Research Communication

(–)-Epicatechin Reduces Blood Pressure and Improves Vasorelaxation in Spontaneously Hypertensive Rats by NO-mediated Mechanism

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Abstract

Studies in humans have found consumption of certain flavanoid-containing foods to be associated with improvement in endothelial function and with reduction of blood pressure (BP). (–)-Epicatechin is a compound representative of the flavanols (a subfamily of flavonoids), abundant in cocoa seeds, which is preserved during the industrialization process to chocolate. The antihypertensive effect of dietary (–)-epicatechin was investigated on spontaneously hypertensive rats (SHRs). Consumption of (–)-epicatechin-supplemented diet (3 g (–)-epicatechin/kg diet) decreased BP in SHR by 27 and 23 mm Hg on days 2 and 6, respectively. On day 6, a 173% increase of nitric oxide synthase (NOS) activity was observed in the aorta of EPI-SHR as compared to nonsupplemented SHR ($P < 0.05$). Responses to acetylcholine (ACh) were then examined in femoral arteries in the absence and the presence of L-NAME, a nonselective NOS inhibitor, to assess the ACh-mediated relaxation ascribed to NO-dependent and -independent mechanisms. Acetylcholine-induced endothelium-dependent relaxation in the femoral artery was significantly higher in EPI-SHR than in SHR, with a predominance of the NO-dependent component of this relaxation. The endothelium-independent relaxation, assayed by using the NO donor sodium nitroprusside, resulted in nonsignificant difference in the three experimental groups, demonstrating an unaffected function of vascular smooth muscle cells. These results give further support to the concept that (–)-epicatechin can modulate BP in hypertension by increasing NO levels in the vasculature.
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Keywords: *flavonoids; hypertension; nitric oxide; phytonutrients; cocoa; chocolate; endothelial dysfunction*

Abbreviations: *ACh, acetylcholine; AUC, area under the curve; BP, blood pressure; eNOS, endothelial nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; SNP, sodium nitroprusside; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.*

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Introduction

Studies in humans have found consumption of certain flavanoid-containing foods to be associated with improvement in endothelial function and with reduction of blood pressure (BP) (1–6). Several meta-analyses have supported an antihypertensive effect of flavonol-rich foods, mostly those derived from cocoa (7–10). A recent meta-regression analysis found a correlation between the BP lowering effect of cocoa-derived products and (–)-epicatechin content (11). (–)-Epicatechin is a flavanol abundant in cocoa seeds, which is preserved during the industrialization process to chocolate.

In humans, pure (–)-epicatechin improved vascular function, as evaluated by measuring flow mediated dilation (12). In

rats, long-term (–)-epicatechin administration prevented DOCA-salt-induced hypertension and endothelial dysfunction via reduction of oxidative stress and activation of aortic endothelial nitric oxide synthase (eNOS) (13). In N^{ω}-nitro-L-arginine methyl ester (L-NAME)-induced hypertension, (–)-epicatechin prevented the reduction of nitric oxide synthase (NOS) activity and the development of oxidative stress during a relatively short-term (4 days) treatment (14,15).

Nitric oxide (NO) is central in the regulation of endothelium-dependent relaxation (16). Reduced availability of vascular NO (eNOS-derived NO) is a major cause of endothelial dysfunction found in atherosclerosis, diabetes, and human hypertension (17,18). An increase in NO steady-state concentration has been proposed as the mechanism by which various natural products reduce BP (revised in ref. (19)). The participation of endothelial dysfunction in the development of hypertension was confirmed in numerous studies using animal models of hypertension, including NO-deficient (or L-NAME-induced) hypertension (20) and adult spontaneously hypertensive rats (SHRs).

The aim of this study was to investigate the effects of dietary (–)-epicatechin on BP, vascular NO production, and endothelial function in adult SHR. As SHR present a model of human essential hypertension, the observed results are relevant for human health and can promote dietary and pharmacological strategies for reducing high BP.

