Original Contribution

Blood pressure-lowering effect of dietary (−)-epicatechin administration in L-NAME-treated rats is associated with restored nitric oxide levels

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ABSTRACT

Epidemiological and intervention studies have shown that the intake of certain chocolates or cocoa products decreases blood pressure (BP) in humans. (−)-Epicatechin is the most abundant flavanol present in cocoa seeds and its derived foods. This work investigates the effects of dietary (−)-epicatechin on BP in rats that received N^{ω}-nitro-L-arginine methyl ester (L-NAME) for 4 days. (−)-Epicatechin administration prevented the 42 mm Hg increase in BP associated with the inhibition of NO production in a dose-dependent manner (0.2–4.0 g/kg diet). This BP effect was associated with a reduction in L-NAME-mediated increase in the indexes of oxidative stress (plasma TBARS and GSSG/GSH[2] ratio) and with a restoration of the NO concentration. At the vascular level, none of the treatments modified NOS expression, but (−)-epicatechin administration avoided the L-NAME-mediated decrease in eNOS activity and increase in both superoxide anion production and NOX subunit p47[phox] expression. In summary, (−)-epicatechin was able to prevent the increase in BP and in oxidative stress and restored NO bioavailability. The fact that (−)-epicatechin is present in several plants usually consumed by humans gives the possibility of developing diets rich in those plants or pharmacological strategies using that flavonoid to diminish BP in hypertensive subjects.

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A high consumption of fruits and vegetables has been associated with a decreased risk for cardiovascular disease in several epidemiological studies and clinical trials [1–6]. Some studies have associated those benefits with general flavonoid intake [7–9] and others with specific flavonoid-containing food consumption, i.e., cocoa or chocolate [10,11]. Regarding vascular function and hypertension, several intervention studies have shown that the intake of certain chocolates or cocoa products decreases blood pressure (BP)[1] in humans [12–16].

The pathophysiology of essential hypertension has been associated with several abnormal endothelial conditions. One of them is the impairment of the endothelium-dependent vasorelaxation caused by a loss of nitric oxide (NO) bioavailability. Under physiological conditions, most NO in the vascular wall is produced at the endothelial cells by the endothelial nitric oxide synthase (eNOS) [17]. Vascular NO steady-state concentration is determined not only by its synthesis but also by its reaction with other chemical species, including superoxide anion. Cellular sources of superoxide anion comprise the mitochondrial electron transport and the activities of NADPH oxidase (NOX), xanthine oxidase, cyclooxygenase/lipoxygenase, and cytochrome P450 [18]. Under particular conditions uncoupled NOS can also generate superoxide anion [19]. The relative physiological importance of the various sources of superoxide anion is highly dependent on the different cell types and conditions. Superoxide anion is mainly generated by NOX activity in the vascular wall at the level of the endothelium, the smooth muscle, and the adventitia [20].

An association between the regulation of vascular function by flavanol-rich foods and BP has been consistently observed. Several clinical trials showed hypotensive and/or antihypertensive effects of cocoa and flavanols, associated with increases in flow-mediated dilation (reviewed in [21]). In select studies a decrease in BP was associated with increases in plasma markers of NO production, i.e., S-nitrosoglutathione [22] and nitrites [23]. The administration of (−)-epicatechin, the most abundant

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[1] Abbreviations used: BP, blood pressure; GSH, reduced glutathione; GSSG, oxidized glutathione; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NNA, N^{ω}-nitro-L-arginine; MDA, malondialdehyde; NOS, nitric oxide synthase; NOX, NADPH oxidase; PBS, phosphate-buffered saline.

flavanol present in cocoa, mimics the effects of cocoa administration in terms of improving flow-mediated dilation and increasing plasma NO metabolites [24]. Additionally, studies in cultured endothelial cells support a role for (−)-epicatechin in the improvement of vascular endothelial function. (−)-Epicatechin could cause an increase in NO bioavailability through an increase in NO generation [25] and/or a decrease in superoxide anion production [26].

This work investigates the effects of dietary (−)-epicatechin administration on BP in a rat model of decreased NO production induced by the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME). We observed that (−)-epicatechin prevented the increase in BP associated with the inhibition of NO production, and such prevention was associated with a reduction in oxidant production and a restoration of NO bioavailability.

Materials and methods

Materials

Primary antibodies for eNOS (sc-654), p47^{phox} (sc-7660), and β-actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated rabbit anti-goat was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). γ-Glutamylglutamate was obtained from MP Biomedicals Corp. (Irvine, CA, USA). L-NAME, (−)-epicatechin, butylhydroxytoluene, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane, β-glucuronidase, sulfatase, heparin sodium salt, bathophenanthroline disulfonate sodium salt, iodoacetic acid, NADPH, nitrate reductase, glucose-6-phosphate dehydrogenase, glucose 6-phosphate, sulfanilamide, *N*-naphthylethylenediamine, reduced glutathione (GSH), oxidized glutathione (GSSG), *N,N′*-dimethyl-9,9′–biacridinium dinitrate (lucigenin), and bovine seroalbumin were from Sigma Chemical Co. (St. Louis, MO, USA). Heptane sulfonic acid was from Taylor Scientific (St. Louis, MO, USA). Dansyl chloride was from Fisher Scientific (Fair Lawn, NJ, USA). L-[^{14}C]Arginine was from PerkinElmer Life and Analytical Sciences (Boston, MA, USA).

