Activation of Endothelial Nitric-oxide Synthase by the p38 MAPK in Response to Black Tea Polyphenols*

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Black tea improves endothelial function in patients with coronary artery disease. We sought to determine the responsible components of black tea and elucidate the underlying cell signaling mechanisms. We exposed porcine aortic endothelial cells to components of black tea and found that the polyphenol fraction acutely enhanced nitric oxide bioactivity. This effect involved endothelial nitric-oxide synthase (eNOS) phosphorylation at Ser-1177 and dephosphorylation at Thr-495, consistent with increased eNOS activity. These effects were calcium-dependent, as removal of extracellular calcium prevented eNOS phosphorylation at Ser-1177, whereas inhibition of intracellular calcium mobilization with TMB-8 blunted Thr-495 dephosphorylation. Black tea polyphenol-induced eNOS activation appeared dependent upon the phosphatidylinositol 3-kinase-Akt pathway, as it was significantly inhibited by LY294002 and a dominant negative Akt, respectively. Pharmacological inhibition of p38 mitogen-activated protein kinase (p38 MAPK) with either SB202190 or SB203580 as well as overexpression of a dominant negative p38 MAPK attenuated both eNOS activation and phosphorylation changes in response to black tea polyphenols. Inhibition of p38 MAPK also blunted Akt activation in response to black tea polyphenols, suggesting that p38α MAPK is upstream of Akt in this pathway. Finally, a constitutively active mutant of MKK6, an upstream kinase for p38 MAPK, enhanced both the basal and stimulated activity of Akt, leading to increased eNOS activity. Taken together, these data identify the p38 MAPK as an upstream component of Akt-mediated eNOS activation.

The general appreciation that oxidative stress is involved in the pathogenesis of cardiovascular diseases has prompted interest in natural classes of antioxidants. One such class of natural antioxidants is the flavonoid family, a group of polyphenolic substances found mainly in fruit, vegetables, wine, and tea (1). Being the second most consumed beverage worldwide aside from water, tea has become a subject of many epidemiological studies, suggesting an inverse relationship between tea consumption and cardiovascular diseases (for review, see Ref 2). One proposed mechanism for the benefit of such dietary flavonoids is their antioxidant properties that include the scavenging of reactive oxygen species (3) and the inhibition of lipid peroxidation (4). This latter property of flavonoids suggests they may inhibit low density lipoprotein oxidation, an event implicated in atherosclerosis (5). However, clinical studies in healthy human subjects indicate that tea flavonoids have a limited impact on this process ex vivo (6).

There is compelling evidence that another target for flavonoids is the endothelium (reviewed in Refs. 1 and 7). Normally, the endothelium regulates vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle proliferation via the release of several paracrine factors, including nitric oxide (NO) (8). These functions of the endothelium are impaired in the setting of atherosclerosis and its associated vascular diseases (8). Moreover, individuals with the greatest impairment of endothelial function are at the greatest risk of cardiovascular events (9), suggesting that endothelial dysfunction is, in part, responsible for the clinical activity of atherosclerosis. In this regard, we have found that endothelial NO bioactivity is enhanced in patients with atherosclerosis by either the acute or chronic administration of black tea (10). The purpose of the current investigation was to determine the components of black tea responsible for this effect and to elucidate the underlying cell signaling events involved in the effect of black tea.

EXPERIMENTAL PROCEDURES

Materials—M-199 medium, 1-glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen. We obtained LY294002, SB202190, SB203580, p38 inhibitor III, W7 hydrochloride, and TMB-8 hydrochloride from Calbiochem. Polyclonal antibodies directed against Akt, phospho-Akt (Ser-473), phospho-GSK-3α/β (Ser-21/9), p38 mitogen-activated protein kinase (p38 MAPK), p38 MAPK (Thr-180 and Tyr-182), phospho-glycogen synthase kinase (GSK3) (Ser-21/9), and anti-hemagglutinin were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies directed against phospho-eNOS (Thr-495) and phospho-eNOS (Ser-1179) were obtained from Upstate Biotechnology.