Experimental Procedures

Animals and Treatment

Adult, 18- to 19-week-old SHR males were divided into two groups (six rats/group): (i) a hypertensive control group (SHR) fed a control diet and (ii) an (–)-epicatechin-supplemented group (EPI-SHR) fed the control diet supplemented with (–)-epicatechin (3 g/kg diet) (Sigma Chemical, St. Louis, MO). The (–)-epicatechin dose was selected based on the previously published experiments (14,15). Age-matched male Wistar–Kyoto rats (WKY, $n = 6$) served as nonhypertensive control group. The rats were housed in rooms with controlled temperature (22–24 °C), humidity (45–60%), and light (12:12-h light/dark cycle). Access to food and tap water was *ad libitum*. Food consumption was 23 ± 2 g/rat/day, corresponding to approximately 250 mg of (–)-epicatechin/kg body weight/day. At the end of the 6-day treatment, the rats were euthanized. The aorta and femoral arteries were excised and immediately used for the indicated determinations. All procedures were approved by the State Veterinary and Food Administration of the Slovak Republic.

BP Determinations

BP was determined in conscious rats by tail-cuff plethysmography (Statham Pressure Transducer P23XL, Hugo Sachs, Germany) as described previously (21). One week before experimentation, the rats were trained for the procedure of BP determination in three sessions. BP was determined before treatment and on days 2 and 6 of treatment between 9:00 and 12:00 h.

NOS Activity

Total NOS activity was measured in aorta homogenates (200 mg of tissue/mL) by the determination of [^{3}H]-L-citrulline formation from [^{3}H]-L-arginine (MP Biomedicals, USA, 50 Ci/mmol) (21). NOS activity was expressed as pmol citrulline/min/mg of protein.

Vascular Relaxation Studies

Vascular relaxation was studied in segments of femoral artery using Mulvany–Halpern myograph (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark) as described previously (21). Measurements were carried out in an oxygenated (5% CO_2, 95% O_2 mixture) working solution (composition in mmol/L: NaCl 118.99, KCl 4.69, $NaHCO_3$ 25, $MgSO_4 \cdot 7H_2O$ 1.17, KH_2PO_4 1.18, $CaCl_2 \cdot 2H_2O$ 2.5, Na_2EDTA 0.03, glucose 5.5), pH 7.4, at 37 °C. The effect of the treatment on relaxant responses was examined by cumulative concentration–response curves to acetylcholine (ACh; 1 nmol/L–10 μmol/L) in the absence and presence of (L-NAME; 300 μmol/L) and sodium nitroprusside (SNP; 1 nmol/L–10 μmol/L). Relaxation responses are expressed as the percentage of relaxation with respect to the maximal contraction (zero relaxation) induced by serotonin (1 μmol/L). Relaxation was alternatively quantified as the area under the curve (AUC) calculated from individual dose–response curves. The NO-dependent component of endothelium-dependent relaxation was calculated as the difference of the AUC between ACh-induced relaxation in the absence and presence of L-NAME.

Statistical Analysis

BP and vascular reactivity were analyzed using two-way ANOVA. Results of NOS activity and AUC were analyzed by one-way ANOVA. Both analyses were followed by the Bonferroni *post hoc* test. Data are presented as mean ± standard error of mean (SEM).

Results

Nonsignificant changes in food consumption were observed within the groups during the 6-day treatment. The food consumption was 29 ± 1 g/rat/day for WKY; 24 ± 1 g/rat/day for SHR and 23 ± 2 g/rat/day for EPI-SHR. Changes in body weight were 18 ± 4, 11 ± 4, and 9 ± 4 g/rat for WKY, SHR, and EPI-SHR, respectively, for the 6-day period of treatment.

Consumption of a diet supplemented with (–)-epicatechin led to a significant reduction in systolic BP in SHR. Systolic BP was lower by 27 and 23 mm Hg in the EPI-SHR as compared with SHR after 2 and 6 days on the diet, respectively (Fig. 1A). On day 6, NOS activity in the aorta of EPI-SHR was significantly higher (2.7-fold increase) than that in SHR (Fig. 1B).

