

Epigallocatechin Gallate, a Green Tea Polyphenol, Mediates NO-dependent Vasodilation Using Signaling Pathways in Vascular Endothelium Requiring Reactive Oxygen Species and Fyn*

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Green tea consumption is associated with reduced cardiovascular mortality in some epidemiological studies. Epigallocatechin gallate (EGCG), a bioactive polyphenol in green tea, mimics metabolic actions of insulin to inhibit gluconeogenesis in hepatocytes. Because signaling pathways regulating metabolic and vasodilator actions of insulin are shared in common, we hypothesized that EGCG may also have vasodilator actions to stimulate production of nitric oxide (NO) from endothelial cells. Acute intra-arterial administration of EGCG to mesenteric vascular beds isolated *ex vivo* from WKY rats caused dose-dependent vasorelaxation. This was inhibitable by L-NAME (NO synthase inhibitor), wortmannin (phosphatidylinositol 3-kinase inhibitor), or PP2 (Src family kinase inhibitor). Treatment of bovine aortic endothelial cells (BAEC) with EGCG (50 μ M) acutely stimulated production of NO (assessed with NO-specific fluorescent dye DAF-2) that was inhibitable by L-NAME, wortmannin, or PP2. Stimulation of BAEC with EGCG also resulted in dose- and time-dependent phosphorylation of eNOS that was inhibitable by wortmannin or PP2 (but not by MEK inhibitor PD98059). Specific knockdown of Fyn (but not Src) with small interfering RNA inhibited both EGCG-stimulated phosphorylation of Akt and eNOS as well as production of NO in BAEC. Treatment of BAEC with EGCG generated intracellular H₂O₂ (assessed with H₂O₂-specific fluorescent dye CM-H₂DCF-DA), whereas treatment with N-acetylcysteine inhibited EGCG-stimulated phosphorylation of Fyn, Akt, and eNOS. We conclude that EGCG has endothelial-dependent vasodilator actions mediated by intracellular signaling pathways requiring reactive oxygen species and Fyn that lead to activation of phosphatidylinositol 3-kinase, Akt, and eNOS. This mechanism may explain, in part, beneficial vascular and metabolic health effects of green tea consumption.

Reciprocal relationships between insulin resistance and endothelial dysfunction contribute importantly to the pathophysiology of diabetes and its cardiovascular complications (1). Vascular actions of insulin to stimulate production of nitric oxide (NO)² enhance capillary recruitment and vasodilation. This leads to increased blood flow to skeletal muscle resulting in increased delivery of glucose and insulin to metabolic targets of insulin action (2). These vasodilator actions of insulin contribute significantly to insulin-mediated glucose uptake (3–6). Synergistic relationships between insulin sensitivity and endothelial function are reinforced because insulin signaling pathways in skeletal muscle, adipose tissue, and liver promoting metabolic actions of insulin are shared in common with insulin signaling pathways in vascular endothelium promoting vasodilator actions of insulin (1).

Green tea is a functional food that may have beneficial health effects to ameliorate cardiovascular and metabolic diseases (7–9). Thus, it may be feasible to identify and characterize nutritional supplements and/or functional foods that simultaneously improve both endothelial function and insulin sensitivity to provide safe and effective adjunctive treatment of diabetes and its cardiovascular complications. Epigallocatechin gallate (EGCG) is a bioactive polyphenol in green tea that makes up one-third of its dry mass (10). EGCG acutely activates specific intracellular signaling pathways regulating metabolic actions of insulin. In hepatocytes, EGCG inhibits gluconeogenesis through activation of PI 3-kinase-dependent pathways (11). In addition, EGCG mediates endothelium-dependent relaxation in aortic rings, whereas inhibitor studies in endothelial cells suggest that EGCG-stimulated activation of eNOS is PI 3-kinase dependent (12). Thus, we hypothesize that EGCG signaling pathways in endothelium may partly overlap with downstream insulin signaling pathways. However, signaling pathways used by EGCG for vasodilation in intact vessels and EGCG signaling pathways for activation of eNOS upstream of

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² The abbreviations used are: NO, nitric oxide; EGCG, epigallocatechin gallate; DAF-2 DA, 4,5-diaminofluorescein diacetate; CM-H₂DCF-DA, 5(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; MVB, mesenteric vascular beds; PI, phosphatidylinositol; WKY, Wistar-Kyoto; NE, norepinephrine; ACh, acetylcholine; PP, perfusion pressure; BAEC, bovine aortic endothelial cells; HAEC, human aortic endothelial cells; siRNA, small interfering RNA; MAP, mitogen-activated protein; ROS, reactive oxygen species; VEGF, vascular epidermal growth factor; L-NAME, N^G-nitro-L-arginine methylester.

PI 3-kinase are presently unknown (and may be distinct from those used by insulin). We now demonstrate novel contributions of reactive oxygen species and Fyn in the vasodilator actions of EGCG resulting from activation of eNOS in endothelium that are distinct from insulin signaling. Elucidating molecular mechanisms underlying vascular actions of EGCG is important for understanding potential health benefits of green tea consumption in patients with diabetes and related cardiovascular diseases that are characterized by both insulin resistance and endothelial dysfunction.

MATERIALS AND METHODS

Animal Experiments—All procedures in animals were performed in accordance with Guidelines and Authorization for the Use of Laboratory Animals (Italian Government, Ministry of Health). Male 12-week-old Wistar-Kyoto (WKY) rats obtained from Harlan Italy (Milan, Italy) were used in all studies.

Drugs—Drugs were obtained from the indicated sources: heparin (Pfizer); insulin (Novo Nordisk); and norepinephrine (NE), acetylcholine (ACh), EGCG (Sigma). Stock solutions of NE (100 mM) and ACh (10 mM) were prepared with distilled water. Final dilutions of these drugs were prepared in modified Krebs-Henseleit solution immediately before use. Stock solutions of EGCG in Me₂SO (10%) were prepared.

Evaluation of Vascular Function ex Vivo—Mesenteric vascular beds (MVB) were isolated from rats as described previously (13). Briefly, MVB mounted in a temperature-controlled moist chamber (type 834/1, Hugo Sachs Elektronik, March-Hungstetten, Germany) were perfused with modified Krebs-Henseleit solution continuously gassed with a mixture of 95% O₂ and 5% CO₂ (pH 7.4). A constant flow rate of 5 ml/min through the MVB was maintained using a peristaltic pump (ISM 833; Hugo Sachs Elektronik, March-Hungstetten, Germany). Drug solutions were infused into the perfusate proximal to the arterial cannula using another peristaltic pump. After an equilibration period (30–40 min), changes in perfusion pressure were measured with a Pressure Transducer System (SP 844 Capto, Horten, Norway) and recorded continuously using data acquisition and analysis equipment (PowerLab System, ADInstruments, Castle Hill, Australia). Intact endothelial function was verified by vasodilation induced by infusion of 1 μM ACh.

Vasodilator Responses in MVB—A steady-state perfusion pressure (PP) of ~120 mm Hg was obtained 30–40 min after initial administration of NE and was maintained by continuous NE infusion (10 μM). Dose-response curves measuring vasodilation (decrease in PP) in response to EGCG were obtained by adding increasing concentrations of EGCG (1–100 μM/4 min perfusion) to the perfusate. Data from each dose-response curve were normalized to PP obtained in WKY rats treated with a maximally stimulating dose of ACh (1 μM, 100% representing initial steady-state perfusion pressure and 0% representing maximal reduction in response to ACh). In some experiments, EGCG-induced relaxation was measured before and after treatment with L-NAME (NOS inhibitor; 100 μM, 30 min), wortmannin (PI 3-kinase inhibitor; 100 nM, 30 min), PD98059 (MEK inhibitor; 10 μM, 30 min), or PP2 (Src family kinase inhibitor; 10 μM, 30 min).