1 The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; PAEC, porcine aortic endothelial cells; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKAPK-2, MAPK-activated protein kinase 2; MKK6, MAPK kinase 6; MKK6β, recombinant adenovirus-expressing activated MKK6; ERK, extracellular signal-regulated kinase (ERK); p38 MAPK, phosphorylated p38 MAPK; Thr-202 and Tyr-204, and anti-hemagglutinin were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies directed against phospho-eNOS (Thr-495) and phospho-eNOS (Ser-1179) were obtained from Upstate Biotechnology.
Role of Black Tea Polyphenols in eNOS Activation

ogy, Inc. (Lake Placid, NY). Anti-eNOS monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). 1-[3H]arginine (1 mCi; 53mCi/mmol) was obtained from PerkinElmer Life Sciences, and cGMP assay kits were from Cayman (Ann Arbor, MI). Black tea polyphenols and black tea fractions were provided by Unilever, Inc. All other reagents were obtained from Sigma.

Endothelial Cell Culture—Porcine aortic endothelial cells (PAECs) were harvested and cultured on fibronectin-coated tissue culture flasks in M-199 medium supplemented with 20% FBS, 50 μg/ml heparin sulfate, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For experiments, confluent PAECs were used between passages 4 and 10. Prior to all experiments, PAECs were placed in serum-free and phenol-free media (Opti-MEM, Invitrogen) for 6 h and then washed twice in HEPESt-buffered physiologic salt solution (PSS) containing 22 mM HEPEs (pH 7.4), 124 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 0.16 mM Na₃PO₄, 0.4 mM NaH₂PO₄, 5 mM NaHCO₃, and 5.6 mM d-glucose. Cells were then incubated in HEPEs-buffered PSS 30 min prior to the addition of the black tea compounds as indicated.

L-arginine to L-citrulline Conversion Assay—Determination of eNOS catalytic activity in intact PAECs was performed by measurement of the conversion of L-arginine to L-citrulline, which is sensitive to L-arginine methyl ester (L-NAMe). Confluent PAECs in 100-mm culture dishes were washed and incubated in PSS for 30 min followed by the addition of 10 μM L-arginine plus 3.3μCi of L-[3H]arginine. PAECs were then treated with the agonist of interest or buffer for 15 min and then washed with PSS and lysed with 200 μl of 100% ethanol, after which 2 ml of stop buffer (20 mM sodium acetate acid hydrolys, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA) was added. The lysates were then subjected to anion exchange chromatography using 2-ml Dowex AG50W-X8 columns (Bio-Rad) equilibrated with stop buffer. The column was washed with 2 ml of stop buffer, and the eluent was collected for the determination of L-[3H]citrulline by liquid scintillation counting. Data are reported as the extent of conversion of L-arginine to L-citrulline that is sensitive to pretreatment of the PAECs for 30 min with L-NAMe (500 μM) and is expressed as dpm/10⁹ PAECs.

Immunoprecipitation and Western Blotting—Confluent PAECs in six-well plates were equilibrated for 30 min in PSS supplemented with 250 μM 3-isobutyl-1-methylxanthine (IBMX) and 250 μM L-arginine in the absence or presence of L-NAMe (500 μM) or the inhibitor of interest. Equilibrated cells were then exposed to the black tea compound or vehicle alone for 30 min before the cells were lysed by the addition of 6% ice-cold trichloracetic acid. Cell lysates were then subjected to centrifugation at 10,000 × g for 15 min at 4 °C, and the supernatant was either resuspended in lysis buffer or incubated in fresh medium with 10% FBS for 24–36 h prior to experimentation.

Immunoprecipitation. For eNOS immunoprecipitation, lysates were incubated with eNOS monoclonal antibody (5 μg/ml) overnight at 4 °C followed by a 1-h incubation with protein A/G-agarose. Following centrifugation, the immunoprecipitates were washed three times with ice-cold trichloroacetic acid. Cell lysates were then subjected to centrifugation for 10 min, and determination of cGMP in the supernatant was performed as described previously (14).

Statistical Analysis—All numerical data are presented as means ± S.E. Western blots shown are representative of three or more independent experiments. For parametric data, comparisons among treatment groups were performed with one-way analysis of variance and an appropriate post hoc comparison. Instances involving only two comparisons were evaluated with a Student’s t test. Instances involving more than two comparisons were performed using analysis of variance. Statistical significance was accepted if the null hypothesis was rejected with p < 0.05.

RESULTS

The Polyphenolic Fraction of Black Tea Activates eNOS—We first assessed dose- and time-dependent effects of black tea on endothelial NO production by the increase in cellular cGMP content (Fig. 1, A and B). Black tea-induced cGMP accumulation appeared to result from eNOS activation, as it was also characterized by the enhanced conversion of L-arginine to L-citrulline by liquid scintillation counting. Data are reported as the extent of conversion of L-arginine to L-citrulline that is sensitive to pretreatment of the PAECs for 30 min with L-NAMe (500 μM) and is expressed as dpm/10⁹ PAECs.

