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ORIGINAL CONTRIBUTION

Endothelium/nitric oxide mechanism mediates vasorelaxation and counteracts vasoconstriction induced by low concentration of flavanols

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Abstract
Purpose At relatively low concentrations, flavanols induce inconsistent effects on isolated arterial tone, sometimes explained as being due to a structure–activity relationship. The aim of our study was to investigate the effects of two flavanols at different doses on arterial functional state.
Methods The effects of two catechins, (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin (EP), on rat-isolated aorta tone were investigated on resting tension and on precontracted preparations, both in the presence and in the absence of endothelium.
Results At resting tension, endothelium-intact preparations, EGCG and EP (0.01–10 μM), induced a slight concentration-dependent, non-significant contraction. On endothelium-denuded preparations, both EGCG and EP induced a concentration-dependent contraction (significance at 0.1 and 1 μM concentrations of the two compounds, respectively). In phenylephrine (PE) (1 μM) precontracted, endothelium-intact preparations, EGCG and EP (0.01–10 μM), induced a concentration-dependent vasorelaxation, reaching significance at 1 μM concentration of both agonists. On endothelium-denuded preparations, EGCG and EP did not significantly affect PE (0.3 μM)-induced tone. In endothelium-intact precontracted preparations, Nω nitro-L-arginine (L-NNA), a nitric oxide synthase (NOS) activity inhibitor, abolished the vasorelaxant effect of EGCG and EP (0.01–10 μM). At high concentrations, EGCG and EP (100 μM) elicited a marked relaxation. This was significantly larger in the presence than in the absence of endothelium or in the presence of L-NNA.
Conclusions Our findings highlight the important role played by an endothelium/NO-mechanism in the regulation of basal tone and in both mediating vasorelaxation and counteracting vasoconstriction induced by low concentrations of flavanols in rat thoracic aorta.

Keywords Flavanols · Flavonoids · Endothelium · Nitric oxide · Vasorelaxation

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Introduction

The majority of human epidemiologic and experimental studies demonstrate the benefits of tea, cocoa and other flavonoid-containing beverages intake [1–3] in reducing the risk of cardiovascular disease (CVD). Studies have examined specific classes of flavonoids and have demonstrated inverse relations between CV risk and intake of flavanols (also referred as catechins or catechin derivatives) [4, 5]. Monomeric flavanols include (−)-epigallocatechingallate (EGCG), (−)-epicatechin (EP), (−)-epigallocatechin (EGC) and (−)-epicatechin-gallate (ECG), EGCG being the most abundant component in tea [1] and EP in cocoa and apples [6, 7]. The mechanisms accounting for the benefits of flavanols remain incompletely defined; however, growing evidence suggests that these nutrients may act by improving endothelial function [1, 4–7]. Flavanols' effect reflects, in part, endothelial production of nitric oxide (NO), a potent vasoprotecting factor, as observed in healthy humans and in patients with coronary artery disease [3–5], resulting from flavanols' induction of an acute increase in flow-mediated

dilatation (FMD) of the brachial artery after tea/cocoa consumption or of individual compounds such as EGCG or EP [8–10]. It is noteworthy that flavanols induce this effect in plasmatic and probably in tissue fluid concentrations as low as 0.15–1.6 μM, which is within the accepted range of flavonoid concentrations needed to achieve a biological effect [11, 12].

A confirmation of NO-mediated vasodilatation induced by flavanols may be provided indirectly by a number of studies in human and animal-cultured endothelial cells, showing that EGCG and EP induce time- and concentration-dependent activation of endothelial NO synthase (eNOS) and production of NO [13, 14]. At present, the major concern about the vasomotor effect of low concentration of flavanols regards the anomaly between the data obtained in both healthy and hypertensive human and animal in vivo studies, which unequivocally show vasodilatation, and the inconsistent effect observed in ex vivo arterial preparations recently summarized by Moore et al. [15]. This inconsistence is thought to be due to a structure–activity relationship of the different flavonoids tested [16–19]. Furthermore, recent evidence that the same compound (such as EGCG), at a similar concentration (about 10 μM), induced on the same preparation [phenylephrine (PE)-precontracted rat aortic rings] potentiation of contraction, relaxation and no effect [20–23], may suggest that inconsistent effects could be due to substantially different experimental procedures used. This hypothesis has been tested in the present study.