Animals and treatments

Male Sprague–Dawley rats (130–140 g) were individually housed in suspended stainless steel cages in a temperature- (23 °C) and photoperiod- (14–10 h light–dark cycle) controlled room. Housing, handling, and experimental procedures followed the rules written in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (DHEW Publication No. (NIH) 85-23, revised in 1996, Office of Science and Health Reports, Division of Research Resources/NIH, Bethesda, MD, USA) and were approved by the Laboratory Animal Use and Care Committees of the University of California at Davis and the University of Buenos Aires. The experiments were performed in four sets of eight rats per experimental group. After a week of adaptation to the diet and housing conditions, treatments were started through the administration of L-NAME in the drinking water (360 mg/L) [27,28] and/or (−)-epicatechin added to the basal diet at the indicated final concentrations. Animals were randomly assigned to three groups: one group (control) received basal diet and water; the second group (L-NAME) received basal diet and water containing L-NAME; the third group (EPI) received basal diet supplemented with (−)-epicatechin and water containing L-NAME. The basal diet was prepared as described [29] and supplemented with 0.2, 1.0, or 4.0 g/kg of (−)-epicatechin at the expense of dextrose as necessary. For some of the experiments, a fourth group receiving basal diet supplemented with (−)-epicatechin (4.0 g/kg) and water was included. To prevent

oxidation and loss of antioxidants, diets were prepared 24–48 h before their use. Animals consumed the diets and fluids ad libitum during the period of time indicated for each experiment. Body weight and food and water consumption were measured daily.

Blood pressure determinations

Systolic BP (SBP) was measured in preconditioned, conscious, prewarmed, and restrained rats by noninvasive tail-cuff plethysmography (SC1000; Hatteras Instruments, Cary, NC, USA). To minimize occasional stress-induced variations in BP, all measurements were taken by the same person in the same environment.

Sample collection

At the end of the experimental periods, animals were anesthetized with CO_2, and blood was collected by cardiac puncture. The animals were euthanized, and hearts and aortas were excised and cleaned of connective and adipose tissue. Blood was divided into two aliquots, one processed for GSH/GSSG determination and the other centrifuged at 6000g for 1 min to obtain the plasma. Aortas were rapidly and carefully dissected, placed into Krebs solution (20 mM Hepes buffer, pH 7.4, containing 119 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 5 mM $NaHCO_3$, 1.25 mM $CaCl_2$, 5.5 mM glucose), and cut into rings of approximately 1 mm for subsequent immunohistochemistry or biochemical procedures.

HPLC analysis of N^{ω}-nitro-L-arginine, malondialdehyde, (−)-epicatechin, and glutathione

HPLC measurements were carried out on an Agilent HPLC 1100 series apparatus with a quaternary pump, autosampler, sample thermostat, and UV/Vis and fluorescence detectors (Agilent Technologies, Santa Clara, CA, USA).

N^{ω}-nitro-L-arginine (L-NNA)
Plasma aliquots were filtered (Centricon filters, MWCO 30,000), and L-NNA was quantified in the filtrate by isocratic reverse-phase HPLC on a C-8 column (mobile phase 90% (v/v) 18.5 mM heptane sulfonic acid and 10% (v/v) acidified methanol, pH 2.7) [30].

Malondialdehyde (MDA)
Immediately after blood centrifugation, plasma samples were supplemented with 0.2% (w/v) butylhydroxytoluene in ethanol and 2.8% (w/v) trichloroacetic acid and the resulting suspension was centrifuged at 4000g for 10 min. The supernatant was mixed with 0.6% (w/v) thiobarbituric acid, heated for 45 min at 90 °C, and then centrifuged at 4000g for 10 min at 4 °C. Derivatized MDA was resolved and quantified by isocratic reverse-phase HPLC on a Supelcosil LC-18 column (Supelco Analytical, Bellefonte, PA, USA; mobile phase 65% (v/v) 50 mM KH_2PO_4 (pH 7.0) and 35% (v/v) methanol). Detection was done fluorimetrically ($\lambda_{ex}=515$ nm; $\lambda_{em}=555$ nm). 1,1,3,3-Tetramethoxypropane was used to prepare the MDA standard [31].

(−)–Epicatechin
Plasma samples were treated with the enzymes β-glucuronidase and sulfatase and extracted as we previously described [32]. Extracted samples were resolved by reverse-phase HPLC on a Supelcosil LC-18 column (Supelco Analytical) with fluorimetric detection ($\lambda_{ex}=276$ nm; $\lambda_{em}=316$ nm) [24].

GSH/GSSG

Immediately after drawing, blood samples were mixed with a preservation solution (100 mM borate, pH 8.5, containing 100 mM serine, 10 mM heparin sodium salt, 1.9 mM bathophenanthroline disulfonate sodium salt, and 20 mM iodoacetic acid) [33]. Samples were centrifuged at 3000g for 30 s. The supernatant was separated and perchloric acid (5% w/v), boric acid (0.2 M), and γ-glutamylglutamate (10 μM) as internal standard were added to it. After derivatization with dansyl chloride, the samples were resolved by HPLC using a Supelcosil LC-NH$_2$ column (Supelco Analytical). A two-solvent gradient (A, 80% (v/v) methanol/water; B, acetate-buffered methanol, pH 4.6) was generated as described [33]. Detection of GSH and GSSG was done fluorimetrically ($\lambda_{ex}=330$ nm; $\lambda_{em}=530$ nm).