Cell Culture and Transfection—Bovine aortic endothelial cells (BAECs) in primary culture were obtained from Cell Applications (San Diego, CA) and maintained in EBM-2 media containing EGM-MV singlequot supplements (Cambrex, Walkersville, MD). All experiments were conducted on BAECs before their fifth passage. For experiments evaluating EGCG treatment, cells were serum-starved overnight and then incubated for 30 min in the absence or presence of various inhibitors prior to EGCG treatment as described in the figure legends. Human aortic endothelial cells (HAECs; Cell Applications) in primary culture were grown in EGM-2 media containing EGM-2 singlequot supplements (Cambrex). HAECs were transiently transfected with 200 nmol of siRNA duplex oligonucleotides (Csk siRNA sense oligonucleotide 5'-GCGAGUGCCUUAUCCAAGAUU-3'; c-Src Smartpool M-003175-03-0005 and Fyn Smartpool M-003140-03-0010 from Dharmacon (Lafayette, CO) using Lipofectamine with Plus reagents (Invitrogen) according to manufacturer's instructions. Two days after transfection, cells were serum-starved for 2 h and then treated without or with EGCG as indicated in the figure legends.

Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was isolated from HAECs using RNeasy mini kit (Qiagen, Valencia, CA). Reverse transcriptase-PCR was performed using one-step reverse transcriptase-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Primers for amplification of Csk are 5'-GGAGCACTACCGCATCATGT-3' (forward), and 5'-CAGCATCACGTCTCCGAAC-3' (reverse); for β-actin, 5'-CTGGCACCCAGCACAATGAAG-3' (forward), and 5'-TAGAAGCATTTGCGGTGGACG-3' (reverse). PCR was performed at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min with 25 cycles. The gene product was analyzed by 1% agarose gel electrophoresis.

Preparation of Cell Lysates and Immunoblotting—Before lysis, cells were briefly washed with ice-cold phosphate-buffered saline. Cells were then scraped in lysis buffer containing 50 mM Tris (pH 7.2), 125 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM Na₃VO₄, 20 mM NaF, 1 mM sodium pyrophosphate, and Complete protease inhibitor mixture (Roche Applied Science). Cell debris was pelleted by centrifugation of samples at 17,000 × g for 10 min at 4 °C. Supernatants were then boiled with Laemmli sample buffer for 5 min and proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with specific antibodies as described in the figures using standard methods. Immunoblots were quantified by scanning densitometry (GE Healthcare).

PI 3-Kinase Activity Assays—BAECs were serum-starved overnight and then treated without or with EGCG (50 μM, 15 min). Cell lysates were then prepared and subjected to immunoprecipitation with specific antibodies against phosphotyrosine (4G10, Upstate, Charlottesville, VA), Cbl (BD Biosciences, San Jose, CA), or Gab-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to standard methods as described (14). PI 3-kinase activity in the immunoprecipitates was detected as follows: for each sample, 10 μg of sonicated phosphatidylinositol substrate (Sigma) was incubated with 50 μl of PI 3-kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 100

Activation of eNOS by EGCG Requires ROS and Fyn

mM NaCl, 0.3 mM EGTA, 10 mM MgCl₂) and 10 μCi of [γ -³²P]ATP for 10 min at 37 °C. The reaction was stopped by adding 100 μl of 0.1 N HCl. Phospholipid was extracted with 200 μl of CHCl₃/CH₃OH (1:1). The organic phase was collected and applied to silica gel thin layer chromatography plates (Whatman) pre-activated with 1% potassium oxalate. Thin layer chromatography was performed in CHCl₃/CH₃OH/H₂O/NH₄OH (60:47:12.3:2). The plates were dried and results were visualized by autoradiography. Quantification was done by PhosphorImager analysis.

Measurement of NO Production—Production of NO was assessed using NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA; Cayman Chemical, Ann Arbor, MI) as described previously (15). Briefly, endothelial cells were grown to 95% confluence in chamber slides (Lab-Tek, Rochester, NY) and serum-starved overnight. Cells were then loaded with DAF-2 DA (final concentration, 1 μM) for 30 min at 37 °C, rinsed 3 times with EBM-2 media and kept in the dark. Cells were then treated without or with insulin or EGCG as indicated in the figure legends. In some experiments, wortmannin (100 nM), L-NAME (100 μM), or PP2 (20 μM) were added 30 min before loading cells with DAF-2 DA. After stimulation, cells were fixed in 2% paraformaldehyde for 5 min at 4 °C. Fixed cells were visualized using an Olympus IX81 inverted epifluorescent microscope with an attached charge-coupled device camera (Retiga Exi, Burnaby, British Columbia, Canada) using appropriate filters with a peak excitation wavelength of 480 nm and a peak emission wavelength of 510 nm. Images were captured using IP Labs Software (Scanalytics, Inc., Fairfax, VA).

Measurement of H₂O₂ Production—Production of intracellular H₂O₂ was assessed using H₂O₂-specific fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA, Invitrogen) using a method modified from Bae *et al.* (16). Briefly, BAECs were plated on chamber slides and incubated in serum- and growth factor-free media overnight. Cells were then washed with Dulbecco's phosphate-buffered saline containing calcium and magnesium before each experiment. Cells were loaded with CM-H₂DCF-DA (5 μM) for 10 min at 37 °C and then washed to remove excess CM-H₂DCF-DA. Cells were then treated with EGCG or H₂O₂ as indicated in the legends to figures followed by washing 3 times with DPBS. Cells were visualized and images were captured as described above for measurement of NO production.

RESULTS

Acute Vasodilator Actions of EGCG—To evaluate vasodilator actions of EGCG *ex vivo*, we isolated MVB from WKY rats. After pre-constriction of MVB with norepinephrine (NE) to a perfusion pressure of ~120 mm Hg, a supramaximal dose of ACh was administered to elicit vasorelaxation and verify endothelial function and integrity. Subsequently, increasing concentrations of EGCG (1–100 μM) were administered to the MVB resulting in dose-dependent reduction in perfusion pressure (Fig. 1, A–D, left panels). Pre-treatment of MVB with L-NAME (NOS inhibitor) completely inhibited vasodilator actions of EGCG (Fig. 1A). Moreover, pre-treatment of MVB with wortmannin (PI 3-kinase inhibitor) or PP2 (Src family kinase inhibitor) also significantly inhibited vasodilator actions of EGCG

