

Endothelial NO Production Is Mandatory for Epigallocatechin-3-Gallate–induced Vasodilation: Results From eNOS Knockout (eNOS^{-/-}) Mice

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Abstract: The underlying mechanisms for the vasodilating effects of the tea catechin epigallocatechin-3-gallate (EGCG) are still not fully understood. Besides nitric oxide (NO)-dependent effects, other modes of action are discussed. To elucidate whether the NO pathway is a prerequisite in mediating vasodilating effects, we investigated EGCG-induced vasorelaxation in isolated aortic rings of endothelial nitric oxide knockout (eNOS^{-/-}) mice. Vasodilation to acetylcholine was fully prevented in aortic rings of eNOS^{-/-} mice, confirming lack of vascular NO production. Vasodilation to the exogenous NO donor sodium nitroprusside was preserved in eNOS^{-/-} mice aortic rings. Low concentrations of EGCG (5–15 μM) resulted in strong vasorelaxation in aortic rings of wild type mice, whereas it was completely absent in eNOS^{-/-} mice. In corroboration, relaxation in response to green tea was significantly inhibited in aortic rings of eNOS^{-/-} mice. These results demonstrate that EGCG-induced vasodilation strongly relies on functional NO synthase in endothelial cells and subsequent stimulation of NO production in vessels.

Key Words: eNOS knockout mice, vasodilation, nitric oxide, aortic rings, EGCG

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INTRODUCTION

Consumption of green tea is inversely associated with risk for cardiovascular diseases and stroke,¹ as well as with lower cardiovascular and total mortality.² Improvement of endothelial function is thought to be one major mechanism for these beneficial effects. Green tea improved flow-mediated dilation in chronic smokers³ and in healthy individuals.⁴ A meta-analysis indicates that green and black tea ingestion increases endothelial-dependent vasodilation.⁵ Because endothelial dysfunction is characterized by reduced availability of nitric oxide (NO), interventions able to stimulate vascular NO production represent a promising tool in the prevention of cardiovascular diseases.

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The catechin epigallocatechin-3-gallate (EGCG) represents the most potent physiologically active tea compound *in vitro*. EGCG has been shown to induce vasodilatory effects in isolated blood vessels in various studies.^{6–12} Besides an NO-dependent mode of action, other NO-independent mechanisms for EGCG-induced vasodilation were also proposed. To elucidate conclusively whether EGCG-induced vasodilation is dependent on NO, we studied effects of EGCG in isolated aortic rings of endothelial NO knockout (eNOS^{-/-}) mice compared with wild type controls.

MATERIALS AND METHODS

Animals

Eight- to 10-week-old male eNOS^{-/-} mice (strain B6.129P2-*Nos3*^{tm1Unc/J}) from Jackson Laboratory were used for the experiments. The background strain C57BL/6J with functional eNOS expression (wild type) served as control. Animals were housed under standard conditions according to institutional guidelines. Water and food was supplied *ad libitum*. Extraction of organs from genetically modified animals was approved by the local authority (Landesamt für Gesundheit und Soziales, Berlin) under the permit number T0026/05.

Vasorelaxation Studies in Isolated Mouse Aortic Rings

Mice were anesthetized by intraperitoneal injection of thiopental (0.3 g/kg body weight). Thoracic aortae from anesthetized mice were rapidly excised, cleaned of connective tissue, and cut into rings of 1 mm in length for organ-chamber experiments. Rings were then mounted on platinum hooks in 10-mL jacketed organ baths containing modified Krebs–Henseleit solution (composition, in mmol/L: NaCl 144, KCl 5.9, CaCl₂ 1.6, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and D-glucose 11.1) and 1 μmol/L of diclofenac. Tension was gradually adjusted to 1 g over 15 minutes. The solution in the bath was maintained at 38°C with a gas mixture of 5% CO₂ and 95% O₂. After equilibration, the reactivity of rings was tested with KCl (40 mM). Nonfunctional rings were discarded and replaced. After repeated washouts, the tension of rings was again adjusted to 1 g. After equilibration, the rings were precontracted with the α₁-receptor agonist phenylephrine (200 nM). To obtain cumulative concentration–response curves, EGCG (Sigma Aldrich, dissolved in water) at

concentrations of 5, 10, and 15 μM was added at 30-minute intervals to the rings. Control rings received the same amount of water. For the relaxation experiments with tea, 1.2 g of Darjeeling green tea (provided by King's Teagarden, Berlin, Germany) was brewed with 100 mL of boiled water for 3 minutes. Rings received the indicated amounts of green tea. Control rings received the same amounts of water. Relaxations to cumulative doses (10 nM–5 μM) of the endothelium-dependent vasodilator acetylcholine were also performed. Maintenance of smooth muscle integrity was confirmed by evaluation of endothelium-independent vasodilation to sodium nitroprusside (SNP, 0.1–100 nM). Vasorelaxation is expressed as percentage of precontraction with phenylephrine. The data represent numbers of individual aortic rings. All experiments were done using 7–8 different animals.