Materials and methods

Standard set of experimental conditions

Male Sprague–Dawley rats (200–300 g), 8–9 weeks old, were killed by decapitation under pentobarbital sodium salt (50 mg/kg) i.p. anesthesia. All procedures in animals were performed in accordance with Guidelines and Authorisation for the Use of Laboratory Animals (Italian Government, Ministry of Health). Lengths of descending thoracic aorta were rapidly removed and placed in a cooled, oxygenated Krebs-Henseleit solution with the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 11.1, (pH = 7.4). The loose connective tissue was carefully removed. The artery was straightened out by slightly stretching and pinning at either end before cutting transverse rings of precisely known length (3–4 mm), using a fixed double-bladed scalpel. Four aortic segments from the same rat, two endothelium-intact and two nominally endothelium-denuded (by gently rubbing the internal surface with filter paper) preparations, were simultaneously mounted on stainless steel hooks and suspended in a water-jacketed 20-mL tissue bath filled with Krebs' buffer maintained at 37 °C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2. The changes in isometric tension of the rings were measured with a digital force isometric transducer connected to a data acquisition system (model: MLT0202; ADInstruments Ltd., Australia). The aortic rings were then equilibrated for 30 min at a resting tension of approximately 1.5–2 g by stretching the vessel gradually until repeated exposure to submaximal concentration of PE induced maximum and repeatable contractions. Optimal resting tension was re-adjusted as necessary. The procedure of stretching the vessel in steps while monitoring the active force development will allow some precision in determining the optimal passive driving force for maximum active force development [24]. On the basis of preliminary complete PE-concentration–response curves (0.1 nM–10 μM), a concentration of 1 μM was selected, which induced about 70–80% of effectiveness (*E*-max % − maximum effective dose producing a desired effect) in the endothelium-intact preparation. In endothelium-denuded tissues, the same concentration of PE induced significantly larger amplitude of contraction ($p < 0.05$). Therefore, in order to obtain responses of similar amplitude in the two preparations, 0.3 μM of PE was used in endothelium-denuded experiments. After the equilibration period, all preparations were maximally contracted with isotonic, high-potassium physiological salt solution (KPSS 127,7 mMol) to establish a referent contraction. The amplitude of PE-induced contractions was 65–70% of high K^+ responses (e.g., about 1.3 g). Once sustained tension induced by PE was obtained, acetylcholine (Ach) (10 μM) was added to evaluate the functional presence of endothelium. Only rings that relaxed more than 70% or less than 10%, in response to Ach, were considered endothelium-intact or endothelium-denuded, respectively.

Protocol I

Contractile effects on resting tension of aortic rings

In each aortic ring, the effect of only one catechin was tested and added in progressively increasing cumulative concentrations (0.01–10 μM), at intervals of approximately 5 min, that is, when the response to each concentration was stable. Vasoconstriction to catechins was expressed as a percentage of the amplitude of contraction induced in each preparation by the initial submaximal concentration of PE. At the end of each experiment, PE usually induced a not significantly different effect as compared with the initial basal value.

Protocol II

Relaxant effect on precontracted aortic rings

In this set of experiments, stimulating α1 adrenergic receptors with PE generated active tone. Activation of the α1 receptor causes Ca^{2+} release from intracellular stores, and this release is responsible for the rapid component of the contractile response. The sustained component of the response appears to be due to Ca^{2+} entry into the smooth muscle cells through nifedipine-sensitive Ca^{2+} channels or through protein kinase C activation [25].

Several observations showed that flavonoids in general, and catechins in particular, induce greater and more repeatable relaxant effects on arterial vessels precontracted with α1-adrenergic receptor agonists than with different contracting agents (high K^+, endothelin 1, etc.) [17, 19, 26], in spite of the lack of α1 receptor antagonist property of catechins [27]. Thus, almost all studies regarding flavonoids vasorelaxant effect on rat aorta were carried out on PE-precontracted preparations [20–23, 26]. When PE contraction reached a steady state, the concentration–effect curves with each catechin were constructed by cumulative administration (0.01–100 μM), at an interval of about 10 min, for example, when the response to each concentration was stable. Only one kind of catechin in each ring was tested. The relaxant effect of catechins was expressed as a percent decrease in the PE-induced control tension (100% relaxation = return to baseline).

Protocol III

Effect of NOS-activity blockade on relaxant responses to catechins

To investigate the potential role of NO on catechin-induced relaxation, this set of experiments was performed only on endothelium-intact rings. In each preparation, the effect of only one catechin was investigated by constructing concentration response curves in the presence or in the absence of Nω nitro-L-arginine (L-NNA), a NOS-activity inhibitor. Cumulative doses of flavanols were added to the tissue bath at 10 min intervals.

Preliminary observations showed that L-NNA (100 μM) alone induced a slow linear increase in tone on endothelium-intact, resting preparations, until reaching (usually after 10–15 min) a stable contractile effect. In the presence of L-NNA-stable tone, PE produced an additional contraction whose net amplitude was significantly greater than in the absence of L-NNA, requiring the reduction in PE concentration (from 1 μM to 0.3 μM) in order to obtain similar amplitude of contraction to that induced in the absence of L-NNA. In a set of experiments, the effects of L-NNA (100 μM) on resting tone and PE-induced contraction were tested in preparations with resting tension adjusted at a fixed value of 0.5 or 0.8 g. In order to evaluate whether the effect of L-NNA was specific (i.e., mediated by the inhibition of NOS-activity), in a set of experiments the effect of oxadiazolo-(4,3) quinoxalin-1-one (ODQ), 10 μM, a heme-site specific inhibitor for NO-activation of muscular soluble guanylate cyclase, was investigated on resting tension, PE-induced contraction and on EGCG- and EP-induced responses [28].

Drugs

The following drugs were used:

EGCG, EP, L-NNA, ODQ, Ach, PE, quinacrine dihydrochloride, dimethyl sulfoxide (DMSO) and pentobarbital sodium salt. They were purchased from Sigma, Milan, Italy. EGCG, EP and quinacrine were initially dissolved in absolute DMSO to prepare a 10^{-2} M stock solution. Further dilutions were made in PSS. The final DMSO concentrations were of 0.05% v/v. Other drugs were dissolved in distilled water, and volumes of <0.02 mL were added to the organ chamber. The concentrations of drugs are expressed as final molar (M) bath concentrations.

