p38 Mitogen-Activated Protein Kinase Activates eNOS in Endothelial Cells by an Estrogen Receptor α-Dependent Pathway in Response to Black Tea Polyphenols

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Abstract—Black tea has been shown to improve endothelial function in patients with coronary artery disease and recent data indicate the polyphenol fraction of black tea enhances endothelial nitric oxide synthase (eNOS) activity through p38 MAP kinase (p38 MAPK) activation. Because the mechanisms for this phenomenon are not yet clear, we sought to elucidate the signaling events in response to black tea polyphenols. Bovine aortic endothelial cells (BAECs) exposed to black tea polyphenols demonstrated eNOS activation that was inhibited by the estrogen receptor (ER) antagonist ICI 182,780, and siRNA-mediated silencing of ER expression. Consistent with this observation, black tea polyphenols induced time-dependent phosphorylation of ERα on Ser-118 that was inhibited by ICI 182,780. Phosphorylation of ERα on Ser-118 was due to p38 MAP kinase (p38 MAPK) as, it was inhibited by SB203580 and overexpression of dominant-negative p38α MAPK. Conversely, constitutively active M KK6 induced p38 MAPK activation that recapitulated the effects of polyphenols by inducing ERα phosphorylation and downstream activation of Akt, and eNOS. The key role of ERα Ser-118 phosphorylation was confirmed in eNOS-transfected COS-7 cells, as polyphenol-induced eNOS activation required cotransfection with ERα subject to phosphorylation at Ser-118. This residue appeared critical for functional association of ERα with p38 MAPK as ERα with Ser-118 mutated to alanine could not form a complex with p38 MAPK. These findings suggest p38 MAP kinase-mediated eNOS activation requires ERα and these data uncover a new mechanism of ERα activation that has broad implications for NO bioactivity and endothelial cell phenotype. (Circ Res. 2005;96:1072-1078.)

Key Words: antioxidants ■ p38 ■ endothelial dysfunction ■ eNOS ■ estrogen receptor

Recent evidence indicate that flavonoids, a group of polyphenolic substances found in tea, fruit, vegetables, and wine favorably impact the endothelium.1,2 Normal endothelial function is critical for regulation of vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle proliferation.3 These actions of the endothelium are mediated via release of several paracrine factors, including nitric oxide (NO).5 These normal functions of the endothelium are impaired in the setting of atherosclerosis and its associated vascular conditions such as hypertension, hypercholesterolemia, and diabetes.3 Impaired endothelial function has important consequences as individuals with the poorest endothelial function are at increased risk of cardiovascular events.4,5 Thus, reversing endothelial dysfunction has become a topic of intense investigation. In this regard, endothelial NO bioactivity is enhanced in patients with atherosclerosis by either the acute or chronic administration of black tea.6 In cultured cells, the black tea polyphenolic fraction promotes both eNOS catalytic activity and NO bioactivity.7 This effect is because of activation of phosphoinositol 3-kinase (PI 3-K) and Akt via a p38 MAPK-dependent mechanism.7 Despite observations that black tea polyphenols enhance endothelial cell NO bioactivity, important questions remain. The upstream components of polyphenol-mediated eNOS activation are not well described and the precise signals linking p38 MAPK to eNOS activation are largely unknown. The purpose of this study, therefore, was to investigate the mechanism of p38 MAPK-mediated eNOS activation by black tea polyphenols.

Materials and Methods

Materials
We obtained ICI 182,780 from Tocris (Ellisville, Mo). Inhibitors of p38 MAPK (SB203580), ERK1/2 (PD98059) and JNK (SP600125) were obtained from Calbiochem (San Diego, Calif). Polyclonal antibodies directed against p38 MAPK, hemagluttinin (HA), ERK1/2, and the phosphorylated forms of p38 MAPK (Thr-180, Tyr-182), ERK1/2 (Thr-202, Tyr-204), JNK (Thr-183, Tyr-185), MAPKAPK-2 (Thr-222), GSK-3α/β (Ser-21/9), and estrogen receptor-alpha (ERα; Ser-118) were obtained from Cell Signaling.
Technology (Beverly, Mass). Polyclonal antibodies directed against phospho-eNOS (Ser1177) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-eNOS monoclonal antibody was obtained from Transduction Laboratories (Lexington, Ky). Antibody against ERα (AER 320) was obtained from Laboratory Vision (Fremont, Calif). L-[3H]arginine (1 mCi, 53 mCi/mmol) was obtained from PerkinElmer Life Sciences (Wellesley, Mass) and eGMP assay kits were from Cayman (Ann Arbor, Mich). Black tea polyphenols, and black tea fractions were provided by Unilever, Inc. All other reagents were obtained from Sigma.