All the following measurements were performed on day 6. To investigate how (–)-epicatechin-associated changes in BP and NOS activity were related to endothelial function, we

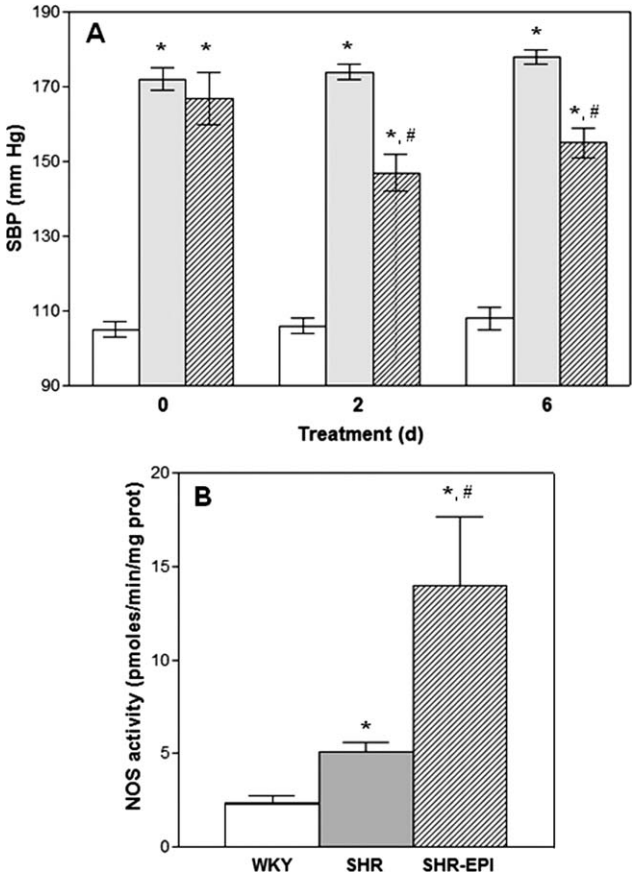

FIG 1

*Effects of (–)-epicatechin supplementation on: (A) BP, and (B), aorta NOS activity in WKY rats (white bars), SHR (gray bars), and EPI-SHR (hatched bars). (–)-Epicatechin was administered in the diet (3.0 g/kg diet) for 0, 2, and 6 days. Values are shown as mean ± SEM (n = 6). *P < 0.05 with respect to the WKY group. #P < 0.05 with respect to the SHR group.*

studied the relaxation of femoral arteries induced by ACh, which requires an appropriate endothelial function. EPI-SHR showed increased ACh-mediated relaxation compared to SHR, concerning both the relaxation at the highest concentrations assayed (1–10 μmol/L) (Fig. 2A) and the AUC of the total relaxation (Table 1). These results indicate that (–)-epicatechin supplementation improves endothelium-dependent relaxation.

Responses to ACh were then examined in femoral arteries preincubated with L-NAME, a nonselective NOS inhibitor, to assess the ACh-mediated relaxation ascribed to NO-independent mechanisms. NO-independent relaxation was significantly increased in EPI-SHR versus SHR only at the highest concentration tested (Fig. 2B), however, not significantly different considering the AUC (Table 1).

The NO-dependent component of ACh-mediated relaxation was calculated as the difference of the AUC between ACh-induced relaxation in the absence and presence of L-NAME. The NO-dependent relaxation component was significantly higher in EPI-SHR than in SHR (Table 1).