Statistical analysis

The estimated parameters are reported as mean ± SEM. For each experimental setting examined, we assessed differences in the induced contractions or relaxations, performing an ANOVA for both EP and EGCG (the response to catechins was expressed as percentage of the PE-induced contraction). All the ANOVA performed show different overall behaviors at different concentrations. Post hoc multiple comparisons using the Newman–Keuls test (Prism version 5, GraphPad Software, USA) were carried out setting the first type error at 0.05 [18]. According to the rat-isolated thoracic aortic ring data, log concentration–response curves were fitted to sigmoids, using nonlinear regression, to calculate sensitivity to flavanols. The sensitivity (pEC50) was measured as a logarithm of the concentration of the flavanols that caused a 50% of the maximal response.

The estimates of pEC50 for EP and EGCG were compared using a two-tailed t test for independent samples, given the different experimental settings. Likewise, the E_{max} estimates were compared.

Results

Contractile effect of EGCG and EP on resting aortic rings

The rat aortic rings lacked spontaneous activity, as reported previously. A single administration of each compound induced a phasic response, consisting of relatively slow contractions that peak in 3–4 min, followed by a slow, profound relaxation with return to the basal tone in about 10 min. DMSO, the vehicle of catechins, at a final concentration of 0.05%, did not modify either the resting tone or the steady-state contractile response to high KCL and PE contractions in either endothelium-intact or endothelium-denuded preparations, or Ach relaxation in endothelium-intact precontracted preparations ($n = 6$, for each experimental condition, data shown in part). Figure 1a, b shows a typical recording of full cumulative dose–response curves to EGCG (0.01–100 μM), both in the presence and in the absence of endothelium. Under both experimental conditions, the contractile response peaked at the concentration of 10 μM. At higher concentrations, EGCG induced slow relaxation. EP (0.01–100 μM) induced qualitatively similar effects (Fig. 1c, d). Figure 2 illustrates a comparison between the concentration-dependent effects of EGCG and EP (0.01–10 μM) in resting conditions, both in the presence and in the absence of endothelium. In endothelium-intact preparations, both EGCG and EP induced contraction, which peaked at 10 μM without reaching statistical significance at a level of 10% of the amplitude induced by PE (1 μM) control contractions. In the absence of endothelium, both EGCG and EP induced a significantly larger contractile effect ($p < 0.05$), which reached statistical significance at a concentration of 0.1 μM and 1 μM of the two compounds, respectively ($p < 0.05$). Similarly, the sensitivity (pEC50) and effectiveness (E_{max}) of EGCG were significantly larger than those of EP ($p < 0.05$). The maximum contraction induced by EGCG was about 40% and that induced by EP 25% of the PE (0.3 μM) control contraction (Table 1). In a preliminary attempt to characterize the unexpected contractile effect of EP, we observed that it was abolished by a relatively low concentration of quinacrine (1 μM), which by itself did not affect the amplitude of contraction induced by PE ($n = 4$, data not shown). In this set of experiments, the concentration response curves to EGCG and EP were constructed up to a concentration of 10 μM, in that both the agonists at higher concentrations elicited vasorelaxation until a return to basal tone.

Relaxant response to EGCG and EP on precontracted rat aortic rings

Figure 1a, b show typical traces of concentration–response curves to EGCG (0.01–100 μM) in the presence and in the absence of endothelium. It is noteworthy the sharp increase in slope of vasorelaxation at concentrations larger than 10 μM independently from the presence or absence of endothelium. Furthermore, the vasorelaxant response to EGCG, mainly at higher concentrations, was preceded by a small and transient contraction in endothelium-intact preparations. This effect was not observed in endothelium-rubbed tissues or in presence of L-NNA.

Qualitatively similar traces were expressed by EP (0.01–100 μM) cumulative dose–response curves (Fig. 1c, d).

Figure 3 compares the effects of EGCG and EP in precontracted condition. In endothelium-intact preparations, both flavanols at lower concentrations (0.01–10 μM) elicited moderate concentration-dependent relaxation, which reaches statistical significance at a concentration of 1 μM of each agonist ($p < 0.05$), being the EGCG's effectiveness significantly larger than that of EP ($p < 0.05$). In endothelium-denuded preparations, the vasodilatory effect of EGCG and EP appeared significantly inhibited ($p < 0.05$). At higher concentrations, EGCG and EP (100 μM) induced marked vasorelaxation, which was significantly larger in the presence of endothelium than in its absence ($p < 0.05$). In endothelium-intact preparations, maximum relaxation induced by EGCG was slightly but significantly larger than that induced by EP ($p < 0.05$) (Table 1).