Cell Culture
Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics and grown on endothelial growth medium (Clonetics, Inc, San Diego, Calif). COS-7 cells (American Type Culture Collection, Rockville, Md) were grown in DMEM supplemented with 10% heat inactivated FBS, 50 µg/mL heparin sulfate, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin as described. For experiments, confluent endothelial cells were used between passages 4 and 8 and treated with physiologically relevant concentrations of black tea polyphenols (100 µg/mL) that we previously demonstrated activate eNOS.8 Before all experiments, cells were placed in serum- and phenol-free media (opti-MEM, Gibco, NY) for at least 16 hours, washed twice in HEPES-buffered physiologic salt solution (PSS), and experiments performed in HEPES-buffered PSS as described.

eNOS Activity Assay
The catalytic activity of eNOS was estimated by the conversion of L-[3H]arginine to L-[3H]citrulline that was sensitive to inhibition by L-nitro-arginine methyl ester (L-NAME). Confluent cells in 100 mm culture dishes were washed and incubated in HEPES-buffered PSS for 30 minutes followed by the addition of 5 µmol/L L-arginine plus 3.3 µCi of L-[3H]arginine. Cells were then treated with the agonist of interest or vehicle for 15 minutes and cells were then lysed and L-[3H]citrulline determined as described.

Immunoprecipitation and Western Blotting
Cells were washed with PSS and incubated in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 µg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride for 30 minutes on ice as described. For ERα immunoprecipitation, lysates were incubated with ERα monoclonal antibody (4 µg/mL) rotating for 16 hours at 4°C followed by a 1-hour protein with A/G-agarose. Following centrifugation, the immunoprecipitates were washed, resuspended in loading buffer, and resolved by SDS-PAGE as described. Resolved proteins were transferred to a nitrocellulose membrane (Hybond; Amersham Biosciences, Inc.) and immunoblotting performed as previously described.7 Densitometric analysis of immunoblots was performed using PDI ImageSystem.

Recombinant Adenoviral Vectors and Transfection
Recombinant adenovirus expressing a HA-tagged constitutively active MKK6 mutant (MKK6bE) was a kind gift from Dr. Jiahua Han, Scripps Institute.8 Adenoviral construct encoding myristoylated, constitutively active Akt (myr-Akt) was a kind gift of Dr. Kenneth Walsh, Boston University. BAECs were infected in medium without FBS for 2 hours, washed, and incubated in fresh medium with FBS for 12 to 24 hours before experimentation.

Plasmid Constructs and Transfection
The construct for human eNOS cDNA was obtained from Dr. Richard Venema, Medical College of Georgia. Expression plasmids for ERα and ERβs18a have been described previously.10 Transfection of COS-7 cells was performed essentially as previously described.8 After transfection, cells were cultured in a phenol red- and estrogen-free media and either vehicle or estrogen was added to effect stimulation of the receptor.

Statistical Analysis
All numerical data are presented as means±SE. Western blots shown are representative of 3 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 2 comparisons were evaluated with a Student’s t test. Statistical significance was accepted if the null hypothesis was rejected with a P<0.05.

Results

Activation of eNOS by Black Tea Polyphenols Involves Estrogen Receptors
To probe pathways involved in black tea polyphenol-induced eNOS activation, we used a pharmacological approach that included the ER antagonist ICI 182,780. As shown in Figure 1A and B, polyphenol-induced eNOS phosphorylation and catalytic activation were both attenuated by ER antagonism. To confirm this finding, we used siRNA to silence ER expression and found it limited black tea polyphenol-induced eNOS phosphorylation on Ser-1177 (human sequence; Figure 1C) and eNOS activation (Figure 1D). Thus, black tea polyphenol-induced eNOS activation involves estrogen receptors.

Black Tea Polyphenols Induce ERα Phosphorylation
Phosphorylation of ERα occurs on multiple residues, including Ser-118.12 To determine whether black tea polyphenols induce ERα Ser-118 phosphorylation, BAECs were treated with black tea polyphenols and probed with a phosphorylation-specific antibody. Under resting conditions, BAECs exhibit little ERα Ser-118 phosphorylation; however, exposure to black tea polyphenols resulted in a time-dependent increase in ERα Ser-118 phosphorylation (Figure 2A). Pharmacological ER inhibition by ICI 182,780 attenuated ERα phosphorylation in response to black tea polyphenols (Figure 2B). These data indicate polyphenols induce ERα phosphorylation in a manner that is inhibited by ICI 182,780.