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Blacks tea polyphenols, which closely approximated the time course of eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495 (Fig. 2A), which is consistent with eNOS activation. We next investigated the upstream kinases involved in eNOS phosphorylation in response to black tea polyphenols.

Black Tea Polyphenols Activate eNOS through a Mechanism Involving the Phosphoinositide 3-Kinase (PI 3-KO)/Akt Signaling Pathway—Phosphorylation of eNOS on Ser-1177 is characteristic of the inactive kinase Akt (18, 19, 21, 22). To probe the involvement of Akt, PAECs were treated with black tea polyphenols, and the lysates were assayed for Akt activation using an antibody specific for activated Akt (i.e. phosphorylated at Ser-473). We observed time-dependent activation of Akt by black tea polyphenols, which closely approximated the time course of eNOS phosphorylation at Ser-1177 (Fig. 2B). To specifically address the role of Akt in black tea polyphenol-induced eNOS stimulation, we overexpressed a dominant negative Akt isoform via adenoviral vector transfection. Dominant negative Akt significantly attenuated black tea polyphenol-induced changes in eNOS Ser-1177 phosphorylation (Fig. 3A),
were incubated for 30 min in PSS containing 250 μM IBMX and 200 μM L-arginine. Cells were then exposed to the indicated concentrations of black tea for 5 min, and cGMP accumulation was determined as described under "Experimental Procedures." B, PAECs were incubated with black tea (100 ng/ml) for the indicated time intervals, and cGMP accumulation was determined. C and D, PAECs were exposed to buffer alone as control (CTL), black tea (TEA; 100 ng/ml), black tea polyphenols (BTP; 100 ng/ml), or A23187 (1 μM) in the presence or absence of 500 μM l-NAME, and L-[3H]citrulline production (C) or cGMP accumulation (D) was determined as described under "Experimental Procedures." Data represent the mean ± S.E. of 10–12 independent experiments; *, p < 0.05 versus with l-NAME.

and its activation was determined both by the increase in cellular cGMP (Fig 3B) and the conversion of L-[3H]arginine to L-[3H]citrulline (Fig. 3C).

Because Akt is a downstream target of PI 3-K (23), we examined the role of PI 3-K in black tea polyphenol-induced eNOS activation. Treatment of PAECs with the PI 3-K inhibitor LY294002 significantly attenuated the black tea polyphenol-induced changes in eNOS phosphorylation and Akt activation (Fig. 4A). This effect had important implications for eNOS activity, because both cellular cGMP accumulation and L-[3H]arginine conversion to L-[3H]citrulline in response to black tea polyphenols were abrogated (Fig. 4, B and C). These data implicate PI 3-K as an upstream signal for both Ser-1177 phosphorylation andThr-495 dephosphorylation in the response to black tea polyphenols.

**Black Tea Polyphenol-induced eNOS Activation Is Dependent on Both Calcium and Calmodulin**—Because eNOS activation can be either calcium-dependent or -independent (24), we investigated the role of calcium on eNOS activation by black tea polyphenols. Stimulation of PAECs with black tea polyphenols in a calcium-free PSS solution selectively attenuated eNOS Ser-1177 phosphorylation with no effect on Thr-495 dephosphorylation (Fig. 5A). This condition induced only a 2-fold increase in cGMP synthesis (p < 0.05 versus without polyphenols) compared with a 5-fold increase in the presence of extracellular calcium (Fig. 5B; p < 0.05 versus with extracellular calcium). In
contrast, inhibition of intracellular calcium release with TMB8 had no impact on Ser-1177 phosphorylation in response to black tea polyphenols and completely abrogated Thr-495 dephosphorylation (Fig. 5A). This condition was associated with a 2.5-fold increase in cGMP (p < 0.05 versus without polyphenols) that was significantly less than that without TMB8 (p < 0.05 versus polyphenols alone; Fig. 5B). The combined inhibition of both intracellular and extracellular calcium transients completely inhibited all responses to black tea polyphenols (Fig. 5B). Thus, both intracellular and extracellular calcium are required for full changes in eNOS phosphorylation and activation in response to black tea polyphenols.