Effects of L-NNA on resting tension and on the vasorelaxant effects of EGCG and EP in precontracted preparations

In endothelium-intact resting tension preparations, L-NNA (100 μM) alone induced a slow increase in tone until a new steady state was achieved, in about 10–15 min, whose amplitude was about 30% of the PE (1 μM) control contraction ($n = 6$, $p < 0.05$, data not shown). In addition, L-NNA (100 μM) significantly potentiated the PE control contraction ($p < 0.05$), requiring a reduction in the concentration of PE from 1 to 0.3 μM in order to compare the effects of EGCG and EP in the absence and in the presence of L-NNA. Conversely, in endothelium-rubbed preparations, L-NNA (100 μM) failed to significantly affect the basal tone, the amplitude of the PE-induced contraction and the responses to EGCG and EP ($n = 6$, data not shown). In the set of experiments where the effect of L-NNA (100 μM) was investigated, on the endothelium-intact preparations whose resting tension was adjusted at

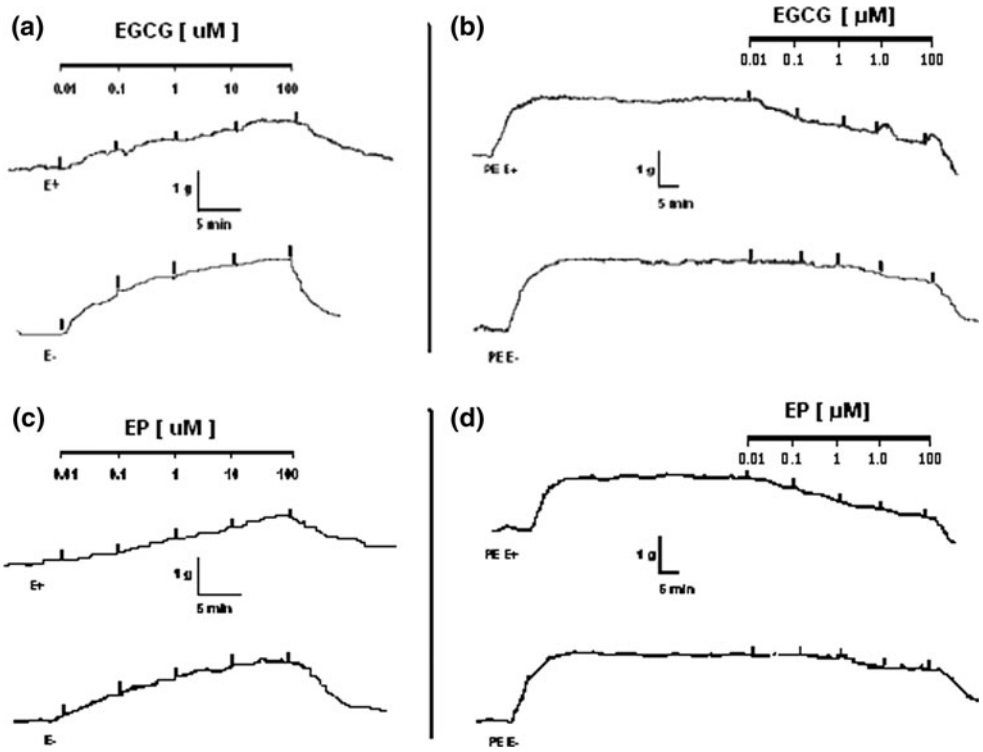

Fig. 1 **a** Tracing showing the concentration-dependent contractile effect of (−)-epigallocatechin-3-gallate (0.01–100 μM) on resting tension rat aortic rings with (E+) or without (E−) endothelium. **b** Tracing showing the concentration-dependent relaxant effect of (−)-epigallocatechin-3-gallate (0.01–100 μM) on phenylephrine-precontracted rat aorta rings with (E+) or without (E−) endothelium. **c** Tracing showing the concentration-dependent contractile effect of epicatechin (0.01–100 μM) on resting tension rat aortic rings with (E+) or without (E−) endothelium. **d** Tracing showing the concentration-dependent relaxant effect of (−)-epicatechin (0.01–100 μM) on phenylephrine (PE)-precontracted rat aorta rings with (E+) or without (E−) endothelium

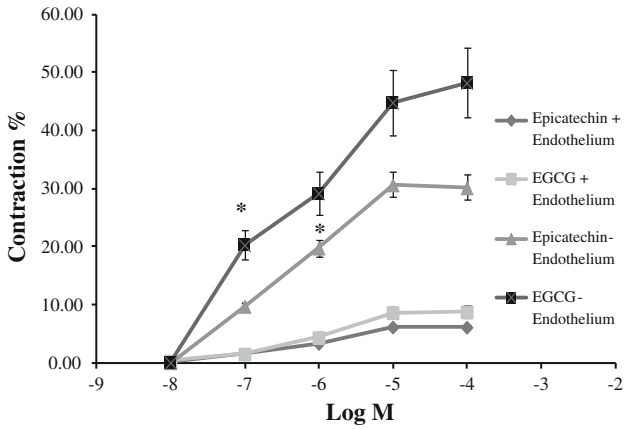

Fig. 2 Concentration-dependent contractile effect of EGCG and EP on resting tension of rat aortic rings with or without endothelium. Results (each point represents the means ± SEM of $n_{EGCG} = 8$ and $n_{EP} = 14$ experiments, significance of differences between two means $p < 0.05$) were expressed as the percentage of the contraction elicited by phenylephrine (1 and 0.3 μM, in endothelium-intact and endothelium-denuded rings respectively). Where no *error bar* is shown, the error is smaller than the symbol. *Ordinate scale*: contraction (percent of control values); *abscissa scale*: catechins concentration (M). *Concentration threshold inducing a significant response ($p < 0.05$)

fixed values of 0.5 and 0.8 g, L-NNA did not significantly affect basal tone. However, at a tension of 0.8 g, it induced a significant increase in the amplitude of PE's control response ($n = 6$, $p < 0.05$ data not shown).