Black Tea Polyphenol-Induced ERα Phosphorylation Involves p38 MAPK
Phosphorylation of ERα on Ser-118 has been linked to activation of extracellular signal-regulated kinase (ERK)12 and we have demonstrated p38 MAPK (p38 MAPK) is required for eNOS activation in response to black tea polyphenols.7 Therefore, to determine the involvement of MAPKs in our system, lysates from black tea polyphenol-treated BAECs were probed for activation of ERK, p38 MAPK, or c-Jun, N-terminal kinase (JNK). As shown in Figure 3A, p38 MAPK was activated in response to black tea polyphenols with no activation of ERK or JNK. Consistent with this observation, only inhibition of p38 MAPK had a material impact on the polyphenol-induced eNOS stimulation (Figure 3B). We next investigated the role of p38 MAPK in ERα phosphorylation. Pharmacological inhibition of p38 MAPK blunted black tea polyphenol-induced ERα Ser-118 phosphorylation and phosphorylation of the p38 MAPK target.
MAPKAPK2 (Figure 4A). We confirmed that ERα phosphorylation was downstream of p38 MAPK as adenoviral transfection with a dominant-negative p38 MAPK mutant prevented black tea polyphenol-induced ERα Ser-118 phosphorylation (Figure 4B). Activation of p38 MAPK proved to be a proximal component of the black tea polyphenol response as dominant-negative p38 MAPK also prevented Akt phosphorylation, eNOS phosphorylation (Figure 4B), and eNOS catalytic activity estimated by the conversion of L-[3H]arginine to L-[3H]citrulline as in “Methods.” Consistent with this observation, constitutive activation of p38 MAPK with the MKK6bE adenovirus produced the same response as black tea polyphenols with regards to phosphorylation of ERα, Akt, and eNOS as well as eNOS catalytic activity as in “Methods.” Immunoblots are representative of 3 independent experiments and composite data are mean±SE. Legend: *P<0.05 vs no additions, †P<0.05 vs corresponding treatment without BTP.

Figure 3. Black tea polyphenol-induced eNOS activation involves p38 MAPK. A, BAECs in HEPES-buffered PSS were incubated with 100 ng/mL black tea polyphenols (BTP) for the indicated time. Cells were then lysed and immunoblotted with antibodies against the phosphorylated (activated) forms of p38 MAPK, ERK1/2, and JNK as indicated. Loading was assessed by immunoblots for total p38 MAPK. B, BAECs were incubated for 30 minutes without (CTL) or with inhibitors for MEK1 (PD98059; 20 μmol/L), p38 MAPK (SB203580; 5 μmol/L), or JNK (SP600125; 10 μmol/L) as indicated. Cells were then treated with BTP (100 ng/mL; 15 minutes) and eNOS catalytic activity determined as the conversion of L-[3H]arginine to L-[3H]citrulline as in “Methods.” Immunoblots are representative of 3 independent experiments and composite data are mean±SE. Legend: *P<0.05 vs corresponding treatment without BTP.

Figure 4. Black tea polyphenols induce ERα phosphorylation. A, BAECs in HEPES-buffered PSS were incubated with 100 ng/mL black tea polyphenols for the indicated time, lysed, and ERα Ser-118 phosphorylation determined by immunoblotting with antibodies specific for the indicated epitopes. B, BAECs were incubated with or without ICI 182,780 or BTP as in Figure 1A. Cells were then lysed and ERα Ser-118 phosphorylation determined by immunoblotting with antibodies against the indicated epitopes. All immunoblots are representative of 3 independent experiments.
observation, BAECs harboring a constitutively active Akt mutant (Myr-Akt) demonstrated eNOS phosphorylation and activation without any effect on ERα or p38 MAPK (Figure 5A and B).

To confirm that p38 MAPK is upstream of ERα, we used siRNA to inhibit ERα expression and found it inhibited black tea polyphenol-induced eNOS Ser-1177 phosphorylation (human sequence) without any effect on p38 MAPK activation (Figure 5C). Collectively, these data establish a linear signaling cascade from p38 MAPK to ERα, PI 3-K/Akt, and eNOS.

Thus, ERα Ser-118 phosphorylation appears critical for signal transduction from p38 MAPK to Akt.

