A Constituent of Green Tea, Epigallocatechin-3-gallate, Activates Endothelial Nitric Oxide Synthase by a Phosphatidylinositol-3-OH-kinase-, cAMP-dependent Protein Kinase-, and Akt-dependent Pathway and Leads to Endothelial-dependent Vasorelaxation*

Epidemiological studies suggest that tea catechins may reduce the risk of cardiovascular disease, but the mechanisms of benefit have not been determined. The objective of the present study was to investigate the effects of epigallocatechin-3-gallate (EGCG), the major constituent of green tea, on vasorelaxation and on eNOS expression and activity in endothelial cells. EGCG (1–50 μM) induced dose-dependent vasodilation in rat aortic rings. Vasodilation was abolished by pretreatment with Nω-nitro-L-arginine methyl ester. In bovine aortic endothelial cells, EGCG increased endothelial nitric oxide (eNOS) activity dose-dependently after 15 min. Treatment with EGCG induced a sustained activation of Akt, ERK1/2, and eNOS Ser1179 phosphorylation. Inhibition of extracellular signal-regulated kinase (ERK)1/2 had no influence on eNOS activity or Ser1179 phosphorylation. Simultaneous treatment of cells with selective inhibitors for cAMP-dependent protein kinase (PKA) and Akt completely prevented the increase in eNOS activity by EGCG after 15 min, indicating that both kinases act in concert. Specific phosphatidylinositol-3-OH-kinase inhibitors yielded identical results. Akt inhibition prevented eNOS Ser1179 phosphorylation, whereas inhibition of PKA did not influence Akt and eNOS Ser1179 phosphorylation. Pretreatment of endothelial cells with EGCG for 4 h markedly enhanced the increase in eNOS activity stimulated by Ca-ionomycin, suggesting that Akt accounts for prolonged eNOS activation. Treatment of cells for 72 h with EGCG did not change eNOS protein levels. Our results indicate that EGCC-induced endothelium-dependent vasodilation is primarily based on rapid activation of eNOS by a phosphatidylinositol 3-kinase-, PKA-, and Akt-dependent increase in eNOS activity, independently of an altered eNOS protein content.

Epidemiological evidence suggests that chronic diseases such as coronary heart disease and stroke have a lower incidence in countries with a high intake of tea. Numerous studies have demonstrated that tea drinking lowers the risk of heart disease and reduces cardiovascular mortality (1–3); others, however, do not support these findings (4, 5). In addition, tea consumption has been inversely associated with the development and progression of atherosclerosis (6).

Tea is second only to water in worldwide usage and contains important phytochemicals, the flavonoids. Tea flavonoids include catechins, quercetin, kaempferol, and numerous other polyphenols (7). Catechins are the major components of tea; in green tea, they represent > 80% of flavonoids, and in black tea, they represent only 20–30% (8).

The suggested mechanisms involved in the prevention of coronary heart disease by green and black tea beverages involve potent antioxidative, antithrombogenic, and antiinflammatory properties of flavonoids, primarily of the major and most active catechin derivative, epigallocatechin-3-gallate (EGCG) (9). In addition, there is experimental and clinical evidence that tea extracts also improve endothelial function (10, 11). Short- and long-term tea consumption has been shown to reverse endothelial dysfunction in patients with documented coronary heart disease (11). Moreover, purified epicatechins from tea evoke endothelium-dependent vasorelaxation in precontracted rat aortic rings by means of nitric oxide (NO) release from the endothelium (10). Although tea catechins may improve endothelial function, the underlying molecular mechanisms are unknown.

Endothelial-dependent NO is produced by endothelial nitric oxide synthase (eNOS). This key enzyme in cardiovascular homeostasis is regulated posttranslationally by means of rapid modulation of eNOS activity by increase in intracellular Ca2+ and/or phosphorylation and on the transcriptional level by changes in eNOS gene expression. In the present study, we show the effects of a natural plant-derived catechin, EGCG, on eNOS expression and activity in endothelial cells, and we elucidate the molecular mechanisms involved.