Statistical Analysis

Values are presented as mean \pm SEM. Statistical calculations were carried out with Student *t* tests for independent samples for pair-wise comparisons of mean values, and with Mann–Whitney rank sum test when comparing medians. Statistical analysis was performed using SigmaStat Version 3. Level of significance was accepted at $P < 0.05$.

RESULTS

Acetylcholine-induced vasodilation was lacking in aortic rings of $\text{eNOS}^{-/-}$ mice (Fig. 1A). Vasodilation to the NO donor SNP was not impaired in $\text{eNOS}^{-/-}$ mice compared with wild type mice. Moreover, we observed a slight but significantly enhanced relaxation to SNP in $\text{eNOS}^{-/-}$ mice (Fig. 1B).

Next, we exposed phenylephrine-precontracted aortic rings to cumulative concentrations of EGCG in a time interval of 30 minutes between applications. Low doses of EGCG (5–15 μM) induced concentration-dependent vasorelaxations in wild type mice. In aortic rings of $\text{eNOS}^{-/-}$ mice, however, EGCG had no effect on vascular tone. An original recording of a representative experiment is shown in Figure 2A. In accordance with our previous results obtained in rats,⁷ treatment of aortic rings from wild type mice with EGCG led to an initial, transient, and reversible contraction. This was followed by a sustained vasodilation. In contrast, neither initial contractions nor any relaxation in response to EGCG could be detected in $\text{eNOS}^{-/-}$ mice (Fig. 2B). The summary of all experiments is shown in Figure 2B. Compared with controls, EGCG induced a significant concentration-dependent vasodilation in wild type mice. EGCG of 5 μM led to a relaxation of 82.3 (± 4.2) % of precontraction, and doses of 10 μM and 15 μM resulted in 56.9 (± 5.0) % and 41.3 (± 5.4) % vasodilation, respectively. However, EGCG completely failed to induce vasodilation in rings of $\text{eNOS}^{-/-}$ mice (Fig. 2). To extend our findings to the whole beverage, green tea was added to aortic rings of wild type and $\text{eNOS}^{-/-}$ mice. Green tea in lower concentrations caused vasodilation only in wild type aortic rings, whereas a slight vasorelaxation was also obtained in $\text{eNOS}^{-/-}$ mice at higher concentrations (Fig. 3).