Figure 4 shows the effects of EGCG and EP (0.01–100 μM) in endothelium-intact, PE-precontracted preparations in the absence and in the presence of L-NNA (100 μM). In the presence of L-NNA, the vasorelaxant effect induced by low concentrations (0.01–10 μM) of both agonists was almost abolished ($p < 0.05$). Similarly, the vasorelaxant effects of higher concentrations of EGCG and EP (100 μM) were significantly reduced, compared with those induced in the absence of L-NNA ($p < 0.05$) and not significantly different from those induced by two flavanol agents in the absence of endothelium (Fig. 3). In a separate set of experiments, ODQ (10 μM) induced a similar effect to that of L-NNA concerning the increase in basal tone, the amplitude of PE-induced contraction, and the inhibition of EGCG- and EP-induced vasorelaxation, at both low and high concentrations, in endothelium-intact preparations ($n = 5$, data not shown). No significant effects were induced by ODQ in the absence of endothelium. These findings suggest that the effects of L-NNA were specific in inhibiting NOS-activity.

Discussion

The present study deals with the acute effects of catechin derivatives, at low concentrations, on arterial tone. Our findings highlight the central role played by an endothelium/NO-mechanism in mediating vasodilatation and in counteracting vasoconstriction acutely induced by low

Table 1 Between/within protocols comparisons of effectiveness (E-max % − maximum effective dose producing contracting or relaxing effect) and sensitivity (pEC50) for epicatechin and epigallocatechin gallate

Protocol	Epicatechin (μM)		Epigallocatechin gallate (μM)		$E_{max\ EP}$ versus $E_{maxEGCG}$	$pEC50_{EP}$ versus $pEC50_{EGCG}$
	E_{max} ± SEM	pEC50 ± SEM	E_{max} ± SEM	pEC50 ± SEM	*p* value	*p* value
Resting condition ($n_{EP} = 14$; $n_{EGCG} = 8$)						
Endothelium	6.39 ± 0.25	6.04 ± 0.11	9.15 ± 0.31	5.95 ± 0.09	ns	ns
No endothelium	30.47 ± 0.74	6.38 ± 0.08	44.60 ± 1.56	6.69 ± 0.13	$p < 0.05$	$p < 0.05$
p value	$p < 0.05$*	$p < 0.05$	$p < 0.05$	$p < 0.05$		
Precontracted ($n_{EP} = 12$; $n_{EGCG} = 10$)						
Endothelium	46.30 ± 2.05	4.72 ± 0.07	50.23 ± 2.74	4.76 ± 0.07	$p < 0.05$	ns
No endothelium	30.83 ± 1.10	4.48 ± 0.06	30.29 ± 1.13	4.49 ± 0.08	ns	ns
p value	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$		
Pre-contracted + endothelium ($n_{EP} = 14$; $n_{EGCG} = 7$)						
No LNNA	45.71 ± 2.06	4.71 ± 0.11	54.26 ± 2.70	4.73 ± 0.12	$p < 0.05$	ns
LNNA	34.15 ± 1.03	4.43 ± 0.07	36.19 ± 1.55	4.39 ± 0.07	ns	ns
p value	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$		

The rows, within each experimental setting, report the comparison of EP with EGCG in terms of E_{max} and pEC50

The columns show the comparison, respectively, within EP and EGCG, of E_{max} and pEC50

* $\alpha = 0.05$

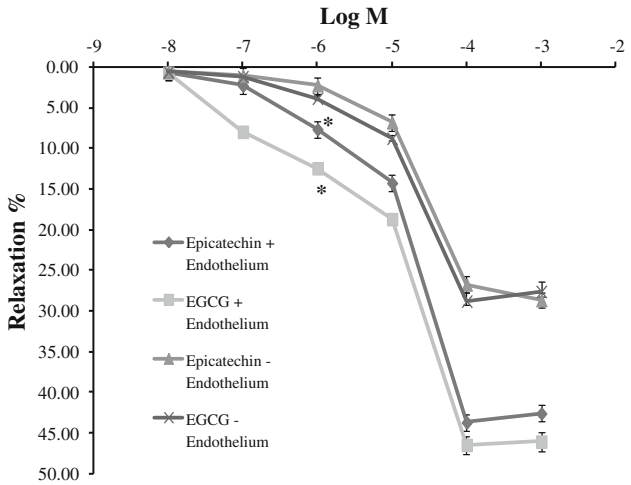

Fig. 3 Concentration-dependent relaxant effects of EGCG and EP on phenylephrine—precontracted rat aorta rings with (+) and without (−) endothelium. Results (each point represents the means ± SEM of $n_{EGCG} = 10$ and $n_{EP} = 12$ experiments, significance of differences between two means $p < 0.05$) were expressed as the percentage of the contraction elicited by phenylephrine (1 and 0.3 μM, in endothelium-intact and endothelium-denuded rings respectively). Where no *error bar* is shown, the error is smaller than the symbol. *Ordinate scale*: relaxation (percent of control values); *abscissa scale*: catechins concentration (M). *Concentration threshold inducing a significant response ($p < 0.05$)