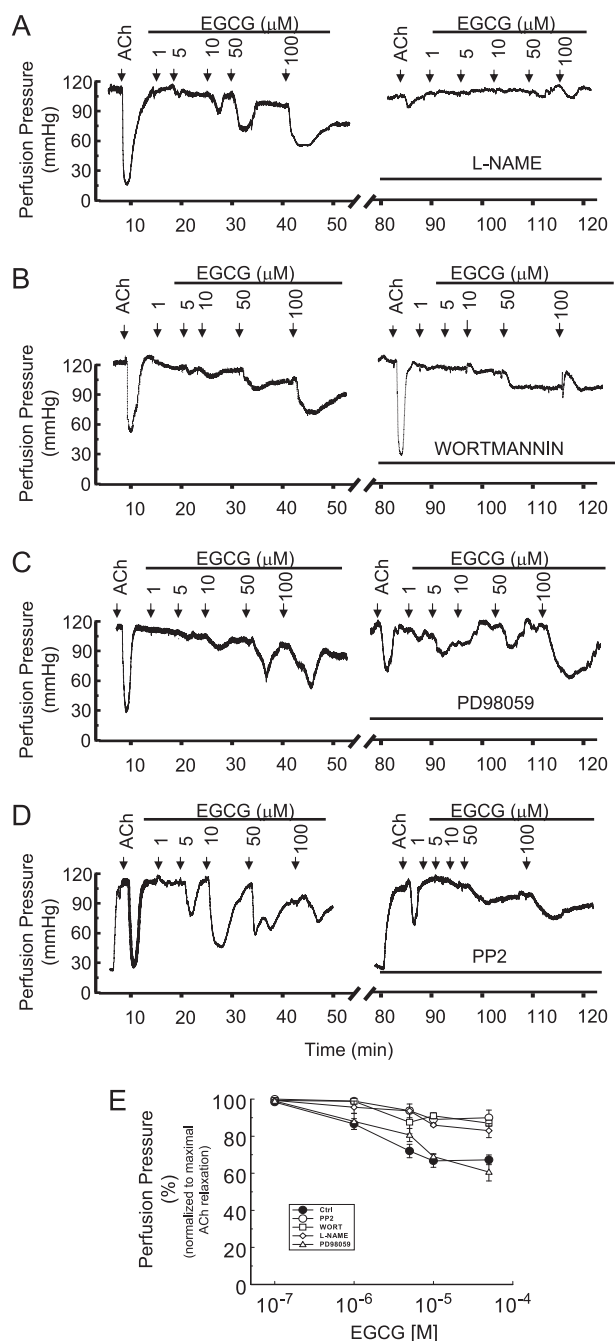


FIGURE 1. Acute vasodilator actions of EGCG require activation of Src family kinases and PI 3-kinase, but not MAP kinase. MVB from 12-week-old WKY rats were isolated as described under "Materials and Methods." Perfusion pressure at 120 mm Hg was maintained by continuous infusion with norepinephrine (NE, 10 μM). Pressure tracings indicate vasodilator responses to ACh (1 μM) and EGCG (1–100 μM) in the absence of inhibitors, and after a 20-min pre-treatment with specific inhibitors of various signaling pathways. Each arrow represents the start of a 30-s (ACh) or 4-min (EGCG) perfusion. Representative tracings from experiments repeated independently at least 5 times are shown for EGCG-induced vasorelaxation in the absence and presence of L-NAME (100 μM) (A), wortmannin (100 nM) (B), PD98059 (25 μM) (C), or PP2 (10 μM) (D). E, quantification of five independent experiments similar to those shown in A–D are plotted as mean ± S.E. Data from each curve were normalized by defining 100% as the initial steady-state perfusion pressure and 0% as the maximal reduction in perfusion pressure obtained after treatment with ACh. Statistical comparisons were made using two-tailed analysis of variance, comparing each curve before and after treatment. The vasorelaxation response to EGCG alone was significantly different when MVB were pretreated with L-NAME ($p < 0.04$), wortmannin ($p < 0.02$), or PP2 ($p < 0.02$), but not with PD98059 ($p > 0.94$).

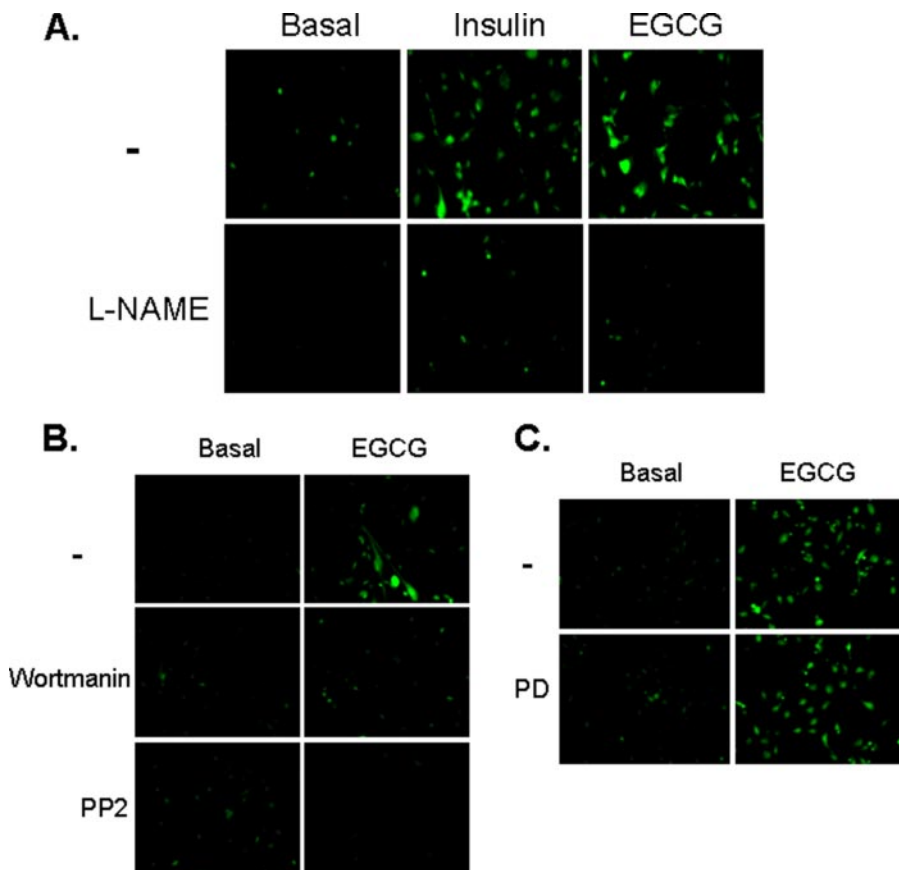


FIGURE 2. EGCG-stimulated production of NO from endothelial cells requires activation of Src family kinases. BAECs were serum-starved overnight and loaded with DAF2-DA as described under "Materials and Methods." Cells were then stimulated without or with insulin (100 nM) or EGCG (50 μ M) for 5 min. Some cells were pretreated with L-NAME (100 μ M) (A), wortmannin (100 nM) (B), PP2 (10 μ M) (B), or PD98059 (25 μ M) (C) 30 min prior to stimulation with EGCG or insulin. After insulin or EGCG treatment, cells were fixed in 2% paraformaldehyde and visualized with an epifluorescent microscope as described under "Materials and Methods." Emission of green fluorescence is indicative of NO production. Experiments shown are representative of those that were independently repeated at least 3 times.

(Fig. 1, B and D, right panels, and E), whereas pre-treatment with PD98059 (MEK inhibitor) did not (Fig. 1, C, right panel, and E). Consistent with our previous studies (13), Me₂SO control alone did not affect vasodilation of our preparation (data not shown). Taken together, these results suggest that EGCG has acute vasodilator actions that are mediated by endothelial production of NO requiring activation of Src family kinases and PI 3-kinase, but not MAP kinase.

EGCG Acutely Stimulates Production of NO from Endothelial Cells—Because endothelial-derived NO is an important physiological vasodilator, we next inquired whether acute EGCG treatment can directly stimulate production of NO from vascular endothelial cells in primary culture. Using the NO-specific fluorescent dye DAF-2 DA, we found that both insulin and EGCG acutely stimulated production of NO in BAEC that was inhibitable by L-NAME (Fig. 2A). EGCG-stimulated production of NO in BAEC was also inhibited by PP2 and wortmannin (Fig. 2B), but not by PD98059 (Fig. 2C). Thus, consistent with its acute vasodilator actions *ex vivo*, EGCG has direct actions in vascular endothelial cells to activate eNOS leading to production of NO that requires activation of Src family kinases and PI 3-kinase but not MAP kinase.