We next examined the role of calmodulin in the black tea polyphenol-mediated eNOS stimulation. Inhibition of calmodulin with W7 had no impact on changes in eNOS phosphorylation induced by black tea polyphenols (Fig. 5C), whereas eNOS activation was completely inhibited as judged by cGMP accumulation (Fig. 5D). Thus, although calmodulin is required for eNOS activation, it is not required for polyphenol-induced changes in eNOS phosphorylation.

The Role of p38MAP Kinase in the Response to Black Tea Polyphenols—Previous data implicate MAP kinases in eNOS activation by high density lipoprotein (25) and H2O2 (26). Therefore, we probed lysates with antibodies specific for the activated forms ERK, p38 MAPK, and c-Jun N-terminal kinase. Lysates from black tea polyphenol-treated PAECs demonstrated rapid phosphorylation of p38 MAPK and its downstream target, MAPKAPK-2 (Fig. 6A), which was temporally reminiscent of eNOS phosphorylation at Ser-1177 (Fig. 2A). Consistent with this notion, pharmacological inhibition of p38 MAPK with either SB202190 or SB203580 attenuated polyphenol-induced eNOS phosphorylation on Ser-1177 (Fig. 6B), and eNOS activation was measured by cGMP accumulation (Fig. 6C) and the conversion of L-[3H]arginine to L-[3H]citrulline (Fig. 6D). In contrast, we did not observe any reproducible activation of ERK or c-Jun N-terminal kinase in response to black tea polyphenols (data not shown).

To confirm the role of p38MAPK, we overexpressed a dominant negative isoform of p38α MAPK and found an inhibition of Akt and eNOS Ser-1177 phosphorylation in response to black tea polyphenols (Fig. 7A) that was associated with a significant inhibition of eNOS activation (Fig. 7B). Using the converse approach, the activation of p38MAPK with a constitutively active mutant of MKK6 (M KK6bE) enhanced both basal and black tea polyphenol-induced Akt activation and eNOS Ser-1177 phosphorylation (Fig. 7C), which is consistent with an observed increased eNOS activity (Fig. 7D). Because there is evidence that Akt can regulate p38 MAPK (27), we examined the relation between Akt and p38 in our...
creased basal p38 MAPK phosphorylation, whereas constitutively active Akt isoforms had any impact on black tea polyphenol-induced p38 MAPK activation (Fig. 9, A) but significantly attenuated Akt phosphorylation and eNOS activation (Fig. 8, B) and MAPKAPK-2 (Thr-222) as well as total p38 MAPK by immunoblotting. Consistent with prior reports (27), these data suggest that p38 MAPK and Akt may share a reciprocal regulatory relationship. Infection of PAECs with MKK6bE lead to activation of p38 MAPK (Thr-180 and Tyr-182) and eNOS phosphorylation (Ser-1177 and Thr-495) by immunoblotting. Total eNOS, Akt, and FLAG (HA-Tag) using immunoblotting with phosphorylation-specific antibodies. Total eNOS, Akt, and HA-Tag (p-HA) were probed for total and phosphorylated eNOS, Akt (P-Akt), and p38 MAPK, and MAPKAPK-2 (P-MAPKAPK) using immunoblotting with phosphorylation-specific antibodies. Total eNOS, Akt, and FLAG (dN P38a) were also assessed by immunoblotting. In panel C, we demonstrated that with buffer alone or with black tea polyphenols (BTP; 100 ng/ml) for 5 min prior to the assessment of eNOS catalytic activity by immunoblotting supernatants for phospho-MAPKAPK-2 and p38 MAPK phosphorylation (Thr-180 and Tyr-182) and eNOS phosphorylation status and total eNOS were determined by immunoblotting as described for Fig. 2A. Activation of p38 MAPK was assessed by immunoblotting supernatants for phospho-MAPKAPK-2 (P-MAPKAPK-2). C and D, PAECs in PSS containing 250 μM IBMX and 200 μM l-arginine were incubated with vehicle (CTL), SB202190 (5 μM), or SB203580 (7.5 μM) as indicated prior to treatment with buffer alone or black tea polyphenols (BTP; 100 ng/ml). After 5 min, cells lysates were analyzed for cGMP (C) or l-[3H]citrulline formation (D) as described in the Fig. 5 legend. Data represent the mean ± S.E. of 8–12 independent experiments and are expressed as the percentage of the values obtained for black tea polyphenols alone. †, p < 0.05 versus control with black tea polyphenols.