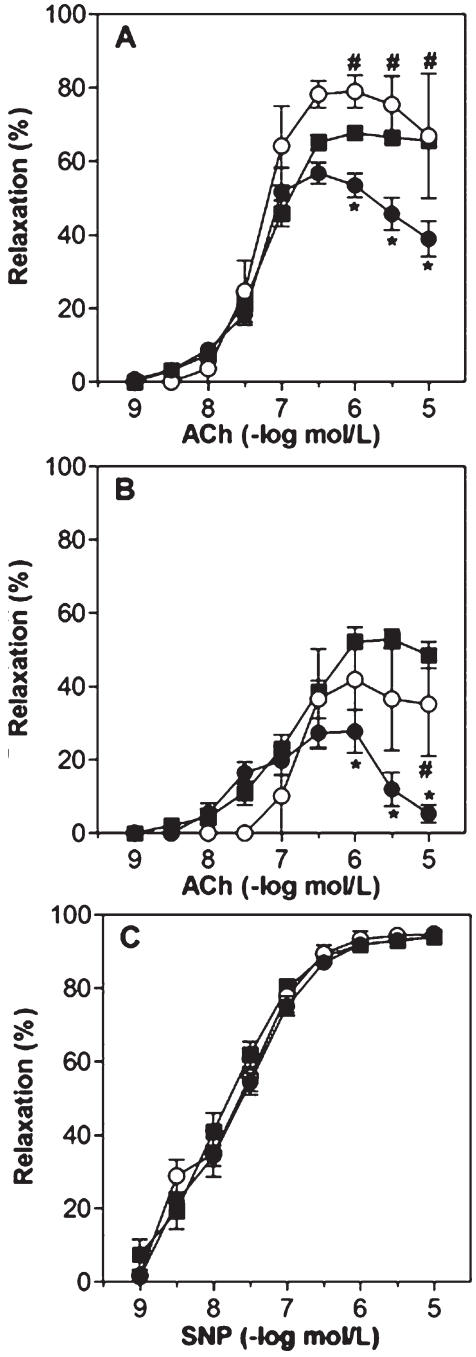

FIG 2

*Effects of (–)-epicatechin supplementation on: (A) endothelium-dependent relaxation; (B) endothelium-dependent relaxation in the presence of L-NAME; and (C) endothelium-independent relaxation in femoral arteries from WKY rats (-■-); SHR (-●-), and EPI-SHR (-○-). Relaxation was induced by cumulative concentrations of ACh (endothelial-dependent relaxation) or SNP (endothelial-independent relaxation) in the femoral arteries previously contracted with serotonin (1 μmol/L). Samples were obtained after 6 days of receiving diets supplemented or not with (–)-epicatechin. Values (percentages of the initial contraction) are shown as means ± SEM (n = 6). *P < 0.05 with respect to the WKY group. #P < 0.05 with respect to the SHR group.*

TABLE 1 ***Total, NO-independent, and NO-dependent ACh-mediated relaxation in femoral artery***

	Relaxation (AUC)		
	Total	*NO independent*	*NO dependent*
WKY	311 ± 8	209 ± 16	102 ± 17
SHR	257 ± 20[a]	111 ± 23[a]	146 ± 16
EPI-SHR	359 ± 35[b]	143 ± 52[a]	216 ± 28[a,b]

(−)-Epicatechin was administered in the diet (3.0 g/kg diet) for 6 days. Femoral arteries were isolated and treated as described in "MATERIALS AND METHODS" section. Values (AUC in arbitrary units) are shown as means ± SEM.

[a]*P < 0.05 with respect to the WKY group.*

[b]*P < 0.05 with respect to the SHR group.*

Finally, the endothelium-independent relaxation was assessed by using the NO donor SNP assay, which tests the function of vascular smooth muscle cells. SNP induced maximal vasodilation at a comparable extent in the three groups of rats studied (Fig. 2C).

Discussion

This study showed that dietary administration of (–)-epicatechin decreased BP and improved endothelial function in SHR on increasing NOS activity in the vasculature. SHRs are a model of human essential hypertension (22) as the development of high BP is associated with an impaired endothelium-dependent vasorelaxation (23–25).

The capacity of (–)-epicatechin to lower BP was found to be quantitatively important. Furthermore, it occurred rapidly, with improvements observed after only 2 days of dietary supplementation. Isolated (–)-epicatechin mimicked the BP lowering response of SHR to (–)-epicatechin-rich foods (26,27). The administration of a single dose (intragastric gavage) of either a cocoa extract (26) or a polyphenol-rich cocoa powder (27) to SHR decreased SBP by approximately 40 mm Hg within 4 h after cocoa administration. In both studies, the BP-lowering effect was transient, with re-established basal SBP values 8 h after administration of the (–)-epicatechin-containing food product. In this study, and consistent with the *ad libitum* supply of dietary (–)-epicatechin, the BP reductions were comparable on days 2 and 6. A similar association between continuous ingestion of (–)-epicatechin and diminished BP was observed upon (–)-epicatechin supplementation in L-NAME-induced hypertension in rats (15). The requirement of a continuous supply of (–)-epicatechin for a significant BP lowering effect is compatible with the transient presence of the ingested (–)-epicatechin in blood. As other flavonoids, blood (–)-epicatechin reaches maximal concentration at about 2 h after ingestion, returning to basal levels after 6–8 h (12,28).