EGCG-stimulated Phosphorylation of eNOS—Insulin-stimulated activation of eNOS requires phosphorylation of eNOS at Ser¹¹⁷⁹ by Akt in a PI 3-kinase-dependent manner (17). Because EGCG mimics some PI 3-kinase-dependent metabolic actions of insulin (11), we evaluated the time course and dose dependence for EGCG-stimulated phosphorylation of eNOS at Ser¹¹⁷⁹. Stimulation of BAEC with EGCG (50 μ M) resulted in a time-dependent increase in phosphorylation of both eNOS and Akt with significant phosphorylation detectable after treatment for 15 min (Fig. 3A). We also observed dose-dependent phosphorylation of eNOS in BAEC after 15 min EGCG treatment with significant phosphorylation occurring at doses of 10 μ M or greater (Fig. 3B). Interestingly, EGCG also acutely stimulated phosphorylation of MAP kinase (Fig. 3, C and D, lane 2). As expected, pre-treatment of BAEC with wortmannin inhibited EGCG-stimulated phosphorylation of Akt and eNOS, but not MAP kinase. Moreover, PD98059 pre-treatment inhibited phosphorylation of MAP kinase in response to EGCG, but not phosphorylation of Akt or eNOS (Fig. 3C). Consistent with the importance of Src family

kinases in EGCG-stimulated production of NO identified in Figs. 1 and 2, pre-treatment of BAEC with PP2 inhibited EGCG-stimulated phosphorylation of Akt and eNOS (as well as MAP-kinase) (Fig. 3D). By contrast, insulin-stimulated eNOS phosphorylation was not affected by PP2 treatment (Fig. 3E). In addition, siRNA-mediated reduction of Csk, a negative regulator of Src family kinases, significantly enhanced EGCG-stimulated phosphorylation of Src family kinases, Akt, and eNOS (Fig. 3F). However, pre-treatment of BAEC with SB203580 (p38 MAPK inhibitor) did not significantly affect EGCG-stimulated phosphorylation of eNOS (data not shown). Taken together, these results suggest that EGCG-stimulated production of NO involves a Src family kinase.

Src Family Kinase Substrates That Bind and Activate PI 3-Kinase in Response to EGCG Treatment—Src family kinases are known to phosphorylate several intracellular substrates including Cbl and Gab-1 on tyrosine residues resulting in the ability of these substrates to bind and activate PI 3-kinase (18, 19). To identify specific Src substrates participating in EGCG-stimulated activation of PI 3-kinase, we treated BAECs without or with EGCG, immunoprecipitated cell lysates with antibodies against phosphotyrosine, Cbl, or Gab-1, and then subjected these immunoprecipitates to a lipid kinase assay using phos-

Activation of eNOS by EGCG Requires ROS and Fyn

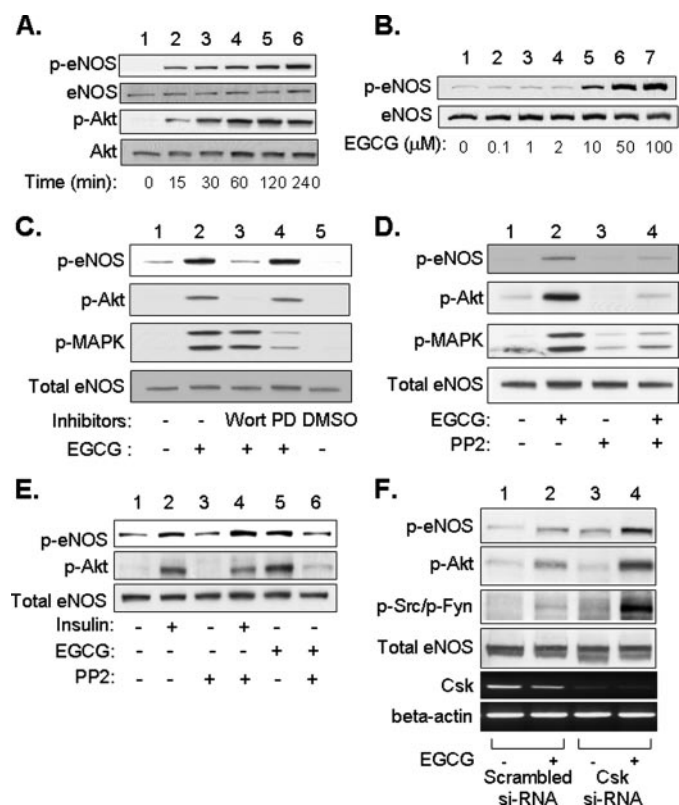


FIGURE 3. EGCG stimulates phosphorylation of eNOS in a time- and dose-dependent manner that requires activation of Src family kinases and PI 3-kinase, but not MAP kinase. BAECs were serum-starved overnight prior to EGCG treatment. In some experiments, cells were pre-treated with wortmannin (100 nM), PD98059 (25 μM), or PP2 (10 μM) 30 min prior to EGCG treatment. Cell lysates were immunoblotted with antibodies against eNOS, Akt, phospho-eNOS (Ser¹¹⁷⁹), phospho-Akt (Ser⁴⁷⁹), or phospho-MAPK (Thr²⁰²/Tyr²⁰⁴) as indicated. **A**, time course of phosphorylation of eNOS and Akt after stimulation of cells with EGCG (50 μM). **B**, treatment of BAEC with EGCG (15 min) stimulated dose-dependent increases in phosphorylation of eNOS. **C**, treatment of BAEC with EGCG (50 μM , 15 min) stimulated phosphorylation of eNOS and Akt that was inhibitable by wortmannin but not by PD98059. **D**, treatment of BAEC with EGCG (50 μM , 15 min) stimulated phosphorylation of eNOS and Akt that was inhibitable by PP2. **E**, after BAECs were serum starved overnight, cells were pre-treated with PP2 (1 μM) 30 min prior to insulin (100 nM, 5 min) or EGCG (10 μM , 15 min). Cell lysates were immunoblotted with the indicated antibodies. **F**, HAECs were transiently transfected with the indicated siRNA constructs as described under "Materials and Methods." 48 h after transfection, cells were serum-starved for 2 h and then treated without or with EGCG (50 μM , 15 min). Cell lysates were subjected to immunoblotting with the indicated antibodies. EGCG-stimulated phosphorylation of Akt and eNOS was enhanced by knockdown of Csk. mRNA levels of Csk were examined by reverse transcriptase-PCR as described under "Materials and Methods." Experiments shown are representative of those that were independently repeated at least 3 times.

phatidylinositol as a substrate (Fig. 4). Increased PI 3-kinase activity associated with phosphotyrosine and Gab-1 containing proteins after treatment of cells with EGCG was inhibited by PP2 pre-treatment. Cbl-associated PI 3-kinase activity was not different after EGCG treatment. Gab-1-associated PI 3-kinase is about 30% of the total phosphotyrosine associated activity. Thus, Gab-1-associated PI 3-kinase activity may contribute to EGCG actions. However, there are likely to be other unidentified Src family kinase substrates that also contribute to this process.