![Fig. 6](image.png)

**FIG. 6.** Role of p38 MAPK in eNOS activation by black tea polyphenols. A, PAECs were treated with 100 ng/ml black tea polyphenols for the indicated times followed by cell lysis and the assessment of phosphorylated forms of p38 MAPK (Thr-180 and Tyr-182) and eNOS phosphorylation status and total eNOS were determined by immunoblotting as described for Fig. 2A. Activation of p38 MAPK was assessed by immunoblotting supernatants for phospho-MAPKAPK-2 (P-MAPKAPK-2). C and D, PAECs in PSS containing 250 μM IBMX and 200 μM l-arginine were incubated with vehicle (CTL), SB202190 (5 μM), or SB203580 (7.5 μM) as indicated prior to treatment with buffer alone or black tea polyphenols (BTP; 100 ng/ml). After 5 min, cells lysates were analyzed for cGMP (C) or l-[3H]citrulline formation (D) as described in the Fig. 5 legend. Data represent the mean ± S.E. of 8–12 independent experiments and are expressed as the percentage of the values obtained for black tea polyphenols alone. †, p < 0.05 versus control with black tea polyphenols.

**FIG. 7.** The role of p38 MAPK in black tea polyphenol-induced eNOS activation. A, PAECs were transfected (multiplicity of infection of 50) with dominant negative p38 MAPKs (dN p38a) or LacZ for 24–36 h. PAECs were then equilibrated in PSS (30 min) followed by treatment with 100 ng/ml black tea polyphenols (BTP) or vehicle for 5 min. The cells were then lysed, and the lysates were probed for the phosphorylation of eNOS (Ser-1177 and Thr-495), Akt (P-Akt), p38 MAPK, and MAPKAPK-2 (P-MAPKAPK) using immunoblotting with phosphorylation-specific antibodies. Total eNOS, Akt, and Flag (dN P38a) were also assessed by immunoblotting. B, PAECs transfected as for panel A were treated with buffer alone or with black tea polyphenols (BTP; 100 ng/ml) for 30 min prior to the assessment of eNOS catalytic activity by l-[3H]citrulline formation as described under “Experimental Procedures.” C, PAECs were transfected (multiplicity of infection of 50) with LacZ or a constitutively active mutant of MKK6 (MKK6bE) for 24–36 h, equilibrated in PSS (30 min), and treated with 100 ng/ml black tea polyphenols or vehicle for 5 min. Cells were then lysed, and the lysates were probed for total and phosphorylated eNOS, Akt (P-Akt), and p38 MAPK (P-p38). Expression of MKK6bE was assessed by immunoblotting for the hemagglutinin tag (HA-Tag). D, PAECs, as described for panel C, were treated with buffer alone or with black tea polyphenols (BTP; 100 ng/ml) for 30 min prior to the assessment of eNOS catalytic activity as described for panel B. Data represent the mean ± S.E. of 6–8 independent experiments and are expressed as the percentage of the values obtained for BTP with the LacZ adenovirus. †, p < 0.05 versus LacZ alone; †, p < 0.05 versus black tea polyphenols with LacZ alone.

**DISCUSSION**

In this study we examined the short term effects of black tea on NO bioactivity in cultured endothelial cells. We found that black tea promoted both eNOS catalytic activity and NO bioactivity in cultured endothelial cells. This activity of black tea could be entirely reproduced by the polyphenol fraction and appeared to involve coordinated eNOS phosphorylation at Ser-1177 and dephosphorylation at Thr-495. Upstream mediators for these events included PI 3-kinase and Akt, as well as a prominent role for p38 MAPK. In support of the latter, we found that both pharmacological inhibition by SB 202190 or SB203580 and the overexpression of dominant negative p38 MAPK significantly inhibited the response to black tea polyphenols. Moreover, we could qualitatively reproduce the effect...
of black tea with a constitutively active mutant of MKK6 that produced p38 MAPK activation independent of polyphenol exposure. The activation of p38 MAPK led to an Akt-dependent increase in eNOS activity. If we consider that the range of black tea polyphenol levels utilized in this study corresponds to that achieved in vivo with the consumption of 1–7 cups of black tea (10, 15), these data suggest that the findings presented here are applicable to clinical studies with black tea.