EXPERIMENTAL PROCEDURES

Cell Culture—Bovine aortic endothelial cells (BAEC) were purchased from Clonetics and maintained in microvascular endothelial cell growth medium (EGM-MV), supplemented with 5% fetal bovine serum, 10 pg/ml epidermal growth factor, 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, and 0.1% gentamicin. For experiments, the cells were seeded onto 6-cm dishes and treated with EGCG (dissolved in water), LY294002, wortmannin, Ca-ionomycin from Sigma (Deisenhofen, Germany), and Schwerpunkt Kardiologie, Angiologie, Pneumologie, Charité, Campus Mitte, Humboldt-Universität zu Berlin, Berlin D-10117, Germany

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1 The abbreviations used are: EGCG, epigallocatechin-3-gallate; eNOS, endothelial nitric oxide synthase; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor; BAEC, bovine aortic endothelial cells; PT3K, phosphatidylinositol-3-OH-kinase; t-NAME, Nω-nitro-L-arginine methyl ester; ERK, extracellular signal-regulated kinase.

1 The abbreviations used are: EGCG, epigallocatechin-3-gallate; eNOS, endothelial nitric oxide synthase; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor; BAEC, bovine aortic endothelial cells; PT3K, phosphatidylinositol-3-OH-kinase; t-NAME, Nω-nitro-L-arginine methyl ester; ERK, extracellular signal-regulated kinase.
Germany), the cell-permeable myristoylated 14–22 amide peptide PKI, H89, and PD98059 from endo Alexis (San Diego, CA) at confluence. Cells were used between passages 4 and 10 for all experiments. For Western blots with phospho-specific antibodies and the cAMP-dependent protein kinase (PKA) assays, cells were starved in medium with 1% fetal bovine serum for 20 h.

**Western Blot Analysis**—After treatment, cells were washed twice with phosphate-buffered saline and lysed in extraction buffer containing 144 mM NaCl, 5.9 mM KCl, 1.6 mM CaCl2, 1.2 mM MgSO4, 4 mM sodium pyrophosphate, 10 mM EDTA, 1 mM phenethylsulfonyl fluoride, 2 mM dithiothreitol, and 1% Triton X-100. For Western blots with phospho-specific antibodies, cells were lysed in buffer containing 20 mM Heps (pH 7.9), 100 mM NaCl, 1 mM Na2VO4, 4 mM sodium pyrophosphate, 10 mM EDTA, 1 mM phenethylsulfonyl fluoride, 10 mM NaF, 0.1 mM okadaic acid, and 1% Triton X-100. Total protein (50 μg per lane) was subjected to SDS-PAGE, and membranes were probed with anti-phospho-eNOS (Ser1177), anti-phospho-Akt (Ser473), and anti-phospho-ERK1/2 (Thr202/Tyr204) from Cell Signaling Technology (Frankfurt, Germany), anti-Akt antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-eNOS from BD Transduction Laboratories (Heidelberg, Germany), secondary anti-mouse from Santa Cruz Biotechnology, and anti-rabbit from Dianova (Hamburg, Germany). Bands were visualized by using either 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma) or the ECL detection system (Amersham, Freiburg, Germany).

**Measurement of eNOS Activity in Intact Cells**—eNOS activity was assessed by formation of L-[3H]citrulline from L-[3H]arginine after separation of the amino acids by cation-exchange chromatography. Endothelial cells were washed twice with phosphate-buffered saline and incubated for 30 min in Hepes buffer, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM Hepes-Na, 10 mM glucose, and 1 mM CaCl2. Stimulation was initiated by the addition of agonists, 10 μM cold t-arginine, and 3 μM t-[3H]arginine. Where indicated, protein kinase inhibitors were present 30 min before and during agonist stimulation. After 15 min, the reaction was terminated with ice-cold stop solution containing 5 mM L-arginine and 4 mM EDTA. Cells were denatured with 96% ethanol and, after evaporation, the soluble cellular fraction was separated from L-[3H]arginine by Dowex chromatography, and L-[3H]citrulline formation was quantified by liquid scintillation counting.