Hypertension and endothelial dysfunction can be associated with reduced NO vascular availability and (–)-epicatechin has been proposed to act by increasing/restoring NO availability (12,14,15,19,29,30).

The determinants of NO availability can be analyzed on considering NO production and NO consumption. An increased NO production can be owing to an increase in NOS protein expression and/or activity. SHRs were found to have a higher aortic NOS activity than WKY (Fig. 1B, (31–33)). This high NOS activity may be considered as a compensatory mechanism in response to the elevation of BP during the development of hypertension in SHR. In this study, aortic NOS was further increased by (–)-epicatechin. In terms of the mechanisms involved, this effect of (–)-epicatechin would not be mediated by an increase in eNOS protein expression, as suggested by the previous results in Sprague–Dawley rats made hypertensive by treatment with L-NAME (15). However, studies in endothelial cell culture demonstrated that (–)-epicatechin increased NOS activity both by increasing phosphorylation in activation sites (Ser_{1177} and Ser_{633}) and by decreasing phosphorylation in inactivation site (Thr_{495}) (34).

Regarding NO consumption, a major way of NO-degradation is its reaction with superoxide anion to produce peroxynitrite (35). Increased production of superoxide anion and increased expression and/or activity of vascular NADPH oxidases (NOXs) were consistently observed in SHR (36–39). In this context, (–)-epicatechin treatment *in vivo* prevented the increases in NOX activities and/or expression of its subunits in DOCA-salt and L-NAME-treated rats (13,15). In addition to these effects, (–)-epicatechin has a chemical structure that allows the scavenging of superoxide and related radicals. Although this direct antioxidant action cannot be disregarded, the actual (–)-epicatechin concentration in blood (low micromolar range) points to an indirect antioxidant effect, for example by decreasing NOX activities, major enzymatic sources of superoxide anion in the vasculature (14). It is important to mention that the interplay between superoxide anion and NO is rather complex, as indicated by an NO-dependent NOX inactivation via *S*-nitrosylation (40) and/or an increased superoxide anion production associated with the uncoupling of NOS (41).

Regarding endothelial function, (–)-epicatechin did not modify vascular smooth muscle cell function, as demonstrated by the fact that vascular relaxation induced by SNP, a direct NO donor, did not differ among the groups studied. Thus, the improvement of vascular function, observed in EPI-SHR, was endothelium dependent. This improvement was mainly mediated by NO, as indicated by the higher contribution of the NO-dependent component to ACh-induced relaxation. The increase of NO availability leads to improved overall relaxation of SHR-EPI rats, reaching the level observed in normotensive WKY rats. Concerning the identity of the chemical species responsible for the *in vivo* observed effect, it was shown that the

parent compound, (–)-epicatechin, has vasorelaxant activity *in vitro* but at higher concentrations than the detected *in vivo* (30–100 μM) (42–44). On the other hand, a mixture of flavanols/metabolites that mimicked the chemical composition of flavanols/metabolites found in human plasma after high-flavanol cocoa consumption (essentially (–)-epicatechin and its metabolites) was effective to induce relaxation in isolated rabbit rings (12).

Conclusions

In summary, dietary (–)-epicatechin administration reduced high BP and improved vasorelaxation in genetically hypertensive rats by improving vascular NO availability. The obtained results provide a rationale for further studies aimed at understanding the mechanisms involved in the actions of (–)-epicatechin on the vasculature. These findings may finally result in nutritional and pharmacological approaches using (–)-epicatechin to control BP levels in humans.

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