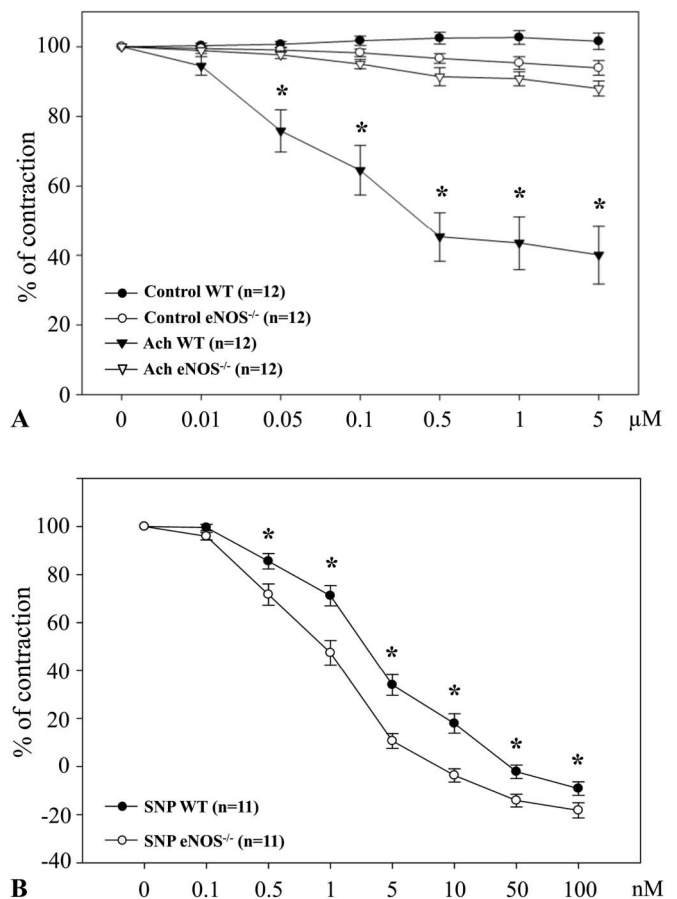
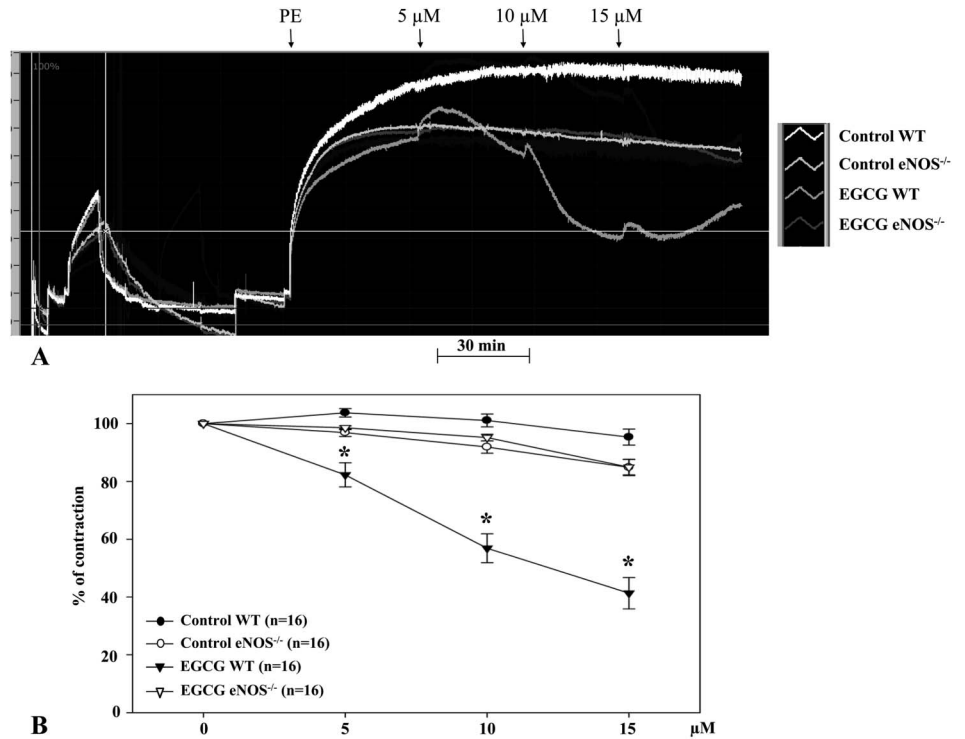


FIGURE 1. Vasorelaxation to Ach in aortic rings of $\text{eNOS}^{-/-}$ and WT mice (A). Graphs show relaxation expressed as a percentage of maximal phenylephrine-induced vasoconstriction. Control rings received the same amount of water. Data are expressed as mean \pm SEM of the indicated number of experiments. * $P < 0.05$ aortic rings of WT versus $\text{eNOS}^{-/-}$ mice at equal concentrations of Ach. B, Cumulative doses of the endothelium-independent vasodilator SNP (0.1–100 nM) were given to the rings to illustrate maintenance of smooth muscle integrity. Data are expressed as mean \pm SEM of the indicated number of experiments. * $P < 0.05$ aortic rings of WT versus $\text{eNOS}^{-/-}$ mice at equal concentrations of SNP. Ach, acetylcholine; WT, wild type.

DISCUSSION

Predominantly, a NO-dependent mechanism for EGCG-induced vasorelaxation has been described, as demonstrated by pharmacological inhibition of endothelial NO production.^{7,10–12} However, alternative modes of action are discussed. The EGCG-induced inhibition of Ca^{2+} influx in smooth muscle cells as a mechanistic factor for vessel relaxation was proposed.⁶ Inhibition of various phosphodiesterase (PDE) isoforms was also suggested as an underlying mechanism for vasodilating effects of EGCG. EGCG reduced the enzymatic activities of isolated PDE1–PDE5 isoenzymes, with strongest inhibitory effects on PDE1 and PDE2. Consequently, the contraction-induced decrease in cyclic nucleotide levels (cAMP and cGMP) in aortic smooth muscle cells was