**PKA Assay**—Measurement of PKA activity was performed with a commercial PKA assay kit (PepTag assay kit, Promega, Madison, WI). Cells were serum-starved in medium with 1% fetal bovine serum for 20 h and stimulated for 15 min with 100 μM EGCG, or EGCG and 20 μM PKI for the specificity of the PKA reaction. After stimulation, cells were lysed in extraction buffer containing 20 mM Tris (pH 7.4), 25 μg/ml aprotinin, 1 mM sodium pyrophosphate, and 1 mM EGTA. We used 5 μg of cell homogenate in the assay. The reactions were performed according to the protocol of the manufacturer. After phosphorylation, the PKA-specific fluorescent peptide substrate kemptide alters the net charge from +1 to −1, and the phosphorylated and non-phosphorylated forms can be separated on agarose gels.

**Vasorelaxation Studies**—Thoracic aortas from male Wistar rats were rapidly excised, cleaned of connective tissue, and cut into rings 2–3 mm in length for organ-chamber experiments. The rings were then mounted on rings were then mounted on the PKA inhibitor PKI (Fig. 3A). After phosphorylation, the PKA-specific fluorescent peptide substrate kemptide alters the net charge from +1 to −1, and the phosphorylated and non-phosphorylated forms can be separated on agarose gels.

**Statistical Analysis**—All values are expressed as mean ± S.E. compared with controls. Vasorelaxation is expressed as a percentage of precontraction with phenylephrine. Statistical analysis was performed by use of ANOVA, Mann-Whitney, or Student’s t test where appropriate. A level of p < 0.05 was considered significant in all statistical tests.

**RESULTS**

To examine whether EGCG could induce vasorelaxation, we exposed phenylephrine-precontracted rat aortic rings to cumulative doses of EGCG. EGCG caused dose-dependent vasorelaxation, starting at 1 μM (Fig. 1A). The original recording of a single experiment is shown that indicates a transient, reversible contraction after each EGCG dose, followed by sustained relaxation. The summary of 7–18 experiments is illustrated in Fig. 1B. EGCG produced vasodilation, which began at 1 μM and reached statistical significance at 10 μM. Pretreatment with 1 mM l-NAME abolished the vasodilation induced by 1–25 μM of EGCG, whereas at 50 μM EGCG, l-NAME had no effect (Fig. 1B). Removal of the endothelium yielded identical results (data not shown), indicating that relaxations of rat aortic rings induced by EGCG is predominantly due to generation of nitric oxide.

To test this assumption, we incubated BAECs with increasing concentrations of EGCG, and measured eNOS activity in intact cells. EGCG caused a dose-dependent increase in eNOS activity after 15 min (Fig. 1C). At 100 μM EGCG, we observed a 4-fold rise in eNOS activity. To investigate whether EGCG can also influence eNOS protein expression, we incubated BAEC with increasing concentrations of EGCG for 72 h. There was no change in eNOS protein content, regardless of the concentration used, which indicates that EGCG has no effect on the level of eNOS protein (Fig. 1D).

It has been shown recently (12) that EGCG can produce H2O2, albeit at low concentrations; and that H2O2 can lead to eNOS activation and vasorelaxation in rabbit aortic rings (13). To exclude a contribution of H2O2 to the observed effects of EGCG, we performed the vasorelaxation studies and eNOS activity assays in the presence of catalase. Preincubation with catalase (1000 units/ml) had no influence on the obtained results, indicating that the effects of EGCG are not due to the production of H2O2 (data not shown).

eNOS is posttranslationally activated by phosphorylation. To look for possible kinases involved in the up-regulation of eNOS activity by EGCG, we assessed Akt and ERK1/2 phosphorylation. EGCG (at 100 μM) induced phosphorylation of Akt (Ser473) and ERK1/2 (Thr202/Tyr204) after 15–30 min (Fig. 2). Whereas the activation of ERK1/2 was transient and returned to basal levels after 6 h, phosphorylation of Akt remained elevated for at least 6 h. Because Akt can phosphorylate eNOS (14), we determined the phosphorylation status for Ser1177 of bovine eNOS. eNOS Ser1177 phosphorylation followed a similar time course as did Akt activation (starting at 15 min) and remained elevated for at least 6 h (Fig. 2). The finding that a prominent increase in eNOS enzyme activity by EGCG is apparent after 15 min, whereas there was only a moderate activation of Akt and eNOS Ser1177 phosphorylation at this time point, suggests that another kinase may be involved in the rapid EGCG-induced eNOS activation. It has recently been shown that PKA can activate eNOS by phosphorylation (15, 16). Pretreatment of cells with PKI (20 μM), a specific PKA inhibitor, partly eliminated the EGCG-stimulated increase in eNOS activity after 15 min (Fig. 3A). Similar results were obtained with another specific PKA inhibitor, H89 (data not shown). To further demonstrate the involvement of PKA in the rapid activation of eNOS by EGCG, we measured PKA activity in BAEC after treatment of cells with EGCG. EGCG at 100 μM led to an activation of PKA within 15 min, which was abolished by the PKA inhibitor PKI (Fig. 3B).