Fyn, but Not Src, Is Necessary for EGCG-stimulated Phosphorylation of eNOS and Production of NO—Of the 11 known members of the Src family, only Src, Fyn, and Yes are thought to

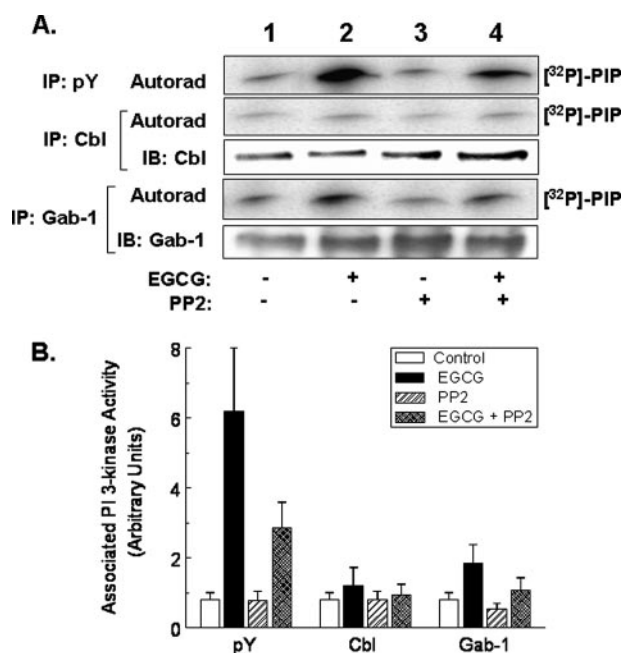


FIGURE 4. Acute treatment of BAEC with EGCG stimulates association of PI 3-kinase activity with phosphotyrosine containing proteins, Cbl, and Gab-1 that requires activation of Src family kinases. BAECs were serum-starved overnight, pre-treated without or with PP2 (10 μM , 30 min) and then stimulated without or with EGCG (50 μM , 15 min). Cell lysates were immunoprecipitated (IP) with antibodies against phosphotyrosine, Cbl, or Gab-1. Immunoprecipitated samples from each group were divided into two aliquots that were subjected to either PI 3-kinase assays or immunoblotting (IB) as indicated. **A**, representative autoradiograms from PI 3-kinase assays and immunoblots are shown for samples immunoprecipitated with the indicated antibodies. **B**, [³²P]PIP products from lipid kinase assays similar to those shown in **A** were quantified using a PhosphorImager. For samples immunoprecipitated with antibodies against Cbl and Gab-1, results were normalized for recovery of these proteins. Tyr(P) (pY) ($p < 0.03$) and Gab-1 ($p < 0.05$) associated PI 3-kinase activity in EGCG and EGCG+PP2 groups are significantly different in Student's *t* test analysis (two-tail), whereas Cbl-associated PI 3-kinase activity was not different ($p > 0.5$). The effect of EGCG alone when compared with control in pY ($p < 0.004$), and Gab-1 ($p < 0.002$) associated PI 3-kinase groups are significantly different, whereas Cbl-associated PI 3-kinase is not significantly different ($p > 0.4$). Mean \pm S.E. of five independent experiments is shown.

be ubiquitously expressed (20). To identify specific Src family members involved with EGCG-stimulated phosphorylation of eNOS and production of NO we used siRNA oligonucleotides to specifically reduce expression of Src or Fyn in HAEC in primary culture. In preliminary experiments, fluorescently tagged control oligonucleotides were used to demonstrate that our transfection efficiency for siRNA in HAEC was greater than 90% (data not shown). When compared with control scrambled siRNA, siRNA targeting Src or Fyn specifically and efficiently reduced expression of the targeted proteins in transfected HAEC (Fig. 5A, bottom panels). Importantly, decreasing Fyn expression substantially inhibited EGCG-stimulated phosphorylation of Akt and eNOS, whereas reducing expression of Src did not (Fig. 5A). Consistent with these results, EGCG-stimulated production of NO was abolished by transfecting cells with siRNA against Fyn, but not Src (Fig. 5C). These results strongly suggest that Fyn is the specific Src family member that is required for EGCG-stimulated activation of eNOS and production of NO in endothelial cells.

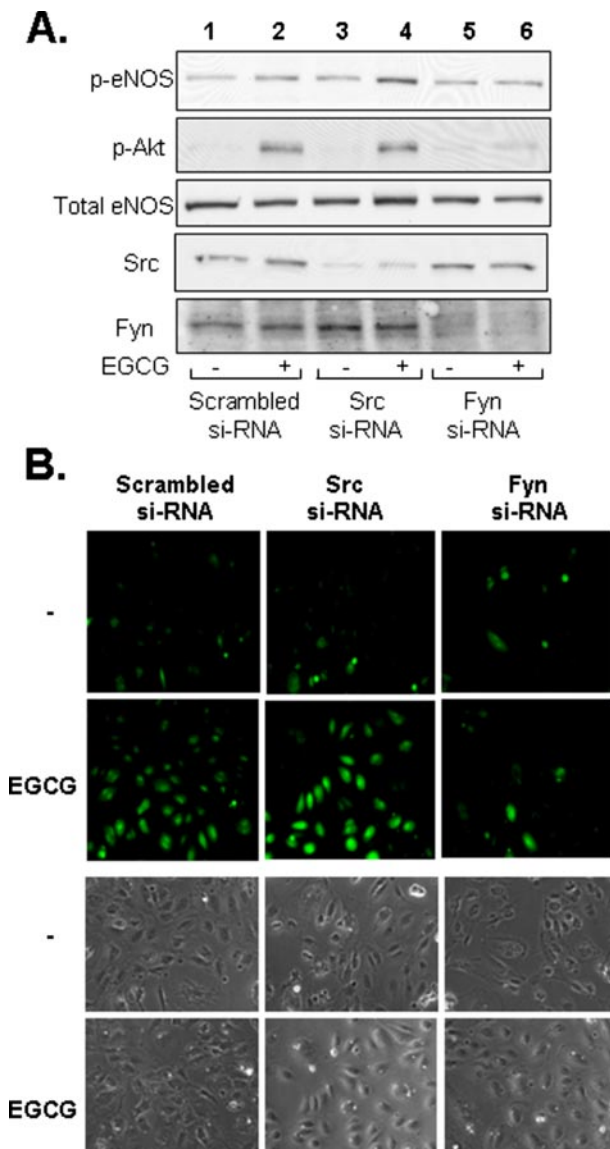


FIGURE 5. siRNA-mediated knockdown of Fyn, but not Src, inhibits EGCG-stimulated phosphorylation of Akt and eNOS as well as production of NO in endothelial cells. HAECs were transiently transfected with the indicated siRNA constructs as described under "Materials and Methods." 48 h after transfection, cells were serum-starved for 2 h and then treated without or with EGCG (50 μ M, 15 min). Cell lysates were subjected to immunoblotting with the indicated antibodies. *A*, EGCG-stimulated phosphorylation of Akt and eNOS was inhibited by knockdown of Fyn, but not Src. *B*, 48 h after transfection with the indicated siRNAs, HAECs were loaded with DAF-2 DA as described under "Materials and Methods" and treated without or with EGCG (50 μ M, 15 min). Cells were then fixed in 2% paraformaldehyde and visualized with an epifluorescent microscope as described under "Materials and Methods." Emission of green fluorescence is indicative of NO production (*upper panels*). *Lower panels* show phase-contrast view of cells. Experiments shown are representative of those that were independently repeated at least three times.