Nitrites and nitrates

NO$_2^-$ and NO$_3^-$ content was determined by Griess colorimetric assay, after enzymatic reduction as described [34]. Plasma samples were processed to make the determination equivalent to GC–MS techniques [35].

Determination of vascular NOS activity

Vascular NOS activity was measured using L-[^{14}C]arginine as substrate in tissue slices [36]. Specific NOS activity was assessed in the presence of 10^{-4} M L-NAME. Nitric oxide production (measured as pmol of L-[^{14}C]citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal periods of time and expressed as pmol/g min.

Determination of vascular NADPH-dependent superoxide anion production

NADPH-dependent superoxide anion production by aorta homogenates was measured using the lucigenin-enhanced chemiluminescence assay [37]. Samples were placed in vials containing warm (37 °C) Krebs solution and NADPH (40 μM) and lucigenin (5 μM) were added to them. Light emission was measured for 10 min using an LKB Wallac 1209 Rackbeta liquid scintillation counter in the chemiluminescence mode, and the area under the curve was calculated and expressed as relative units/mg protein.

Western blots

Aorta homogenates were added with 5× solution of SDS-sample buffer (200 mM Tris–HCl buffer, pH 6.8, containing 5% (w/v) SDS, 50% (v/v) glycerol, 12.5% (v/v) β-mercaptoethanol, and 0.025% (w/v) bromophenol blue), and they were then heated at 95 °C for 2 min. Sample aliquots containing 30 or 70 μg protein (for eNOS or p47^{phox}, respectively) were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run simultaneously. Membranes were blotted for 2 h in 5% (w/v) nonfat milk and incubated overnight in the presence of the corresponding antibodies (1:500 dilution) in 5% (w/v) bovine serum albumin in 10 mM sodium phosphate, 145 mM NaCl, pH 7.6 (PBS buffer) containing 0.1% (v/v) Tween 20. After incubation for 90 min at room temperature in the presence of the corresponding horseradish peroxidase-conjugated secondary antibody, complexes were detected using the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Films were scanned and densitometric analysis was performed using ImageJ (National Institutes of Health). Protein band densities were normalized to β-actin content.

Immunohistochemistry

Aortas were postfixed in a solution of 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature, followed by through washing in PBS and cryoprotection in 30% (w/v) sucrose in PBS for 24 h. Tissues were frozen at −80 °C and subsequently sectioned (16 μm) using a Leica CM 1850 cryotome (Leica Microsystems, Buffalo Grove, IL, USA). For immunohistochemistry, cryotome sections were rinsed twice with PBS, followed by PBS 0.3% (v/v) Tween 20 (PBST), and then blocked for 2 h at 37 °C with a solution containing 5% (w/v) bovine serum albumin in PBST. Incubation with the primary antibody for p47^{phox} (1:50) was done overnight at 4 °C in a humidified chamber. Sections were incubated with a Cy3-conjugated rabbit anti-goat antibody (1:1000) for 2 h, and cell nuclei were stained with Hoechst 33342 [38]. Microscopic observations were done in a laser spectral confocal microscope (Olympus FV 1000; Olympus Corp., Tokyo, Japan) provided with a Cool-Snap digital camera, to evaluate colocalization of green elastin autofluorescence and p47^{phox} protein. Integrated optical density was measured as relative to area and was determined for each condition in five randomly selected fields. Evaluation of the data was carried out using the ImagePro Plus version 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data were analyzed by one-way analysis of variance using StatView 5.0 (SAS Institute, Cary, NC, USA). Fisher's least significant difference test was used to examine differences between group means. A $p < 0.05$ was considered statistically significant. Data are shown as means ± SEM.

Results

Initial and final body weights, heart weight, cardiac weight index, and the average amount of beverage and food consumed during the experimental period are shown in Table 1. All these parameters were not significantly affected by the treatments.

The amounts of L-NAME and (−)-epicatechin consumed were calculated considering food and beverage daily consumption. Rats in the L-NAME and EPI groups consumed 39 ± 1 and 40 ± 1 mg of L-NAME/kg body wt/day, respectively. The plasma concentration of the L-NAME active metabolite, L-NNA, was similar in the L-NAME and EPI groups (Table 1). Rats that did not receive L-NAME had no detectable levels of L-NNA in plasma. In the EPI group the calculated amount of (−)-epicatechin consumed was 304 ± 16 mg/kg body wt/day, which resulted in a plasma (−)-epicatechin level of 7.0 μM (sum of free (−)-epicatechin plus sulfate and glucuronide derivatives) (Table 1). Rats that did not

Table 1
Body weight, cardiac weight index, beverage and food consumption, and N^{ω}-nitro-L-arginine and (−)-epicatechin concentrations in plasma from the treatments.