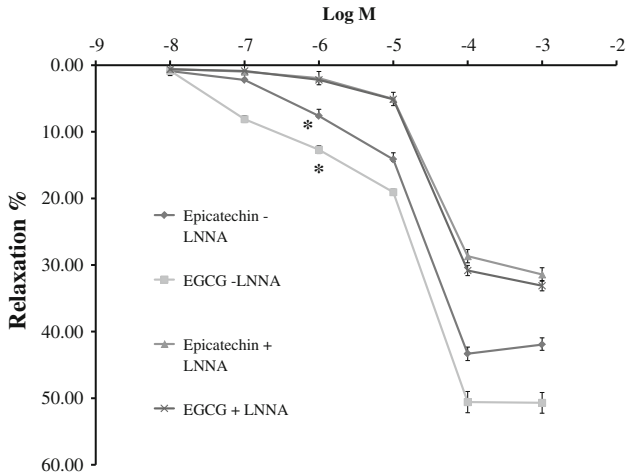

Fig. 4 Concentration-dependent relaxant effects of EGCG and EP on phenylephrine—precontracted rat aorta rings with endothelium with and without L-NNA. Results (each point represents the means ± SEM of $n_{EGCG} = 7$ and $n_{EP} = 14$ experiments, significance of differences between two means $p < 0.05$) were expressed as the percentage of the contraction elicited by phenylephrine (1 and 0.3 μM, in the absence and in the presence of L-NNA respectively). Where no *error bar* is shown, the error is smaller than the symbol. *Ordinate scale*: relaxation (percent of control values); *abscissa scale*: catechins concentration (M). *Concentration threshold inducing a significant response ($p < 0.05$)

concentrations of catechins in the precontracted and resting rat aorta, respectively (Table 1).

While the endothelium-dependent vasodilatory effect induced by catechins has been described in the rat aorta, to our knowledge the potent endothelial-dependent modulation of their direct contractile effect on the musculature has not been previously reported. According with this, we observed that in resting conditions, both EGCG and EP

induced a significant contractile effect that was counteracted in endothelium-intact preparations. The same condition was observed evaluating relaxing effects. The presence of the endothelium guaranteed a significant vasorelaxation that was abolished or reduced in endothelial-denuded rat aortic rings or inhibiting eNOS (Table 1). Suggesting a possible structure-dependent difference in affecting arterial function, the contracting and relaxing effects reported as sensitivity (pEC50) and effectiveness (E_{max}) were slightly, but in some cases significantly, larger after EGCG than EP (Table 1).

Thus, the relevant vasodilatatory role of endothelium in determining the effect of catechins observed in this study deserves additional detailed comment.

Contractile effect of EGCG and EP

Previous studies reported that catechins, and EGCG in particular, at low concentrations (<10 μM), did not affect basal arterial tone of either endothelium-intact or endothelium-denuded rat aortic preparations [20, 29] and that a single administration of EGCG, at larger concentrations, independently of the presence or absence of endothelium, induced a phasic response [27, 29] consisting in a contraction followed by relaxation. Furthermore, cumulative concentration–response curves to EGCG (3–300 μM) and EP (30–300 μM) induced contraction independently of the presence or absence of endothelium [27, 29]. There are inconsistencies and contradictions as to the site and mode of action of EGCG on rat aorta contraction. These incongruences include inactivation of endothelium-derived NO [20], increasing smooth vascular cell membrane permeability to Ca^{2+} [29], and activation of multiple signaling pathways due to the production of H_2O_2 in muscular cells [27]. In the present study, both EGCG and EP (0.01–10 μM), in rat aortic endothelium-intact preparations, induced a slight concentration-dependent contraction, whose maximal amplitude did not reach statistical significance. Conversely, both catechins induced a marked contraction in the absence of endothelium, whose significance was obtained at concentrations as low as 0.1 μM for EGCG and 1 μM for EP (Table 1). At higher concentrations, both agonists induced a marked vasorelaxation. As a contractile effect elicited by low concentrations of EGCG has not been previously observed in the absence of endothelium, it is difficult to explain the anomaly of our findings. This could tentatively be related to the experimental procedures, leading to a substantial sensitization of the muscular contractile machinery. Indirect support of this hypothesis could be: (1) the significant increase in basal tone (~30% of the PE control contraction) induced by L-NNA alone; (2) the finding that the maximal contractile effect of EGCG was obtained at a concentration (10 μM) much lower than that reported in the Shen et al. [27] and Alvarez-Castro et al. [29] studies (300 μM). However, it is noteworthy that Shen et al. [27] constructed non-cumulative concentration curves to avoid the phenomenon of desensitization of the flavonoid compounds described mainly at high concentrations and that Alvarez-Castro et al. [29] constructed cumulative concentration curves in partially K^+-depolarized preparations and at much longer intervals than in the present study [27, 29]. In particular, the present finding that EP induced a substantial contractile effect, albeit significantly less potent than that induced by EGCG, was not expected (Table 1). To our knowledge, no previous observation has been reported in the literature regarding a contractile effect induced by EP on the ex vivo resting arteries. In Shen's study [27], where the contractile properties of different flavanols were investigated on the rat aorta, EP and ECG at concentrations of up to 300 μM were without effect on the resting tone of endothelium-intact or endothelium-denuded preparations. This suggested that the epigallol structure in B ring (three contiguous hydroxyl groups) of the catechin molecules was determinant in inducing the observed contractile effect of EGCG and EGC. At present, we are investigating the potential mechanisms that explain the EP-contractile effect observed in this study, which may be favored, at least in part, by the possible sensitization of muscular contractile machinery induced by our experimental procedure. Preliminary data showed that the contractile effect induced by EP was abolished by a low concentration of quinacrine (1 μM). Quinacrine and analogs, such as mepacrine, have been previously observed to inhibit contractile response of rat aorta induced by agents activating phospholipase A_2, including EGCG and EGC [27]. The activation of phospholipase A_2 induces prostanoid products, which in turn stimulate thromboxane-prostanoid receptors and cause contractions.