Having shown the involvement of both kinases in rapid and sustained eNOS activation, we investigated whether common upstream signaling pathways are involved. Akt can be activated by means of phosphatidylinositol-3-OH-kinase (PI3K)
recently, a PI3K-dependent, PKA-dependent pathway for eNOS phosphorylation in BAEC was described (16). To investigate whether PI3K is involved in rapid EGCG-dependent eNOS activation, BAEC were pretreated with LY294002 (30 μM) before stimulation with EGCG (100 μM) for 15 min. As shown in Fig. 3C, inhibition of PI3K completely prevented EGCG-induced eNOS activation. Wortmannin, another inhibitor of PI3K, yielded identical results (data not shown), demonstrating the requirement of PI3K for eNOS activation by EGCG.

To formally exclude a contribution of ERK1/2 to the increase in eNOS activity after 15 min, cells were pretreated with PD98059 (10 μM), an inhibitor of ERK1/2. PD98059 had no influence on EGCG-induced increase in eNOS activity after 15 min (Fig. 3C). Furthermore, EGCG-induced eNOS Ser1179 phosphorylation after 1 h was abolished by LY294002, whereas PD98059 was without effect (Fig. 3D), which indicates that the transient ERK1/2 activation does not contribute to either rapid (after 15 min) or sustained (after 1 h) eNOS activation.

To examine whether the activation of PKA lies upstream of Akt activation, we measured Akt and eNOS phosphorylation after inhibition of PKA. Pretreatment of BAEC with PKI had no effect on EGCG-induced Akt and eNOS Ser1179 phosphorylation after one hour, suggesting that both kinases act in parallel (Fig. 4A). However, SH-5 (20 μM), an inhibitor of Akt (17), abolished both Akt and eNOS phosphorylation (Fig. 4A), revealing that Akt is responsible for the sustained eNOS Ser1179 phosphorylation by EGCG. In line with the above results, simultaneous treatment of cells with PKI and SH-5 completely prevented the increase in eNOS activity after 15 min, which indicates that both kinases act in concert. SH-5 on its own led only to partial inhibition (Fig. 4B).

To analyze whether prolonged eNOS phosphorylation leads to a more active enzyme, we incubated BAEC with 100 μM EGCG for 4 h and measured basal and stimulated eNOS activity in intact cells. No EGCG was present before and during the percentage of maximal phenylephrine-induced vasoconstriction. Data are expressed as mean ± S.E. *p < 0.05 versus vehicle-treated rings.

Fig. 1. EGCG produces vasorelaxation in rat aortic rings and increases eNOS activity in endothelial cells. A, original recording of a single experiment of EGCG-induced vasorelaxation in phenylephrine-precontracted rat aortic rings showing an initial, reversible contraction after each EGCG dose, followed by sustained relaxation. B, summary of 7–18 experiments. Cumulative doses of EGCG were added to phenylephrine-precontracted rat aortic rings. Selected rings were pretreated with 1 mM L-NAME. Graphs show relaxation expressed as a percentage of maximal phenylephrine-induced vasoconstriction. Data are expressed as mean ± S.E. *p < 0.05 versus vehicle-treated rings.

Fig. 2. Time course of EGCG-induced phosphorylation of ERK1/2, Akt, and eNOS Ser1179 in endothelial cells. BAEC were treated for the indicated times with 100 μM EGCG. Western blots were probed with phospho-specific antibodies against ERK1/2 (Thr202/Tyr204), Akt (Ser473), and eNOS Ser1179. Anti-eNOS antibody served as a control for equal loading of proteins. Results are representative of three separate experiments.
PKA-specific fluorescent peptide substrate kemptide after separation on agarose gels. A representative result from three independent experiments is shown.