Parameter	Control	L-NAME	EPI
Initial weight (g)	152 ± 2	152 ± 2	152 ± 3
Final weight (g)	171 ± 3	182 ± 7	179 ± 8
Heart weight/body weight	4.2 ± 0.4	4.0 ± 0.1	3.9 ± 0.2
Beverage consumption (ml/day)	27 ± 2	25 ± 2	24 ± 2
Food consumption (g/day)	17 ± 1	16 ± 1	14 ± 2
Plasma N^{ω}-nitro-L-arginine (μM)	nd	39 ± 9	50 ± 7
Plasma (−)-epicatechin (μM)	nd	nd	7.0 ± 0.9

In the EPI group (−)-epicatechin was administered at 4.0 g/kg of diet. Measurements were done after 4 days of treatment. nd, value under the detection limit in the assayed condition. Values are shown as means ± SEM ($n=8$ per group).

receive (−)-epicatechin had no detectable plasma (−)-epicatechin. To further evaluate potential interactions between L-NAME and (−)-epicatechin, an additional experimental group of rats received (−)-epicatechin-supplemented diet and nonsupplemented water. In this group (−)-epicatechin plasma concentration at the end of the 4-day treatment was similar to that measured in the EPI group (Table 1). These results show that there were no significant interactions between L-NAME and (−)-epicatechin absorption.

SBP was similar in the three experimental groups at the beginning of the treatment and did not significantly change along the treatment period in the control group (Fig. 1A). At the end of the 4-day treatment, the L-NAME group showed a significant increase in SBP with respect to day 0 (140 ± 10 vs 98 ± 4 mm Hg). In the EPI group (at a dose of 4.0 g/kg diet) the increase in SBP induced by L-NAME was prevented, being the SBP at day 4 106 ± 7 mm Hg (Fig. 1A). The antihypertensive effect of (−)-epicatechin on the L-NAME-induced increase in SBP was dose-dependent (Fig. 1B).

Fig. 2 shows the effects of (−)-epicatechin removal from the diet after treating the rats with L-NAME and (−)-epicatechin for 4 days. Under this experimental condition, two groups of rats

were treated with L-NAME and fed with either a basal diet or a (−)-epicatechin-supplemented diet (1.0 g/kg diet). As expected, after 4 days of treatment, SBP in rats receiving simultaneously L-NAME and (−)-epicatechin was significantly lower than in the L-NAME group (112 ± 6 vs 139 ± 6 mm Hg, respectively, $p < 0.01$). After cessation of (−)-epicatechin administration, SBP increased progressively reaching, after 4 days, values similar to those of L-NAME-treated group. These results constitute a proof of concept that (−)-epicatechin is responsible for the observed decrease in SBP.

Because of the lack of specificity of the oxidative stress markers, we combined GSH and GSSG, and MDA measured by HPLC, to appraise levels of plasma oxidation. The GSSG/GSH^2 ratio was 67% higher in the L-NAME group compared to the control group (Fig. 3A). This higher ratio is due to increased GSSG, given that GSH levels were not significantly affected by the treatments. In the EPI group the GSSG/GSH^2 ratio was similar to that of the control group. Plasma MDA concentration was 31% higher in the L-NAME group compared to the control and EPI groups (Fig. 3B). These results indicate that the systemic oxidative stress induced by L-NAME treatment was prevented by (−)-epicatechin dietary administration.

NO availability was evaluated by measuring plasma NO metabolites. The L-NAME group showed lower levels (−48%) of NO metabolites in plasma than the control group, as expected based on NOS inhibition by L-NAME. Plasma concentrations of NO metabolites were similar in the control and EPI groups (Fig. 4). To examine whether treatments affected vascular NOS, both enzyme activity and protein levels were measured in aorta. NOS activity was decreased in the L-NAME group compared to the control and EPI groups (Fig. 5A), but similar eNOS expression was observed in the three experimental groups (Fig. 5B).

The relevance of aortic NOX was studied by measuring NADPH-driven superoxide anion-dependent lucigenin chemiluminescence and the expression of the p47^{phox} NOX subunit. NADPH-stimulated chemiluminescence was significantly higher (56%) in the L-NAME group compared to both control and EPI groups (Fig. 6A). The expression of p47^{phox} was 73% higher in the L-NAME group than in the control and EPI groups (Fig. 6B). p47^{phox} expression in aorta was also studied by immunohistochemistry. L-NAME treatment led to a 58% increase in p47^{phox}
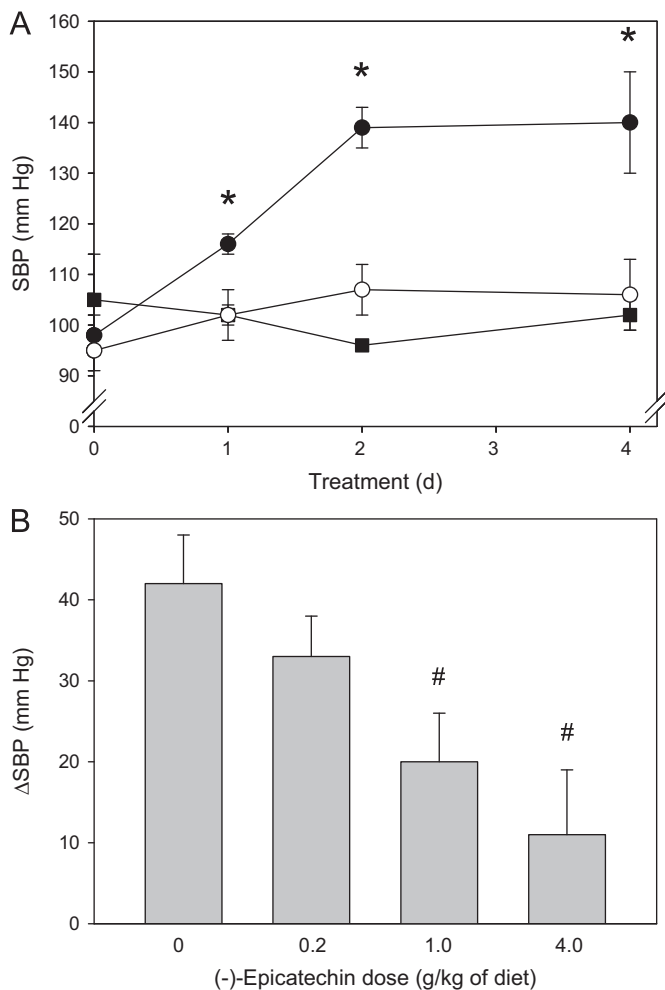