**P38 MAPK-Mediated eNOS Activation Requires ERα Ser-118**

To establish that black tea polyphenol-induced eNOS activation requires ERα, we conducted experiments in COS-7 cells...
that lack ERs. In COS-7 cells transfected with eNOS alone, neither estradiol nor black tea polyphenols stimulated eNOS activity, whereas A23187 enhanced eNOS activity by 3-fold (Figure 6A). However, in COS-7 cells cotransfected with eNOS and ERα, both estradiol and black tea polyphenols significantly increased eNOS activity and the response to A23187 was not altered (Figure 6A). Black tea polyphenol-mediated p38 MAPK activation was observed both in the presence and absence of ERα transfection in COS-7 cells (Figure 6A).

To further define the role of ERα Ser-118 in black tea polyphenol-induced eNOS activation, we used an ERα mutant (ERαs118a) containing an alanine in place of serine at position 118 (ERαs118a) that is not subject to phosphorylation. As shown in Figure 6B, COS-7 cells transfected with ERαs118a and eNOS showed significant attenuation of eNOS stimulation in response to black tea polyphenols compared with cells harboring the wild-type ERα. These data indicate that ERα Ser-118 is required for black tea polyphenol-induced eNOS stimulation.

ERα and p38 MAPK Are Functionally Associated in Response to Black Tea Polyphenols

To probe for the existence of a functional complex including both p38 MAPK and ERα, COS-7 cells cotransfected with eNOS and wild-type ERα, were treated with black tea polyphenols and the lysates were immunoprecipitated with antibodies directed against ERα. We then probed the precipitates for p38 MAPK and found a time-dependent increase in the pellet p38 MAPK with a corresponding decrease in supernatant p38MAPK. (Figure 7A). Similarly, the converse experiment involving immunoprecipitation of p38 MAPK from black tea polyphenol exposed COS-7 cells demonstrated evidence for ERα in the pellet and a corresponding decrease in the supernatant (Figure 7B). This functional association between p38 MAPK and ERα could not be detected in cells cotransfected with eNOS and ERαs118a (Figure 7C and D). Immunoprecipitation of ERα from BAECs also demonstrated polyphenol-induced complex formation between ERα and p38 MAPK (data not shown). Collectively, these data support the notion that ERα is an upstream mediator of Akt and eNOS activation by black tea polyphenols. Moreover, the data also suggest that p38 MAPK activates ERα in response to black tea polyphenols.

Discussion

In this study we found that ERα plays a key role in mediating the activation of eNOS in response to black tea polyphenols. In particular, we found that ERα is phosphorylated on Ser-118 in response to black tea polyphenol-mediated p38 MAPK activation. This ERα phosphorylation is required for p38 MAPK downstream signaling to Akt and eNOS that mediates black tea polyphenol-induced eNOS activation. We were able to implicate Ser-118 as a critical residue in this process as we observed ERα Ser-118 phosphorylation in response to black tea polyphenols and mutation of this residue abrogated polyphenol-induced eNOS activation in COS cells. This residue also appeared important for the functional association of p38 MAPK with ERα as mutants lacking this site did not associate with p38 MAPK in response to black tea polyphenols. The data suggest a novel adaptor function of ERα that integrates p38 MAPK signaling with downstream targets.

The rapidity of the response to black tea polyphenols suggests a nongenomic role for ERα in our system. These findings are in agreement with previous studies indicating that ERα mediates nongenomic eNOS activation in response to estradiol.13–15 Ligand engagement of ERα induces its association with the p85α-subunit of phosphoinositide 3-kinase (PI 3-K) and c-Src leading to Akt activation.15,16 This event promotes eNOS phosphorylation at Ser-1177,17 a key event that stimulates enzyme activation18,19 and enhances
its sensitivity to calcium.\(^{20}\) The data presented here add to this body of literature by identifying p38 MAPK as an upstream effector of ER\(\alpha\) that mediates rapid eNOS activation. Moreover, we have strong evidence that p38 MAPK induces ER\(\alpha\) phosphorylation in a ligand-independent manner as molecular activation of p38 MAPK with MKK6bE recapitulated both ER\(\alpha\) phosphorylation and eNOS activation in the absence of polyphenols. To our knowledge, this is the first demonstration of rapid, nongenomic effects of ER\(\alpha\) stimulation that is ligand-independent. Typically, ligand-independent ER\(\alpha\) activation has been restricted to the transcriptional functions of this nuclear hormone receptor in response to stimuli such as epidermal growth factor,\(^{21}\) insulin-like growth factor,\(^{21}\) and the cyclins A\(^{22}\) and D1.\(^{23}\) Thus, the data provided here indicate a novel ER\(\alpha\) function as a consequence of ligand-independent activation.