FIGURE 2. Original recording of a single experiment of EGCG-induced vasorelaxation in phenylephrine-precontracted aortic rings of eNOS^{-/-} and WT mice (A). Control rings received the same amount of water. The graphs show an initial transient contraction after each EGCG dose in WT aortic rings that is followed by sustained relaxation. No change in vessel force was seen in aortic rings of eNOS^{-/-} mice. B, Cumulative concentrations of EGCG were added to phenylephrine-precontracted aortic rings of eNOS^{-/-} and WT mice. Shown are the results as percent of phenylephrine-induced contractions. Data are expressed as mean ± SEM of the indicated number of experiments. **P* < 0.05 aortic rings of WT versus eNOS^{-/-} mice at equal concentrations of EGCG. WT, wild type.



reversed by EGCG.⁸ In addition, an increase in endothelial prostacyclin production by EGCG was reported at the cellular level.¹³ We demonstrate in our study that the vasodilating effects of EGCG strongly rely on the presence of eNOS and thus NO production in endothelial cells. However, several mechanisms can result in stimulation of cellular NO production, for example, the generation of reactive oxygen species.^{10,11}

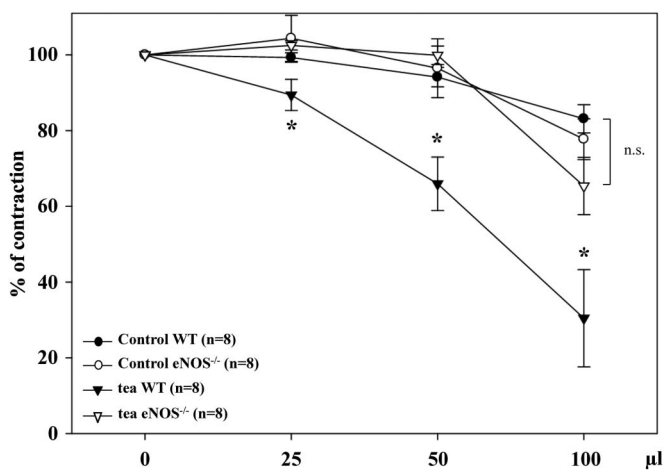


FIGURE 3. Cumulative doses of green tea were added to phenylephrine-precontracted aortic rings of eNOS^{-/-} and WT mice. Data are expressed as mean ± SEM of the indicated number of experiments. **P* < 0.05 aortic rings of WT versus eNOS^{-/-} mice stimulated with equal amounts of green tea. n.s., not significant; WT, wild type.

Besides vasodilation, a number of studies describe vasoconstrictive effects of EGCG. Does EGCG induce rather vasorelaxation or vasoconstriction? A transient Ca²⁺-dependent contractile response to EGCG was observed in resting rat aortae (without precontraction).¹⁴ EGCG-induced contractions involved the formation of H₂O₂, which results in Ca²⁺ influx in smooth muscle cells through nonvoltage-dependent Ca²⁺ channels.¹⁵ The apparent contradiction between these opposite effects (vasoconstriction vs. relaxation) could be attributed to the time-course in EGCG-induced changes in vessel tone. EGCG results in vasoconstriction^{7,8,12,14,15} that is transient in resting rings^{8,14,15} or followed by vasodilation in precontracted vessels.^{7,8,12} An initial activation of Ca²⁺ influx into smooth muscle cells through voltage-operated Ca²⁺ channels followed by inhibition of voltage-operated Ca²⁺ channels was suggested as potential explanation for biphasic actions of EGCG on vessel tone.⁹ Interestingly, this biphasic mode of action was completely absent in eNOS^{-/-} mice in our study. Neither vasoconstriction nor vasodilation was observed. The lack of EGCG-induced contractions was also obtained after pharmacological inhibition of eNOS and after removal of the endothelium in rat aortae.^{7,12} The reason for this endothelial-dependent and NO-dependent transient vasoconstriction induced by EGCG is unknown at present. Relaxations to SNP were slightly more pronounced in eNOS^{-/-} mice. This would not be surprising because loss of endothelial NO likely increases sensitivity to exogenous NO.

In summary, irrespective of potential upstream signaling molecules and pathways involved, our study clearly demonstrates that the EGCG-induced changes in vessel tone

(both initial contractions and subsequent relaxations) are exclusively dependent on the presence of eNOS and endothelial NO production. This may confer EGCG a potential favourable profile of action.

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