Fig. 1. Effect of dietary (−)-epicatechin on systolic blood pressure (SBP). (A) Evolution of SBP in the control (■), L-NAME (●), and EPI (○) groups. L-NAME was administered in the drinking water and (−)-epicatechin was administered in the diet (4.0 g/kg diet). (B) Variations in SBP (ΔSBP=SBP at day 4 − SBP at day 0) in rats treated with L-NAME and various doses of (−)-epicatechin in the diet. Values are expressed as means ± SEM ($n=8$ per group). *$p < 0.05$ respect to the control and EPI groups. #$p < 0.05$ respect to the L-NAME group.
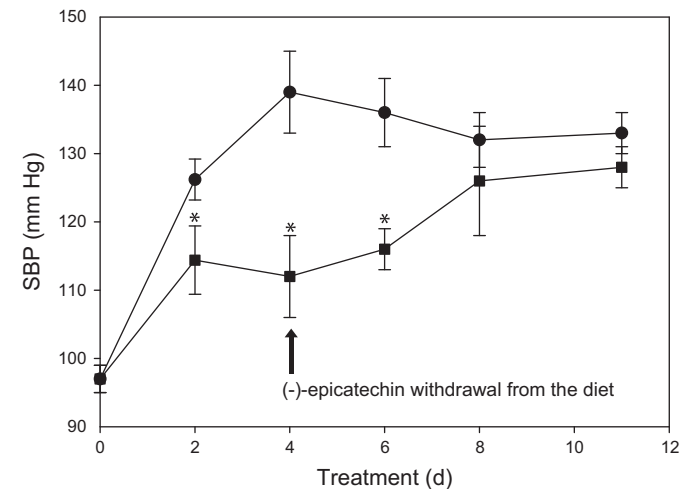
Fig. 2. Effect of (−)-epicatechin withdrawal on systolic blood pressure (SBP). Evolution of the SBP in the L-NAME (●) and EPI (■) groups. L-NAME was administered in the water and (−)-epicatechin was administered in the diet (1.0 g/kg diet). Rats in the EPI group were changed after 4 days to a basal diet for the next 7 days. Values are expressed as means ± SEM ($n=4$ per group). *$p < 0.05$ with respect to the L-NAME group.
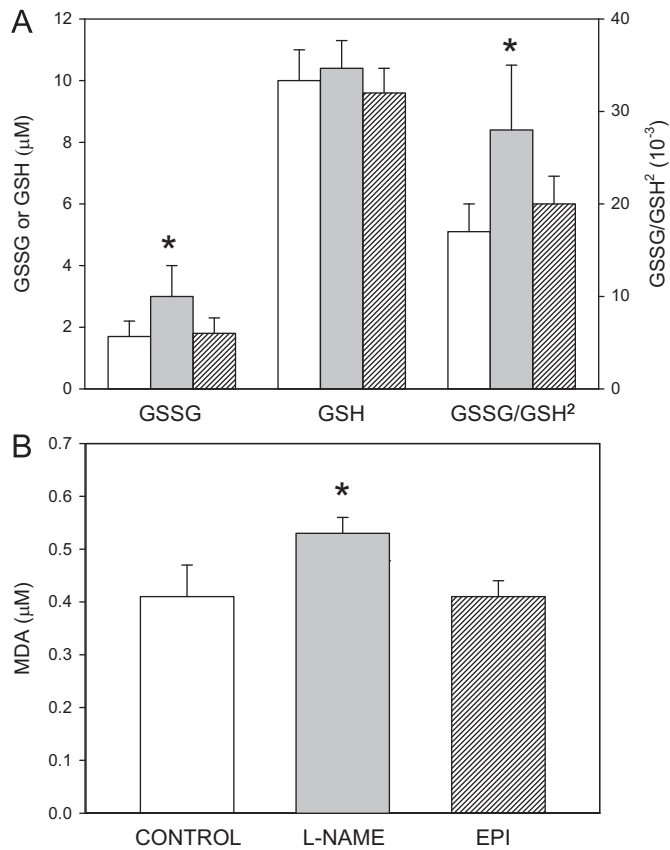
Fig. 3. Effects of dietary (−)-epicatechin on oxidative stress markers. (A) Plasma GSH, GSSG, and GSSG/GSH^2 ratio and (B) malondialdehyde (MDA) levels in control (white bars), L-NAME (gray bars), and EPI (striped bars) groups. Determinations were done after 4 days on the corresponding treatment as described under Materials and methods. Values are expressed as means ± SEM ($n=8$ per group). *$p < 0.05$ with respect to the control and EPI groups.
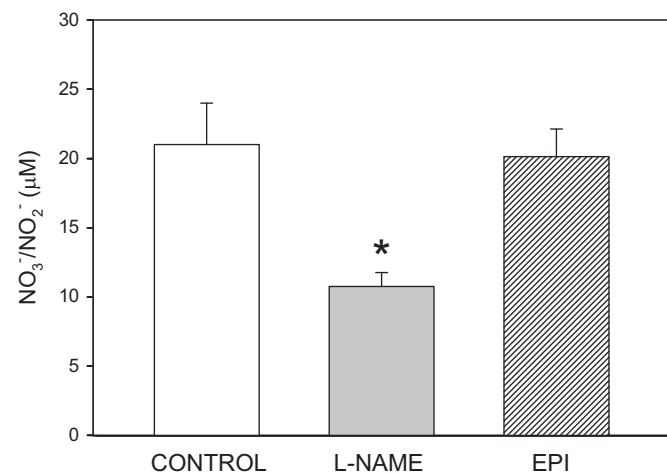
Fig. 4. Effects of dietary (−)-epicatechin on plasma nitric oxide metabolites. Plasma nitrate/nitrite (NO$_3^-$ and NO$_2^-$) content in control, L-NAME, and EPI groups. Determinations were done after 4 days on the corresponding treatments as described under Materials and methods. Values are expressed as means ± SEM ($n=8$ per group). *$p < 0.05$ with respect to the control and EPI groups.