**C**, BAEC were pretreated or not with the PI3K inhibitor LY294002 (30 μM) or the ERK1/2 inhibitor PD98059 (10 μM) for 30 min. After stimulation with 100 μM EGCG for 15 min, eNOS activity in intact cells was measured. *, p < 0.05. **ns**, nonsignificant.

**D**, cells were stimulated for one hour with 100 μM EGCG, and Western blots were probed with phospho-specific antibodies against ERK1/2, Akt, and eNOS Ser1179. Anti-eNOS antibody served as control for equal loading. Data are representative from three separate experiments.

**Fig. 3.** Effect of kinase inhibitors on eNOS activity and phosphorylation in BAEC. **A**, cells were pretreated or not with the PKA inhibitor PKI (20 μM) for 30 min and stimulated with 100 μM EGCG for 15 min. eNOS activity in intact cells was measured by conversion of L-[3H]citrulline from L-[3H]arginine. *, p < 0.05. **B**, measurement of PKA activity in BAEC. Cells were pretreated or not with PKI (20 μM) for 30 min and stimulated with 100 μM EGCG for 15 min. Pos. and neg. indicate the phosphorylated versus non-phosphorylated forms of the PKA-specific peptide substrate kemptide after separation on agarose gels. A representative result from three independent experiments is shown. **C**, BAEC were pretreated or not with the PI3K inhibitor LY294002 (30 μM) or the ERK1/2 inhibitor PD98059 (10 μM) for 30 min. After stimulation with 100 μM EGCG for 15 min, eNOS activity in intact cells was measured. *, p < 0.05. **ns**, nonsignificant. **D**, cells were stimulated for one hour with 100 μM EGCG, and Western blots were probed with phospho-specific antibodies against ERK1/2, Akt, and eNOS Ser1179. Anti-Akt antibody served as control for equal loading. Data are representative from three separate experiments.
EGCG and eNOS

Discussion

The present study demonstrates that endothelium-dependent vasorelaxation induced by the tea-derived catechin EGCG occurs in response to a potent, dose-dependent activation of eNOS in endothelial cells. The resulting increase in eNOS activity is observed within a few minutes, suggesting post-translational regulation of eNOS as an underlying mechanism. Improvement of vascular endothelial function by flavonoids may be an important mechanism by which tea reduces cardiovascular risk (11). The normal endothelium plays a critical role in regulating vasomotor tone, and it is well established that impaired endothelium-derived nitric oxide activity is associated with the progression of atherosclerosis. Recent evidence has demonstrated that short- and long-term tea consumption reverses endothelial dysfunction in patients with documented coronary heart disease, thereby providing a putative mechanism responsible for the beneficial effect of tea in these patients. Our vasoreactivity studies and previous experiments reinforce this clinical evidence by suggesting that purified epicatechins from tea evoke endothelium-dependent vasorelaxation in precontracted rat aorta by means of NO release from the endothelium (10). However, experimental data on the vascular effects of catechins are not consistent. Reports have described no effect on vasoreactivity (18) as well as even the opposite action of inhibition of acetylcholine-induced endothelium-dependent vasodilation (19). Differences in experimental setup (e.g., incubation time, dose, species) may explain these inconsistent data. A recent experimental study (20), moreover, has demonstrated that epicatechins exert vasoconstriction on resting arteries. These dual properties may partly explain the biphasic vascular effects observed in our vasoreactivity studies (transient vasoconstriction followed by sustained relaxation) in rat aortic rings after EGCG exposure.

In accordance with EGCG-induced endothelium-dependent vasodilation in the rat aorta, which could be up to 25 μM of EGCG blocked by pretreatment with l-NAME, we observed a dose-dependent increase in eNOS activity in BAEC after 15 min.

EGCG can generate H₂O₂ (12, 20), and H₂O₂ can lead to eNOS activation and vasorelaxation in rabbit aortic rings (13). However, our experiments in the presence of catalase excluded an involvement of H₂O₂ to the obtained results.

To examine possible mechanisms leading to eNOS activation in BAEC after treatment with EGCG, we investigated the role of potential protein kinases activated by EGCG. Our study demonstrates that ERK1/2, Akt, and PKA are involved in the cellular response after EGCG stimulation. A recent study has shown that MAPK/ERK1/2 can induce eNOS activation (21). However, our experiments indicate that ERK1/2 is not involved in either rapid or sustained EGCG-induced eNOS activation in BAEC.