EGCG-stimulated Phosphorylation of eNOS Requires Production of Reactive Oxygen Species (ROS)— H_2O_2 is an important mediator of intracellular signaling initiated by cell surface receptors (16, 21–23). Moreover, H_2O_2 is capable of stimulating activation of Akt through a Src family kinase-dependent mechanism in some cell types (24). Therefore, we next examined whether H_2O_2 plays a role in EGCG-stimulated activation of eNOS in endothelial cells. BAEC loaded with the H_2O_2 -spe-

cific fluorescent dye CM- H_2 DCF-DA were treated with EGCG (50 μ M) for 0–15 min. We observed green fluorescence in the cells indicative of H_2O_2 production that increased from 5 to 15 min after EGCG treatment (Fig. 6A). Increases in intracellular H_2O_2 in response to treatment of BAEC with either EGCG or H_2O_2 were abrogated by pre-treatment of cells with the ROS scavenger *N*-acetylcysteine (Fig. 6B). Under similar conditions, increased tyrosine phosphorylation of Fyn at Tyr⁴¹⁸ (a proxy for Fyn activation) in response to EGCG stimulation was significantly inhibited by pre-treatment of cells with *N*-acetylcysteine (Fig. 6C). Likewise, increased phosphorylation of Akt and eNOS in response to EGCG stimulation was significantly inhibited by pre-treatment of cells with *N*-acetylcysteine (Fig. 6D). However, *N*-acetylcysteine treatment did not substantially affect insulin-stimulated phosphorylation of Akt or eNOS (Fig. 6E). These results suggest that EGCG-stimulated production of ROS (including H_2O_2) in endothelial cells is a part of the intracellular signaling cascade upstream from PI 3-kinase distinct from insulin signaling.

Exploration of Putative Upstream Mediators of EGCG Action—The laminin receptor has been implicated as a specific cell-surface receptor for EGCG that mediates some of its biological actions (25, 26). When we overexpressed or reduced expression of laminin receptors in endothelial cells we did not observe any significant effects to alter EGCG-mediated phosphorylation of Akt or eNOS (data not shown). Thus, it seems unlikely that the laminin receptor is mediating vasodilator actions of EGCG in endothelial cells. Because EGCG mimics some vascular actions of insulin (Figs. 1–3), we also evaluated the possibility that EGCG may be transactivating the insulin receptor. When we treated BAEC with insulin or EGCG, we only observed tyrosine phosphorylation of the insulin receptor in response to insulin, but not EGCG (Fig. 7A). Therefore, it is unlikely that EGCG-stimulated production of NO is mediated by transactivation of the insulin receptor. Similarly, to help rule out the possibility that vasodilator actions of EGCG are mediated by the VEGF receptor, we treated BAEC without or with EGCG or VEGF in the absence or presence of the VEGF receptor inhibitor VTI (Fig. 7B). Although both EGCG and VEGF stimulated phosphorylation of Akt and eNOS, only the effects of VEGF, but not EGCG, were inhibited by VTI. Thus, EGCG-stimulated production of NO is unlikely to be mediated by transactivation of the VEGF receptor.

DISCUSSION

Metabolic diseases including diabetes and obesity often cluster together with cardiovascular diseases including hypertension and coronary heart disease in a metabolic syndrome that is characterized by both insulin resistance and endothelial dysfunction (27, 28). Some epidemiological studies have linked green tea consumption with significant reductions in cardiovascular and metabolic diseases as well as overall mortality (7–9, 29, 30). However, molecular mechanisms underlying putative health benefits of green tea consumption are poorly understood. Green tea consists of unfermented leaves of *Camellia sinensis* that contain a number of bioactive polyphenols. EGCG, the major polyphenol in green tea, makes up ~30% of the solids in green tea. Red wine, black tea, and dark choco-