immunostaining levels in the aorta compared to the control and EPI groups (Fig. 7A). The localization of the increased NOX protein was observed mostly at the media layer.
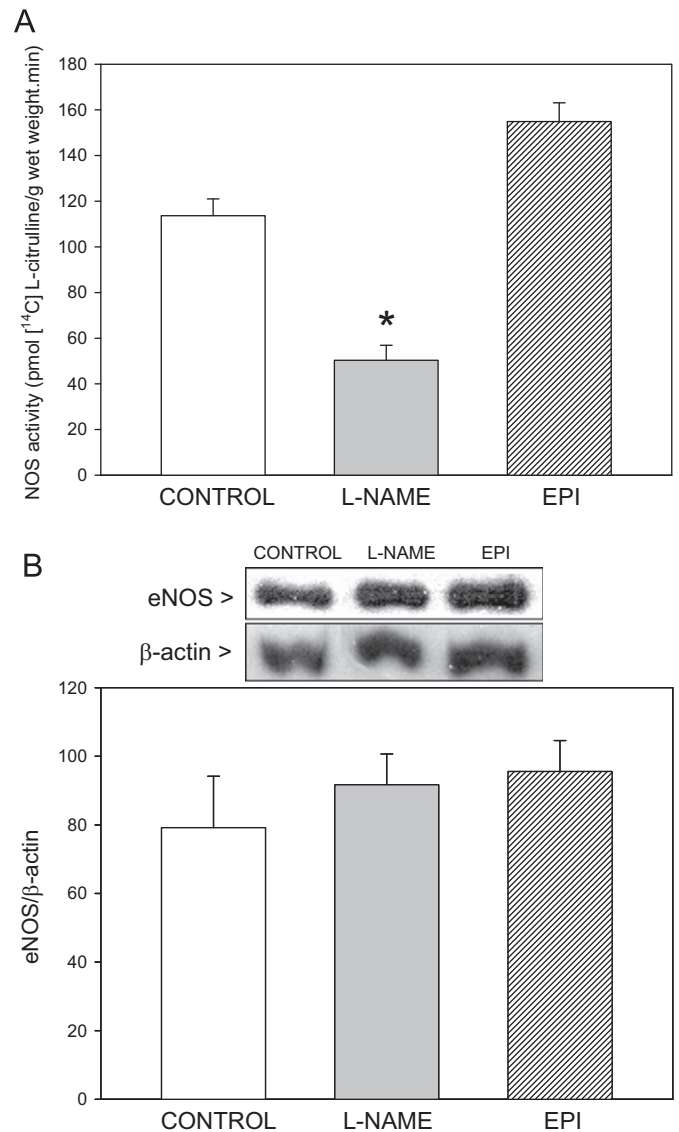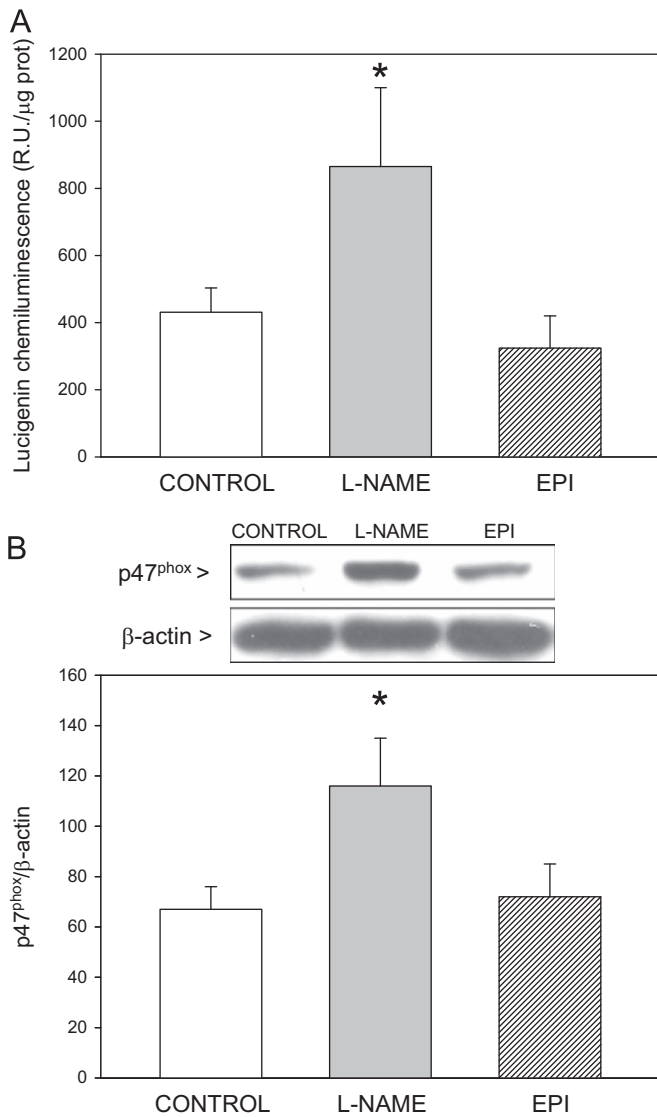

