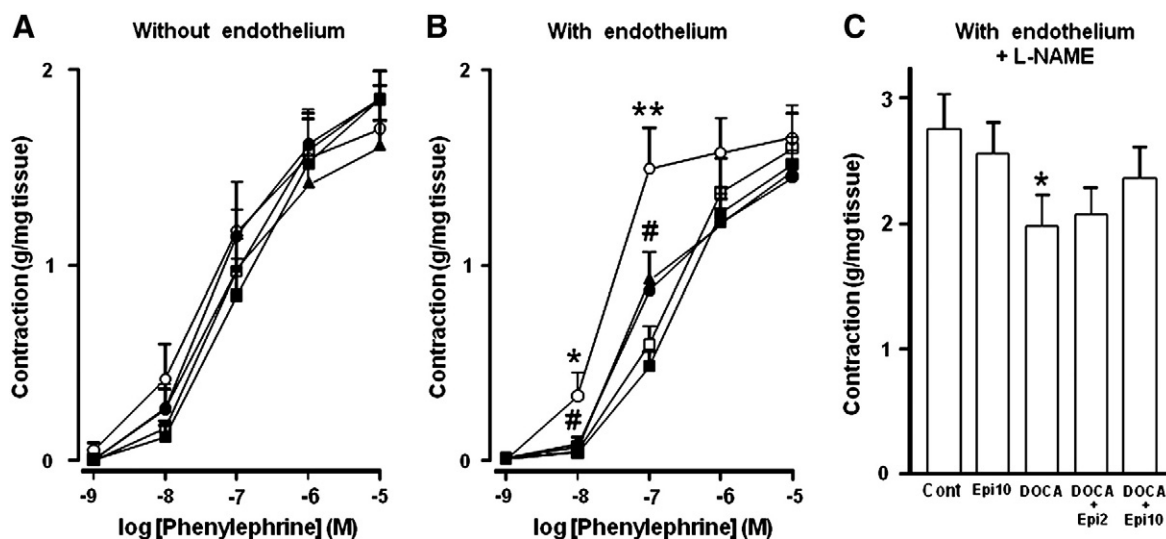


Fig. 8. Effects on contractile responses. Vasoconstrictor responses induced by phenylephrine in aortic ring (A) without and (B) with endothelium and (C) with endothelium plus L-NAME 10^{-4} M in aortic rings from all experimental groups. Values are expressed as means \pm SEM ($n = 9$ or 10). Experimental groups: control (\square ; $n = 10$), DOCA (\circ ; $n = 9$), epicatechin 10 mg kg^{-1} (\blacksquare ; $n = 10$), DOCA-epicatechin 2 mg kg^{-1} (\bullet ; $n = 10$), and DOCA-epicatechin 10 mg kg^{-1} (\blacktriangle ; $n = 9$). * $P < 0.05$ and ** $P < 0.01$ compared to the control group. # $P < 0.05$ compared to the DOCA group.

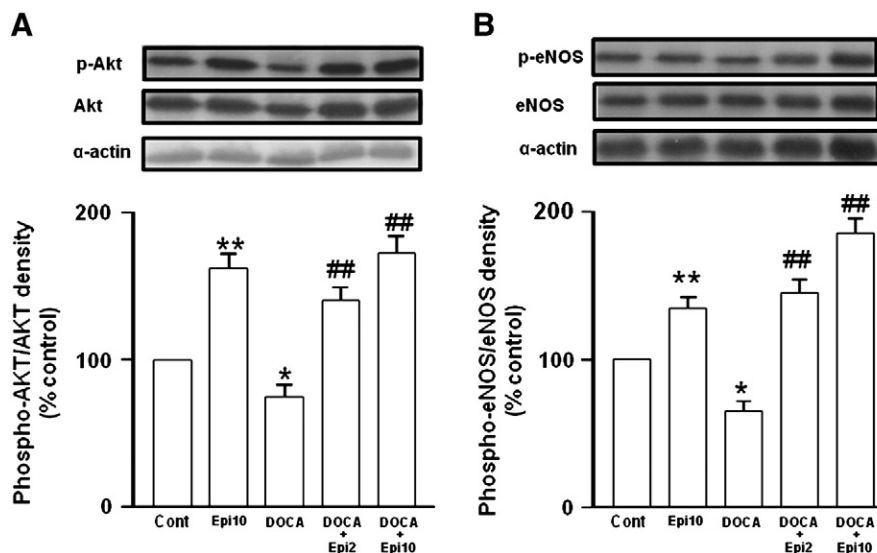


Fig. 9. Effects of epicatechin on Akt and eNOS pathways. (A and B) Representative bands and histograms represent densitometric values of phospho-Akt and phospho-eNOS relative to total Akt and eNOS protein levels, respectively, in aortic rings from all experimental groups. Values are expressed as means \pm SEM ($n = 4-6$). Groups: control (Cont; $n = 10$), (–)epicatechin (Epi10, 10 mg kg^{-1} , $n = 10$), DOCA-salt ($n = 9$), DOCA-salt + Epi2 (2 mg kg^{-1} , $n = 10$), and DOCA-salt + Epi10 (10 mg kg^{-1} , $n = 9$). * $P < 0.05$ and ** $P < 0.01$ compared to the control group. ## $P < 0.01$ compared to the DOCA group.

Science and Education and Manuel Gómez-Guzmán holds a studentship from the Junta de Andalucía.

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