Activation of eNOS by EGCG Requires ROS and Fyn

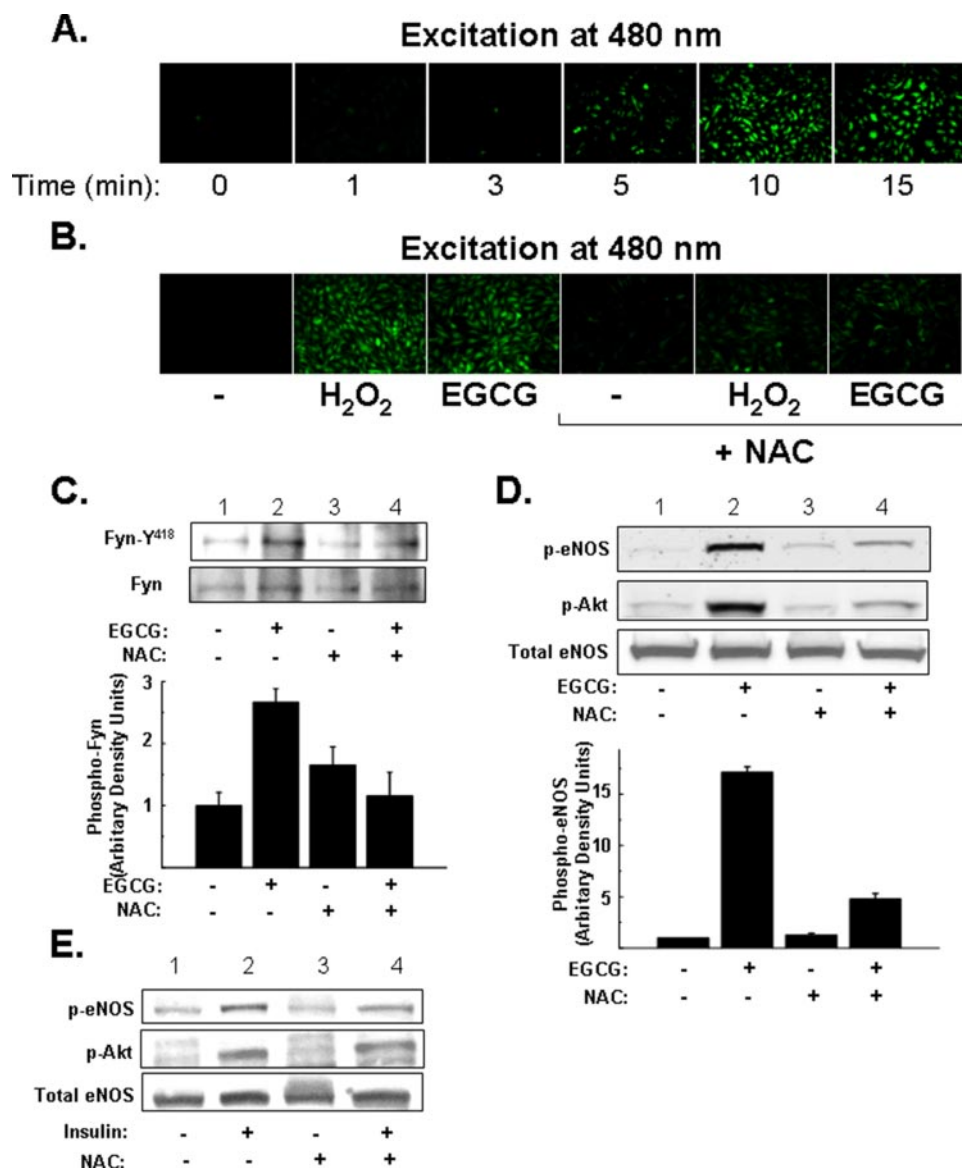


FIGURE 6. EGCG-stimulated phosphorylation of eNOS requires production of reactive oxygen species. *A*, BAECs were serum-starved overnight and then loaded with CM-H₂DCF-DA as described under "Materials and Methods." Cells were stimulated with EGCG (50 μM) for the times indicated. Emission of green fluorescence is indicative of H₂O₂ production. Experiments shown are representative of those that were repeated independently twice. *B*, BAEC prepared as in *A* were treated with EGCG (50 μM, 15 min) or H₂O₂ (1 μM, 5 min) in the absence or presence of *N*-acetylcysteine pre-treatment (NAC, 10 mM, 2 h). Emission of green fluorescence is indicative of H₂O₂ production. Experiments shown are representative of those that were independently repeated at least 3 times. *C*, BAECs were serum-starved overnight and then treated with EGCG (50 μM, 15 min) in the absence or presence of *N*-acetylcysteine pre-treatment (NAC, 10 mM, 2 h). Cell lysates were then immunoprecipitated with anti-Fyn antibody followed by immunoblotting with phospho-specific anti-Fyn (Tyr⁴¹⁸) antibody (upper panel). The same blot was stripped and then re-probed with anti-Fyn antibody (lower panel). Immunoblots from 3 independent experiments were quantified by scanning densitometry, normalized for total Fyn amount, and then data were plotted as the mean ± S.E. *D*, BAECs were serum-starved overnight and then treated with EGCG (50 μM, 15 min) or H₂O₂ (1 μM, 5 min) in the absence or presence of *N*-acetylcysteine pre-treatment (NAC, 10 mM, 2 h). *E*, BAECs were serum-starved overnight and then treated with insulin (100 nM, 5 min) in the presence or absence of *N*-acetylcysteine pre-treatment (2 mM, 2 h). Cell lysates were immunoblotted with specific antibodies as indicated. Immunoblots from three independent experiments were quantified by scanning densitometry, normalized for total eNOS amount, and then data were plotted as the mean ± S.E.

late contain polyphenolic compounds related to EGCG including resveratrol, epicatechin gallate, and epicatechin that have vascular actions related to activation of eNOS (31–34). Drinking 8–10 cups of green tea is sufficient to increase serum levels of EGCG into the micromolar range (35). In some animal and cellular studies, EGCG has negative regulatory effects on

energy metabolism that include stimulation of fat oxidation and weight loss, inhibition of fatty acid synthase, and decreased appetite (36–39). Interestingly, EGCG also mimics metabolic actions of insulin to inhibit gluconeogenesis in hepatocytes (11). Because shared insulin signaling pathways in metabolic and vascular tissues contribute to linking metabolic and cardiovascular homeostasis (1), we investigated potential mechanisms for EGCG to improve cardiovascular health.

Acute Vasodilator Actions of EGCG—In the present study, we demonstrated that EGCG has direct NO-dependent effects to acutely mediate vasodilation in MVB from WKY rats that requires activation of Src family kinases and PI 3-kinase, but not MAP kinase. These results regarding the signaling pathways in intact vessels extends a previous report that found relaxation of aortic rings in response to EGCG is blocked by the NOS inhibitor L-NAME (12). With respect to vasodilator actions of insulin, we previously elucidated a complete biochemical signaling pathway that involves the insulin receptor phosphorylating IRS-1 that then binds and activates PI 3-kinase resulting in activation of PDK-1 and Akt, that then directly phosphorylates eNOS (17, 40–42). Thus, only some, but not all, of the signaling pathways mediating vasodilator actions of EGCG are shared in common with insulin (1) (Fig. 8). The magnitude of vasodilation in response to EGCG that we observed in MVB from WKY rats is comparable with that observed with EGCG treatment of MVB from SHR rats (43). By contrast, insulin-resistant, hypertensive SHR rats have impaired vasodilator responses to insulin when compared with normotensive WKY rats without insulin resistance (13, 44). Taken together with the observa-

tion that signaling pathways regulating vasodilator actions of EGCG are not completely overlapping with those of insulin, these results raise the possibility that vasodilator actions of EGCG may have beneficial effects on endothelial dysfunction related to insulin resistance. Indeed, we recently demonstrated that chronic treatment of SHR rats with EGCG for 3 weeks

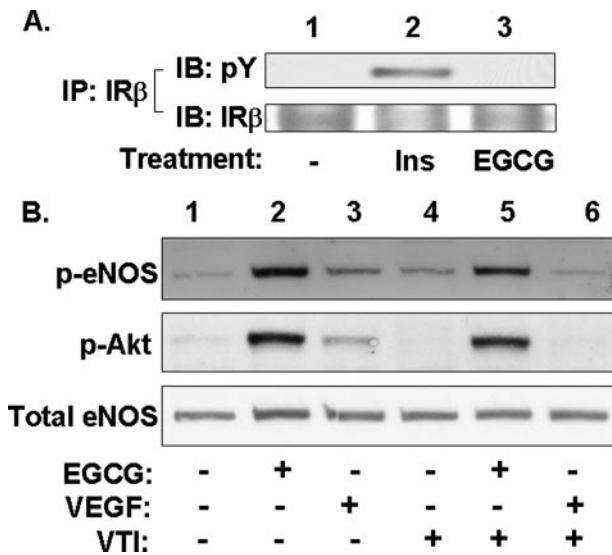


FIGURE 7. Acute treatment of BAEC with EGCG does not transactivate insulin or VEGF receptors. *A*, BAECs were serum-starved overnight and then treated without or with insulin (100 nM, 5 min) or EGCG (50 μM, 15 min). Cell lysates were immunoprecipitated (IP) with antibodies against the β-subunit of the insulin receptor and then immunoblotted (IB) with antibodies against phosphotyrosine (pY) or the β-subunit of the insulin receptor. *B*, BAECs were serum-starved overnight, pre-treated without or with VTI (10 μM, 30 min), and then treated without or with VEGF (100 nM, 2 min) or EGCG (50 μM, 15 min). Cell lysates were subjected to immunoblotting with the indicated antibodies. Representative immunoblots are shown from experiments that were repeated independently at least three times.

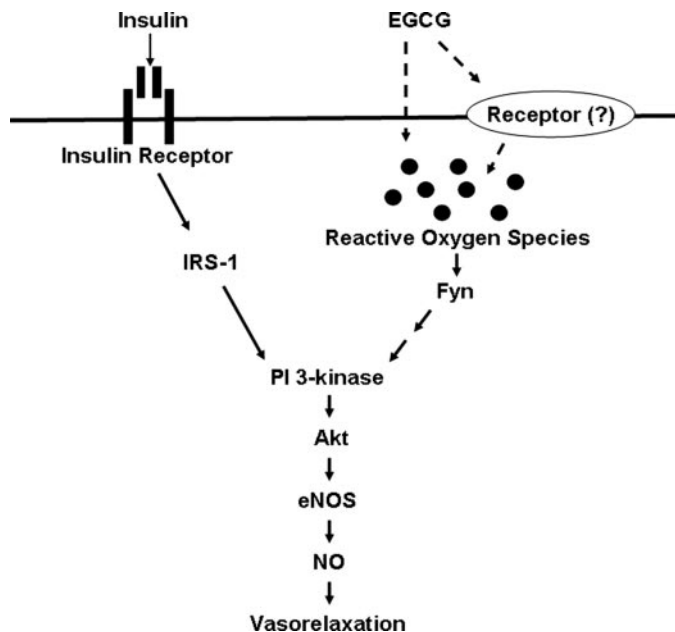


FIGURE 8. Schematic diagram of EGCG signaling pathway in vascular endothelium that converges with insulin signaling pathways related to production of NO and vasorelaxation.

lowers blood pressure, improves endothelial dysfunction and insulin resistance, and protects against myocardial ischemia/reperfusion injury in a genetic model of the metabolic syndrome (43).