Fig. 5. Effect of dietary (−)-epicatechin on aorta NOS activity and eNOS expression. (A) NOS activity in aorta slices from control, L-NAME, and EPI groups. (B) Western blot for eNOS in aortic homogenates from control, L-NAME and EPI groups. β-Actin levels were measured as loading controls. Determinations were done after 4 days on the corresponding treatments as described under Materials and methods. Values are expressed as means ± SEM ($n=4$ per group for NOS activity, and $n=8$ for eNOS expression). *$p < 0.05$ with respect to the control and EPI groups.

Discussion

This work shows that the dietary administration of (−)-epicatechin prevents or attenuates the increase in BP induced by L-NAME in rats. The mechanisms associated with this effect were investigated considering that L-NAME treatment increases BP by causing NO deficiency and oxidative stress.

The effects of the dietary administration of flavonoids on vascular health have been tested at various doses. In rodent models, flavonoids have been provided with the diet at concentrations of up to 30 g/kg diet [39–42]. The 0.2–4.0 g of (−)-epicatechin/kg diet used in this study is high compared with human consumption but has no toxic actions. In this regard, the present daily consumption provides an amount of (−)-epicatechin significantly lower than the NOAEL (no observed adverse effect level) established for rats fed other flavonoids, i.e., rutin [43] or green tea catechins [44].

Fig. 6. Effect of dietary (−)-epicatechin on aorta NADPH-dependent superoxide anion production and p47phox expression. (A) Superoxide anion production measured by lucigenin chemiluminescence in aortic homogenates from control, L-NAME, and EPI groups. (B) Western blot for p47phox in aortic homogenates from control, L-NAME, and EPI groups. β-Actin levels were measured as loading controls. Determinations were done after 4 days on the corresponding treatments as described under Materials and methods. Values are expressed as means ± SEM ($n=8$ per group). $*p < 0.05$ with respect to the control and EPI groups.

Fig. 7. Localization of p47phox protein in aorta. (A) Representative images of immunohistochemistry of p47phox protein. Left column shows p47phox protein (red). Central column shows elastin autofluorescence (green). Right column shows the p47phox protein (red) merged with elastin autofluorescence (green). In all the images blue fluorescence indicates nuclear stain with Hoechst. (B) After quantitation p47phox red fluorescence levels were referred to area. Determinations were done after 4 days on the corresponding treatments as described under Materials and methods. Values are shown as means ± SEM ($n=4$ per group). $*p < 0.05$ with respect to the control and EPI groups.

Chronic blockade of NO synthesis by L-NAME-mediated NOS inhibition is a well-known model of hypertension. Even though this model cannot be easily extrapolated to human hypertension conditions, it provides the possibility of reducing the causes of increased BP to a single factor: the decrease in NO bioavailability. Although the doses of L-NAME used in this work are within the range commonly used [45–47], the length of the treatment was markedly shorter than the periods generally used (4 days vs 4–12 weeks). This short-term treatment allows a NO-dependent increase in BP while preventing the long-term effects that can follow a sustained NO deficit, e.g., chronic inflammation and cardiac remodeling.

The administration of (−)-epicatechin seems to be responsible for the prevention of L-NAME-induced increase in BP as supported by the following findings of this study: (i) the presence of (−)-epicatechin in blood after its dietary administration, (ii) the dose-dependent antihypertensive effect of (−)-epicatechin,

and (iii) the requirement of a sustained (−)-epicatechin presence to prevent the development of high BP. The observed dose-dependent action of (−)-epicatechin is consistent with the results of a meta-regression analysis on the effects of human cocoa consumption on BP [48]. A recent study using a 4-week treatment with L-NAME and a low (−)-epicatechin administration (10 mg/kg body wt/day via gavage) did not find a prevention of the increase in BP by (−)-epicatechin [49]. However, the same (−)-epicatechin treatment was effective in attenuating a BP increase in DOCA-salt animals [50]. These and our present observations stress that the approaches to inducing hypertension and/or to supplementing with (−)-epicatechin are relevant for the results to be obtained in terms of BP modulation.