Despite recent reports demonstrating polyphenol-induced ERK activation,\(^{24}\) we did not find a role for ERK in our system (Figure 3). Possible explanations for this observation might include the fact we used a tea polyphenol extract rather than a red wine extract or authentic resveratrol.\(^{24}\) Moreover, our data fit best with a ligand-independent mechanism rather than polyphenol binding to the estrogen receptor. In this regard, previous data depict ligand-independent ER\(\alpha\) activation as an ERK-mediated phenomenon that involves the ER\(\alpha\) Ser-118 residue.\(^{21}\) The data presented here add a new facet to this body of literature in that ER\(\alpha\) is a target of the p38 MAPK in endothelial cells. There is one prior report of p38 MAPK-mediated ER\(\alpha\) phosphorylation in a study that involved endometrial carcinoma cells and Thr-311 as the p38 MAPK target.\(^{25}\) In contrast, our report involves endothelial cells and Ser-118 appears to be the site of phosphorylation in response to black tea polyphenols. This discrepancy could be a function of the different cell types between these studies. Alternatively, it is possible that our study also involved p38 MAPK phosphorylation of ER\(\alpha\) on Thr-311 that facilitated ER\(\alpha\) Ser-118 phosphorylation by some other, as yet unrecognized, kinase. However, we did exclude ERK as mediating Ser-118 phosphorylation in this study, but other kinases do target this site including cdk7.\(^{26}\) Thus, determining the precise molecular events surrounding black tea polyphenol-mediated ER\(\alpha\) phosphorylation will require further study.

Evidence from this work does distinguish estradiol-mediated eNOS activation from that observed with black tea polyphenols. Although both agents induce ER\(\alpha\) Ser-118 phosphorylation, this event was only critical for polyphenol-induced eNOS activation. In COS-7 cells transfected with ER\(\alpha\) harboring an alanine at position 118 (ER\(\alpha\)s118a), we observed intact eNOS activation in response to authentic estradiol, whereas the black tea polyphenol response was attenuated compared with wild-type ER\(\alpha\). These data are in keeping with the known role of Ser-118 in mediating ligand-independent ER\(\alpha\) function with little role in ligand-dependent responses.\(^{10}\) The results of our study may also be related to the presence of other phosphorylation sites on ER\(\alpha\) such as Ser-167, Thr-311, and Ser-522 that were not the subject of this investigation. In this regard, Ser-522 has been proven important for cancer cell-mediated metastasis.\(^{25}\) The data presented here indicate that p38 MAPK has a role in regulating the activity of a nuclear hormone receptor, ER\(\alpha\). Although not described for ER\(\alpha\), there is some precedent for nuclear hormone receptors to be modulated by p38 MAPK. For example, in the setting of cytokine stimulation, PPAR\(\gamma\) coactivator-1 is phosphorylated by p38 MAPK and this event regulates the control of genes involved in energy expenditure.\(^{27}\) Phosphorylation of the glucocorticoid receptor by p38

**Figure 7.** Functional association of p38 MAPK and ER\(\alpha\). A, COS-7 cells cotransfected with eNOS and ER\(\alpha\) were treated with black tea polyphenols (BTP; 100 ng/mL) for the indicated times. After treatment, cells were lysed, ER\(\alpha\) immunoprecipitated, and both the pellet (PLT) and supernatant (SNT) subjected to immunoblotting as indicated. B, Cells were treated as in (A) except p38 MAPK and both the pellet and supernatant immunoblotted with antibodies to the indicated epitopes. C, COS-7 cells were cotransfected with eNOS and mutant ER\(\alpha\) (ER\(\alpha\)s118a), treated with BTP (100 mg/mL) for the indicated times, and subjected to immunoprecipitation and immunoblotting as in (A). D, COS-7 cells were cotransfected and treated with BTP as in (C) followed by immunoprecipitation and immunoblotting as in (B). All blots are representative of 3 independent experiments.
MAPK appears to reduce receptor activity and this may be involved in feedback regulation of the receptor in the setting of inflammation. The precise nature of how p38 MAPK interacts with ERα is not clear from this work, but our experiments do suggest these 2 proteins occur in a common complex in response to polyphenol stimulation. Further investigation will be required to determine the molecular determinants of this event.

In summary, the data presented here indicate that black tea polyphenols stimulate eNOS activity largely through nongenomic ligand-independent activation of ERα in vascular endothelial cells. The mechanisms involve a p38 MAPK-induced phosphorylation of ERα on Ser-118, which in turn leads to the activation of the PI 3-K/Akt pathway and eNOS. Along with the implications regarding estrogen and vascular endothelial function, the present findings are important to the mechanisms underlying the rapid activation of estrogen receptors in vascular endothelium.

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