Signaling Pathways Regulating EGCG-stimulated Production of NO in Endothelial Cells—In vascular endothelial cells, EGCG-stimulated production of NO required ROS generation and activation of Fyn that then lead to activation of PI 3-kinase/

Akt/eNOS. EGCG treatment of endothelial cells stimulated phosphorylation of eNOS at its Akt phosphorylation site in a time- and dose-dependent manner. Our findings on this PI 3-kinase dependence are consistent with Lorenz *et al.* (12) who showed the inhibitor LY29402 blunts EGCG-stimulated phosphorylation and activation of eNOS in cultured endothelial cells. However, our findings regarding the requirement of ROS and Fyn emphasize that EGCG uses distinct mechanisms from those of insulin upstream from PI 3-kinase.

Because our assay for NO production in endothelial cells is only qualitative, we cannot correlate EGCG dose responses for NO production with vasodilator responses in MVB *ex vivo*. It is also difficult to compare temporal responses between our *ex vivo* experiments and *in vitro* ROS formation or NO production. There are limitations in comparing time courses of two very different types of experimental preparations due to differences in the sensitivity of *in vitro* and *ex vivo* experiments. We were able to detect eNOS phosphorylation and NO production at earlier time points such as 5 min but chose to show 15 min because the signal was stronger at that point. Nevertheless, both *ex vivo* and *in vitro* EGCG effects are relatively acute.

Our finding that Fyn is a signaling molecule upstream of PI 3-kinase required for EGCG-stimulated phosphorylation of eNOS and production of NO is a novel and important feature that distinguishes vasodilator actions of EGCG from those of insulin. Fyn can activate PI 3-kinase either by directly binding to the p85 regulatory subunit of PI 3-kinase (45, 46), or by phosphorylating intracellular substrates including Cbl and Gab-1 that then bind and activate PI 3-kinase (20). When we examined potential substrates that may link Fyn with activation of PI 3-kinase in response to EGCG in endothelial cells, we found substantial PI 3-kinase activity associated with phosphotyrosine proteins (this may represent Fyn itself). We also found ~30% of PI 3-kinase activity associated with Gab-1 but not with Cbl. Consistent with these results, Gab-1 plays an important role in mediating effects of shear stress to stimulate activation of eNOS through ligand-independent activation of the VEGF receptor that involves Src family kinases (18). Cbl has also been implicated previously in activation of PI 3-kinase in response to shear stress in endothelial cells (47), but Cbl does not seem to be significantly involved in EGCG stimulated PI 3-kinase activity.

Using specific inhibitors and siRNA constructs, we helped to rule out roles for Src, MAP kinase, and p38 MAP kinase in EGCG-stimulated phosphorylation of eNOS and production of NO. Src family members play important roles in VEGF-mediated angiogenesis and activation of eNOS (48–52). In addition, Src and Yes help to regulate vascular permeability (52, 53), whereas Fyn may play a role in tube formation by endothelial cells (54, 55). Interestingly, although Src has been implicated in activation of eNOS by estrogen (56), high density lipoprotein (57), and shear stress (58), these studies generally rely on the Src family kinase inhibitor PP2 or expression of dominant negative Src mutants that may also interact with other Src family members. Our experiments helped to rule out a role for Src *per se* by using specific siRNA oligonucleotides that do not significantly affect other Src family members such as Fyn. We investigated MAP kinase because although both EGCG and insulin stimulate phosphorylation of MAP kinase, this is not involved with

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insulin-stimulated production of NO (1, 13). With respect to p38 MAP kinase, black tea polyphenols (that have some overlap with green tea polyphenols) stimulate phosphorylation of eNOS and production of NO, in part, through activation p38 MAP kinase (59).

Exploring Potential Mediators of Vascular Actions of EGCG Upstream from ROS and Fyn—The laminin receptor (67 kDa) is one specific cell-surface receptor for EGCG that mediates some of its biological actions (25, 26). However, when we overexpressed or inhibited expression of the laminin receptor, we did not observe any significant differences in EGCG-stimulated phosphorylation of Akt or eNOS. Thus, the laminin receptor does not appear to mediate vasodilator actions of EGCG. Because EGCG stimulates production of NO from endothelial cells using signaling mechanisms partially overlapping with those of insulin, we also examined whether treatment of endothelial cells with EGCG activates the insulin receptor (assessed by insulin receptor tyrosine phosphorylation). We were unable to detect phosphorylation of the insulin receptor in response to EGCG treatment. Therefore, it seems unlikely that vasodilator actions of EGCG are mediated by transactivation of insulin receptors. In other cellular contexts, EGCG treatment of hepatocytes stimulates tyrosine phosphorylation of the insulin receptor (11). Differences between these results and our results may be due to cell-type specific differences between hepatocytes and endothelial cells. Activation of Src family kinases have been implicated in ligand-independent and -dependent activation of VEGF receptors mediating angiogenesis and activation of eNOS (18, 49–52). However, in our studies, a specific inhibitor of VEGF receptor signaling did not significantly affect EGCG-stimulated phosphorylation of Akt or eNOS. Thus, our results with EGCG are unlikely to be due to transactivation of the VEGF receptor.

Several receptor tyrosine kinases including the platelet-derived growth factor receptor and epidermal growth factor receptor mediate their signaling, at least in part, by generation of H₂O₂ (60) that can then go on to activate Src family kinases including Fyn (61). Interestingly, ROS are implicated in mediating effects of EGCG to mimic metabolic and vascular actions of insulin in hepatocytes (11), and vascular endothelial cells (this study). When compared with EGCG, the signaling pathway used by insulin in endothelial cells upstream from PI 3-kinase seems to be distinct. Although in some cell types insulin can produce H₂O₂ that leads to activation of PI 3-kinase and Akt (62, 63), the role of ROS in insulin signaling in vascular endothelial cells has not been reported. In the present study, we found that scavenging ROS does not substantially affect insulin-stimulated phosphorylation of Akt and eNOS (Fig. 6E). In the case of EGCG, there may be unidentified specific cell surface receptors for EGCG that are involved in generation of ROS. However, another possibility is that EGCG may directly generate ROS (including H₂O₂) in a receptor-independent fashion either by autooxidation (64) or by acting as a pro-oxidant *in vivo* at physiological pH (65). Thus, despite many claims that EGCG is an antioxidant *in vitro* (66), beneficial bioactivity of EGCG may in fact depend on its ability to behave as a pro-oxidant in physiological contexts that initiates acute intracellular signaling events such as those described in the present study. Clearly,

further studies are required to elucidate signaling mechanisms upstream from ROS that mediate vasodilator actions of EGCG.

Conclusions—We have identified novel roles for ROS and Fyn in activating PI 3-kinase/Akt/eNOS to mediate NO-dependent vasodilator actions of EGCG in vascular endothelium that are partially overlapping with mechanisms regulating vasodilator actions of insulin. Given the importance of reciprocal relationships between insulin resistance and endothelial dysfunction in the pathophysiology of diabetes and cardiovascular diseases, our findings may be relevant to understanding molecular mechanisms responsible for beneficial effects of green tea consumption to improve metabolic and cardiovascular health as well as overall mortality.

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Epigallocatechin Gallate, a Green Tea Polyphenol, Mediates NO-dependent Vasodilation Using Signaling Pathways in Vascular Endothelium Requiring Reactive Oxygen Species and Fyn

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