Sufficient NO is associated with normal vasodilation and, consequently, normal BP. Then, a failure to generate NO or an enhanced NO consumption can lead to hypertension. In the model currently used, the diminished NO bioavailability observed in the L-NAME group would be mainly the result of NOS inhibition. However, it can also result from an increased NO oxidation to

peroxynitrite secondary to an increased production of superoxide anion because of a higher NOX activity [51].

Thus, the mechanisms involved in the restoration by (−)-epicatechin of appropriate NO steady-state concentrations can include: (i) an increase in eNOS activity or expression, (ii) a direct superoxide anion scavenging and/or prevention of NOX-mediated oxidative stress (free radical propagation), and/or (iii) a diminished activity of NOX and/or expression. Concerning a modulation of eNOS, the obtained results show that the reduction in vascular NOS activity by L-NAME was reversed by (−)-epicatechin; nevertheless, its expression was not modified by the treatments. A direct effect of (−)-epicatechin on eNOS activity by displacing L-NNA from its binding site in eNOS would not be possible under the studied conditions. The plasma concentrations of L-NNA were higher than the reported IC_{50} (39–50 μM vs 3.5 μM), whereas (−)-epicatechin plasma concentrations were significantly lower than the corresponding IC_{50} (7 μM vs > 500 μM), precluding a competitive effect [52,53]. An action of (−)-epicatechin regulating NOS activity by modulating NOS phosphorylation cannot be ruled out from our experiments. Along this line, it was shown that (−)-epicatechin increased eNOS phosphorylation at activation sites in human coronary artery endothelial cells in culture and mesenteric arteries ex vivo [25,54].

(−)-Epicatechin has a chemical structure that allows reaction with superoxide anion and other radicals, e.g., hydroxyl and peroxyl radicals. However, the (−)-epicatechin concentration detected in plasma would not be consistent with an antioxidant effect as free radical scavenger [55,56]. Even though those direct antioxidant reactions are thermodynamically favored, the observed plasma (−)-epicatechin concentration, ca. 7 μM, would imply rates of reaction with free radicals that are relatively low compared with those of other physiological antioxidant substances, e.g., ascorbate or GSH [57–59]. Then, indirect antioxidant mechanisms, probably related to specific flavanol– and/or (−)-epicatechin–protein [60,61] and (−)-epicatechin–lipid interactions [62–64], can better explain the observed in vivo effects of this compound decreasing systemic oxidative markers (plasma $GSSG/GSH^2$ ratio and MDA concentration).

Excessive superoxide anion production seems to be responsible for the oxidative stress observed in several rat models of hypertension, e.g., male [65] and female [66] spontaneous hypertensive (SHR), Otsuka Long-Evans Tokushima Fatty [67], DOCA-salt [50], two-kidney two-clip hypertensive [68], Dahl salt-sensitive [69], and stroke-prone spontaneously hypertensive [70]. L-NAME treatments are also associated with an increase in NOX activity and/or expression in aorta [50,71]. Even 2 weeks of treatment with L-NAME [72] leads to an increase in NOX subunit mRNA levels, and 7 days of treatment causes an increase in NOX activity in aorta [71]. We observed these effects of L-NAME on NOX activity and $p47^{phox}$ expression, even in the short length of our study.

The possibility of a cross talk between the pathways of expression/activation of eNOS and NOX is usually associated with a superoxide anion-dependent uncoupling of NOS to produce more superoxide anion [73–75]. The opposite situation, NO regulating NOX activation, could be operative under the present L-NAME-dependent conditions. It is postulated that appropriate NO levels control superoxide anion production via NOX S-nitrosylation [76]. Then, (−)-epicatechin administration would promote eNOS activation, maintaining NO steady-state levels able to control NOX activity and superoxide anion production.

As was previously observed in other models of hypertension, L-NAME induced increases in NOX expression in smooth muscle cells from the aorta media layer [65,77,78]. This could be of relevance for the local and systemic production of superoxide, given that in the vasculature the amount of smooth muscle cells is

relatively much higher than the amount of endothelial cells. The inhibition of superoxide production by (−)-epicatechin could be of high relevance in keeping low the vascular and systemic oxidative stress associated with hypertension. These concepts could be extended to other pathophysiological conditions in which both NO and superoxide have a significant participation, e.g., inflammatory processes and ischemia–reperfusion [79,80].

With polyphenols being a heterogeneous family that includes thousands of different compounds, it is important to mention that current research is oriented toward understanding specific effects of an isolated compound. Theoretically other flavanols, e.g., tea catechins, or other flavonoids, e.g., quercetin, can share some of the physiological effects and/or biochemical mechanisms of (−)-epicatechin because they share similar chemical moieties. The structure–function research of a series of catechins, polyphenols, and/or phytonutrients will require new and significant research considering the possibilities of relevant molecular interactions.

In summary, (−)-epicatechin was able to prevent L-NAME-induced increases in BP and in oxidative stress by restoring NO bioavailability. Given that (−)-epicatechin is present in several plants usually consumed by humans it is feasible to develop diets rich in those plants to diminish BP in hypertensive people. Additionally, the fact that we used pure (−)-epicatechin opens up a pharmacological application of this compound to treat hypertension and other vascular diseases.

Acknowledgments

References

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