In this regard, there is data showing that the polyphenols found in black tea have physiological actions on the endothelium in vivo. Patients with coronary artery disease exhibit impaired endothelial function in both the coronary (28) and brachial (29) arteries. We have demonstrated previously that both acute and chronic tea consumption improves flow-mediated endothelium-dependent, arterial relaxation in the brachial artery (10). In light of observations that brachial artery flow-mediated dilation is dependent on endothelium-derived NO (30), the data presented here provide some insight into these clinical studies. Indeed, flow-mediated activation of eNOS involves Akt-dependent phosphorylation (22), and our data indicate that black tea polyphenols also induce Akt activation (Fig. 2B). Thus, one might speculate that black tea and shear could produce synergistic Akt activation, thereby explaining the effect of black tea on endothelial function. Thus, our data provide evidence for direct effects of black tea polyphenols on endothelial cells that could, in part, explain the clinical indicating observations that tea improves endothelial function in patients with vascular disease.

Our findings that the polyphenolic fraction of black tea promotes eNOS activity are in agreement with published data on other sources of polyphenols. For example, resveratrol is a polyphenol found in red wine that increases both the expression and activity of eNOS in endothelial cells (31). A recent study with the green tea constituent epigallocatechin-3-gallate demonstrated activation of eNOS through a mechanism that involved both Akt and protein kinase A (32). With regard to the former, we found that the polyphenolic fraction of black tea activated Akt in a PI-3K-dependent manner. The major contribution of this study is the identification of p38 MAPK as an upstream mediator of Akt activation in response to polyphenols. Indeed, these findings prompt speculation that p38 MAPK may be involved in the regulation of eNOS activity in response to other stimuli as well.

Although it is generally thought that MAP kinases do not play a crucial role in eNOS regulation, our studies are reminiscent of reports on estrogen demonstrating that it enhances endothelial NO release in a MAP kinase-dependent manner (33). Although that data implicated ERK in estrogen-mediated eNOS activation, we only observed p38 MAPK activation in response to black tea polyphenols. Consistent with this observation, the inhibition of p38 MAPK attenuated eNOS activation in response to black tea polyphenols, whereas ERK inhibition had no impact (data not shown). The p38 MAPK has been generally shown to be activated in response to inflammatory cytokines, endotoxins, and osmotic stress, but its role in endothelial cells is incompletely defined. Our data suggest a novel role for p38 MAPK as an upstream mediator of Akt-dependent eNOS activation. It is important to note, however, that we could only approximate the extent of polyphenol-induced eNOS activation with constitutively active MKK6. The precise mechanism(s) for these observations is not clear, but may indicate that p38 MAPK and Akt activate eNOS in a cooperative manner rather than a strictly linear kinase cascade. A full accounting of the p38 MAPK role in eNOS regulation will require further study.

We observed that the effect of black tea polyphenols involved coordinated phosphorylation of eNOS on Ser-1177 and dephosphorylation at Thr-495, two events that have been reported previously to correlate directly with eNOS activity status (17, 20, 34). However, the relative importance of Ser-1177 phosphorylation versus Thr-495 dephosphorylation with respect to the stimulation of eNOS activity remains controversial. Our data shed some light on this issue, as transfection with dominant negative Akt abolished only eNOS Ser-1177 phosphorylation, yet eNOS catalytic activation was inhibited by ~50% (Fig. 3, B and C). Considering that dominant negative Akt did not alter polyphenol-induced Thr-495 dephosphorylation, these data suggest that dephosphorylation provides an independent contribution to eNOS stimulation. In support of this hypothesis,
the inhibition of intracellular calcium release with TMB8 abrogated Thr-495 dephosphorylation without altering events at Ser-1177, and this manipulation also produced ~50% inhibition of the response to black tea polyphenols. These data suggest that one cardiovascular benefit of black tea polyphenols, suggesting that calcineurin is not involved in black tea polyphenol-induced Thr-495 dephosphorylation. Experiments with calciulin A were confounded by the toxicity of the compound to endothelial cells. Thus, the nature of the calcium-dependent phosphatase induced by black tea polyphenols remains to be uncovered.

In summary, the data presented here indicate that black tea stimulates eNOS catalytic activity largely as a consequence of its polyphenolic fraction. The mechanisms of black tea polyphenol-induced eNOS stimulation involve p38 MAPK-mediated activation of the PI 3-kinase/Akt pathway that leads to the concerted phosphorylation of eNOS at Ser-1177 and dephosphorylation at Thr-495. This action of black tea polyphenols affords calmodulin-dependent eNOS activation and enhanced NO bioactivity. These data suggest that one cardiovascular benefit of black tea is that the activation of eNOS that may, in part, explain clinical trials supporting reduced cardiovascular disease as a function of increased black tea consumption.

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