Treatment of cells with specific protein kinase inhibitors established a role of PKA and Akt in eNOS activation. Phosphorylation of Akt (Ser[1179]) and eNOS (Ser[1179]) occurred in parallel and resulted in sustained, long-term activation of eNOS. Inhibition of Akt prevented phosphorylation of eNOS at Ser[1179] in accordance with the well established role of Akt in eNOS phosphorylation (14). Sustained eNOS Ser[1179] phosphorylation leads to augmented enzyme activation, as evidenced by increased Ca-ionomycin stimulation after treatment of cells with EGCG for 4 h. Inhibition of PKA partially attenuated the rise in eNOS activity after 15 min. The following findings substantiate the involvement of PKA in the rapid activation of eNOS enzyme activity by means of phosphorylation: (i) two specific independent inhibitors of PKA (PKI and H89) at least partially inhibited rapid eNOS activation by EGCG after 15 min; (ii) activation of PKA occurred within 15 min after treatment of BAEC with EGCG, as shown in the PKA assay; and (iii) inhibitors of PI3K completely prevented up-regulation of eNOS activity after 15 min, whereas there was only a modest phosphorylation of eNOS Ser[1179] at this time point.

A recent report (16) has demonstrated that PKA stimulates eNOS Ser[1179] phosphorylation in response to shear stress, and, in another study, it was likewise shown that PKA also phosphorylates and activates eNOS at Ser[635] (15). Inhibition of PKA did not affect Akt and eNOS Ser[1179] phosphorylation, which indicates that PKA did not mediate the phosphorylation of this site in our study. It also suggests that these two kinases do not depend on each other, and that there is no cross-talk between these signaling pathways. Simultaneous blocking of both kinases abolished the rise in eNOS activity after 15 min, which evidences a concerted action of PKA and Akt. Furthermore, complete prevention of the rapid increase in eNOS activity and Akt-dependent eNOS phosphorylation by inhibition of PI3K suggests that PI3K lies upstream and is required for activation of both kinases. The PKA-mediated phosphorylation and activation of eNOS by means of an upstream PI3K-dependent pathway in endothelial cells has only recently been described (16). However, no clear mechanistic insight into the mechanism of PKA activation by PI3K could be provided. One possible mechanism of PKA activation by PI3K could involve the interaction of PKA with different protein kinase A anchoring proteins after phosphorylation. For example, after phosphorylation of the regulatory subunit of PKA by CDK1, the kinase changes its subcellular localization by association with protein kinase A anchoring protein 95 (22). Phosphorylation of the regulatory subunit of PKA by PI3K or other upstream kinases could likewise alter its cellular localization and bring the PKA in closer proximity to the eNOS target in caveolae. An interaction and regulation of the catalytic subunit of PKA with caveolin-1 in caveolae, the main intracellular compartment for eNOS protein localization, has been demonstrated (23). However, at present there is no established mechanism as to how EGCG could activate PKA by means of a PI3K-dependent pathway. Further studies are required to address this question.

The results of our study show that EGCG activates eNOS by parallel and additive actions of PI3K/PKA- and Akt-dependent phosphorylation. Treatment of BAEC with increasing concentrations of EGCG for 72 h did not change eNOS protein expression.

In the present study, we provide the first experimental evidence that the tea polyphenol EGCG rapidly activates the key enzyme of vascular homeostasis eNOS and induces endothelium-dependent vasodilation. It remains to be elucidated whether these in vitro data may be assigned to the in vivo situation. Plasma catechin concentrations after ingestion of green and black tea in humans lie within the order of magnitude of −1 μM (24, 25). Although the concentrations of EGCG in our cell culture model were above this value, we observed a slight NO-dependent vasodilation in rat aortic rings at 1 μM. The effects of catechins in vitro may accordingly be relevant to the improvement of endothelial function and atherosclerosis in vivo.