Relaxant effects of EGCG and EP

Previous investigations into the effect of relatively low concentrations of catechin derivatives in ex vivo precontracted arterial preparations showed a prevalent, moderate, endothelial-dependent vasorelaxation mediated by the production of NO and, sometimes, of vasodilatory prostaglandins [5, 21, 22]. However, on rat aortic PE-precontracted, endothelium-intact tissues, EGCG, at concentrations of up to (10 μM), has been observed by Lorenz et al. [21, 22] to induce endothelium-dependent and NO-mediated relaxation, by Sanae et al. [20] potentiation of contraction, endothelium-dependent by NO-mediated inactivation and by Lim et al. [23] no effect. Such contradictory effects could be due, at least in part, to different experimental conditions. For example, by comparing the modality and the extent of

passive force exerted, one can observe that Lorenz et al. [21, 22] gradually stretched the tissue for up to 2 g of tension in over 1 h, while Sanae et al. [20] adjusted the tension at 1 g and Lim et al. [23] at 0.5 g.

With regard to EP, at low concentrations, it induced inconsistent effects on isolated precontracted aortic rings. Schroeter et al. [5] observed vasorelaxation in the rabbit aorta by an endothelium-dependent, NO-mediated mechanism, at concentrations similar to those that increased FMD in healthy humans in the same study. However, a recent study in the rat aorta observed that EP, at a concentration of up to 10 μM, did not affect PE-induced tone [22].

In the present study, both EGCG and EP (0.01–10 μM) elicited a concentration-dependent, moderate relaxation on PE-precontracted, endothelium-intact preparations, being the EGCG's effect significantly larger than that of EP ($p < 0.05$). This effect reached statistical significance at a concentration of 1 μM of both catechins and was almost abolished by the removal of endothelium (Table 1).

Our data regarding EGCG's effect are in agreement with those reported by Lorenz et al. [21, 22], whose experimental procedures are similar to those employed in the present study and at variance with results previously reported [20, 23]. However, our results are at variance with those of Lorenz et al. [22] as concerns EP's effect. The lack of vasodilatory response to EP could be unexpected for at least two reasons. The first regards the molecular structure of EP, which is the only flavanol sharing with flavonols (for example: quercetin) a catechol group (two hydroxyls at C_3 and C_4 of the B ring) and the presence of an OH group in C_3 of the C ring, OH substitution, considered relevant by Chan et al. [18] in determining (endothelium-dependent and in part NO-mediated) vasodilatation induced by flavonols, at very low concentrations (0.1–10 μM), in the rat aorta. It is noteworthy that the relaxant effects of flavonoids are reported to have the following potency: flavonols > flavones > flavanols [16]. In healthy humans, pure dietary flavonoids quercetin and EP augment NO products, at a plasma concentration of ~3 μM/L [30]. The second consideration regards the recent observation of Ramirez-Sanchez et al. [14] that EP induces a marked production of NO in human endothelial cultures, with the maximal effect at a concentration of 1 μM, correlated with that reported to induce vasodilatory and cardioprotective action in both animals and humans. In this study, EP's activation of eNOS is considered to be dependent on an action exerted at the cell membrane level, leading to accumulation of inositol phosphate and intracellular calcium [14]. This is also the mechanism of action of the physiological stimulators of eNOS, such as bradykinin or estrogens. Huang et al. [31] previously observed that purified green tea EP, at larger concentration and a longer period of incubation, induced an increase in $[Ca^{2+}]$ in human endothelial cells and a significant increase in cGMP tissue content and vasodilatation in isolated rat mesenteric arteries, abolished by removal of the endothelium and eNOS inhibition.

Conversely, EGCG is considered to activate eNOS through different mechanisms involving the endocellular production of reactive oxygen species (ROS), mainly through an autoxidant process critically dependent on conjugated hydroxyl functions, such as those on the B ring and gallate moiety of EGCG [13, 32].

In the present study, at higher concentrations (100 μM), both EGCG and EP induced a profound relaxation, due to a combination of endothelial-mediated and direct action on smooth muscle, the maximum effect being significantly larger in the endothelium-intact than in endothelium-rubbed preparations (Table 1). This observation confirms previous evidence that flavonoid compounds, including flavanols, mainly at high concentrations, induce profound vasorelaxation through a direct action on vascular smooth muscle, mediated by an array of mechanisms including the inhibitory action on protein kinase C, on cAMP or cGMP phosphodiesterases, and/or on Ca^{2+} influx through voltage-sensitive Ca^{2+} channels [16, 17, 33]. Furthermore, the present finding that EGCG, in endothelium-intact preparations, induced a significantly larger relaxation than that of EP (Table 1), confirms previous observations indicating that EGCG is more effective than EP in rat aorta [34].

Another interesting difference between vasorelaxant effects of two flavanols is that only those induced by EGCG, mainly at higher concentrations, are preceded by a small and transient contraction in endothelium-intact preparations. This effect was not observed in endothelium-rubbed tissues, nor in the presence of L-NNA (Table 1). A similar transient contraction, preceding profound relaxation, induced by EGCG, was reported on the rat endothelium-intact aorta, but not in the presence of eNOS inhibition [21]. Thus, this effect of EGCG appears to be different in nature compared to contraction that the catechin, at low concentration, induces in endothelium-deprived resting preparations. At relatively high concentrations, EGCG could critically produce an amount of ROS, mainly superoxide anion, capable of inactivating NO released by endothelium, as observed by Sanae et al. [20] in the same preparation, before the activation of eNOS, probably through the same mechanism of action. The absence of contraction preceding the EP-induced vasorelaxation may indicate that its activation of eNOS may not be mediated by ROS production.

Effects of L-NNA on spontaneous and evoked NO-release

In the present study, L-NNA, in addition to eliciting a potentiation of PE-induced contraction, a common effect of

eNOS inhibitors due to the suppression of spontaneous release of NO [35], provoked a significant increase in basal tone (of about 30%) on endothelium-intact resting preparations, usually not observed in rat aorta and mesenteric arteries [20, 36].

The latter effect could suggest a substantial activation of endothelial function due to experimental conditions, including the procedure of selecting the "normalization" of the active development force of aortic rings, leading to an inappropriate level of passive force [37]. Indeed, in the set of experiments, where the effect of L-NNA was investigated on preparations whose resting tension was adjusted at fixed values of 0.5 and 0.8 g, no increase in basal tone was observed; even though, at 0.8 g of tension, a significant increase in the amplitude of PE's control response was observed. These findings indirectly confirm the notion of a highly regulated endothelial release of NO, also in ex vivo conditions, where physical stimuli (probably linked to smooth muscle tone) may play an important role in regulating the basal release of NO. In the presence of L-NNA, the vasodilatatory effect of both EGCG and EP (0.01–10 μM), observed on endothelium-intact preparations, was almost abolished. This finding indirectly indicates that a minimal role, if any, may be played by direct action of catechins on either smooth muscle or on endothelial cells, the latter leading to the release of vasorelaxant materials different from NO. Furthermore, the maximal vasorelaxation induced by higher concentrations of flavanols (100 μM) was significantly reduced. More frequently, in the presence of eNOS inhibition and/or in the endothelium removal, an increase in sensitivity, but not of effectiveness of flavonoids, has been observed, indicating a predominant direct effect on smooth muscle induced by higher concentrations of these compounds [18, 19].

Thus, also this finding may further confirm the particularly relevant role played by the endothelial NO-mechanism in inducing the vasorelaxant effect of catechins observed in this study.

Conclusions

In this study, EGCG and EP, at concentrations achievable in the human plasma and probably in tissue fluid, strongly activated an endothelium/NO-mechanism that appears to mediate the vasorelaxation of precontracted preparations and to counteract their direct vasoconstriction on resting smooth muscle (Table 1). In both experimental conditions, EGCG appears more effective than EP, even though its sensitivity is larger only as regard the contractile effect, suggesting the existence of a structure–activity relationship in the effect of flavonoids, as previously observed. However, such a crucial role exerted by the endothelium/NO-mechanism on the effect of catechins might be partly due to experimental procedures used, taking into account the apparent high functional interconnection (positive feedback) between endothelial cells (including NO-release) and the level of the underlying smooth muscle tone. In spite of these possible limitations, it seems reasonable to conclude that at least some of the present findings may be of physiological, and possibly clinical, significance concerning the beneficial effects of low concentrations of catechins, given endothelial integrity, in preventing CVD, as observed in epidemiological and experimental studies in vivo. Damage to the endothelium will result in an increased sensitivity and responsiveness of the muscular layer to the contractile effects of both endogenous and exogenous vasoconstrictors, including flavanols, and consequently, in decreasing the vasorelaxant properties owned of the latter. Two very recent studies support this hypothesis. In the first, EGCG also at low concentration (1μM) caused remarkable contractions in the aged SHR aorta and induced much smaller increases in tension in preparations of the normotensive WKY [38]. In the second study, carried out in aging mice with established arteriosclerosis, it has been reported that a late, chronic catechins treatment is deleterious to the residual function of the endothelium and to the regulation of vascular tone [39].

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