In summary, we postulate that the plant-derived catechin EGCG leads to endothelium-dependent vasorelaxation and acts as a natural activator of eNOS in endothelial cells by increasing its protein phosphorylation. The resulting amelio-
ration of endothelial function may in part explain the protective effects of tea-derived flavonoids on cardiovascular diseases. Our data may prove helpful in the development of new natural drugs to improve endothelial function and thereby prevent cardiovascular diseases.

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REFERENCES
1. Nakachi, K., Matsuyama, S., Miyake, S., Suganuma, M., and Imai, K. (2000) Biofactors 13, 49–54
2. Arts, I. C., Hollman, P. C., Feskens, E. J., Bueno de Mesquita, H. B., and Kromhout, D. (2001) Am. J. Clin. Nutr. 74, 227–232
3. Mukamal, K. J., Maclure, M., Muller, J. E., Sherwood, J. B., and Mittleman, M. A. (2002) Circulation 105, 2476–2481
4. Rimm, E. B., Katan, M. B., Ascherio, A., Stampfer, M. J., and Willett, W. C. (1996) Ann. Intern. Med. 125, 384–389
5. Woodward, M., and Tunstall-Pedoe, H. (1999) J. Epidemiol. Community Health 53, 481–487
6. Geleijnse, J. M., Launer, L. J., Hofman, A., Pols, H. A., and Witteman, J. C. (1999) Arch. Intern. Med. 159, 2170–2174
7. Kris-Etherton, P. M., and Keaney, J. F., Jr. (2002) Circ. Res. 90, 1449–1457
8. Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. (1997) Crit. Rev. Food Sci. Nutr. 37, 693–704
9. Vinson, J. A., and Dabbagh, Y. A. (1998) FEBS Lett. 433, 44–46
10. Huang, Y., Chan, N. W. K., Lau, C. W., Yao, X. Q., Chan, F. L., and Chen, Z. Y. (1999) Biochim. Biophys. Acta 1427, 322–328
11. Duffy, S. J., Kenney, J. F., Jr., Holbrook, M., Okusa, M., Swerdloff, P. L., Frei, B., and Vita, J. A. (2001) Circulation 104, 151–156
12. Hong, J., Lu, H., Meng, X., Ryu, J. H., Hara, Y., and Yang, C. S. (2002) Cancer Res. 62, 7241–7246
13. Thomas, S. R., Chen, K., and Kenney, J. F., Jr. (2002) J. Biol. Chem. 277, 6017–6024
14. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 601–605
15. Michell, B. J., Harris, M. B., Chen, Z., Ju, H., Venema, V. J., Blackstone, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) J. Biol. Chem. 277, 42544–42551
16. Boo, Y. C., Sorescu, G., Boyd, N., Shiojima, I., Walsh, K., Du, J., and Jo, H. (2002) J. Biol. Chem. 277, 3388–3396
17. Kozikowski, A. P., Sun, H., Broman, J., and Dennis, P. A. (2003) J. Am. Chem. Soc. 125, 1144–1145
18. Andriambelson, E., Magnier, C., Haan-Archipoff, G., Lobstein, A., Anton, R., Bereta, A., Stoclet, J., and Andraantsitohaina, R. (1998) J. Nutr. 128, 2324–2333
19. Sanae, F., Miyai, Y., Kizu, H., and Hyashi, H. (2002) Life Sci. 71, 2555–2562
20. Shen, J. Z., Zheng, X. F., Wei, E. Q., and Kwan, C. Y. (2003) Clin. Exp. Pharmacol. Physiol. 30, 88–95
21. Mineo, C., Yuhanna, I. S., Quan, M. J., and Shaul, P. W. (2003) J. Biol. Chem. 278, 9142–9149
22. Landovitz, H. B., Carlson, C. R., Steen, R. L., Vassebein, L., Herberg, F. W., Tasker, K., and Collas, P. (2001) J. Cell Sci. 114, 3255–3264
23. Razani, B., Rubin, C. S., and Lisanti, M. P. (1999) J. Biol. Chem. 274, 26353–26360
24. Yang, C. S., Chen, L., Lee, M. J., Balentine, D., Kuo, M. C., and Schantz, S. P. (1998) Cancer Epidemiol. Biomarkers Prev. 7, 351–354
25. Umegaki, K., Sugisawa, A., Yamada, K., and Higuchi, M. (2001) J. Nutr. Sci. Vitaminol. 47, 402–408