The vascular endothelium mediates many important physiological functions, such as responses to shear stress, angiogenesis, vascular remodeling, inflammation, and coagulation. It also participates in metabolic and synthetic processes. A key modulator of endothelial cell activity is nitric oxide (NO), which under physiological conditions is mainly produced by the endothelial nitric oxide synthase (eNOS) isoform. NO regulates vascular tone, proliferation of vascular smooth muscle cells, and hemostasis, among other important functions. Disruptions in the physiological production of NO triggers endothelial cell dysfunction, resulting in an increased susceptibility to CVD.

Cardiovascular diseases (CVD) are a leading cause of morbidity and mortality, affecting Western industrialized countries as well as developing countries. CVD imposes major direct and indirect costs on health care systems, ranging from hospitalizations, drugs, and rehabilitation services to losses of productivity due to premature mortality and short- and long-term disability. The incidence of CVD continues to increase; thus, it is important to identify potential therapeutic agents to prevent and treat CVD.

CVD is importantly linked to endothelial cell dysfunction. Endothelial cell dysfunction is associated with conditions, such as obesity, smoking, diabetes, and hormonal changes. The vascular endothelium mediates many important physiological functions, such as responses to shear stress, angiogenesis, vascular remodeling, inflammation, and coagulation. It also participates in metabolic and synthetic processes. A key modulator of endothelial cell activity is nitric oxide (NO), which under physiological conditions is mainly produced by the endothelial nitric oxide synthase (eNOS) isoform. NO regulates vascular tone, proliferation of vascular smooth muscle cells, and hemostasis, among other important functions. Disruptions in the physiological production of NO triggers endothelial cell dysfunction, resulting in an increased susceptibility to CVD.

The induction of NO synthesis by flavonoid-containing compounds has received widespread attention, as their effects appear to positively impact CVD. Epidemiological studies indicate that the regular dietary intake of plant-derived foods and beverages high in flavonoids is inversely associated with the incidence of CVD. Although these compounds are pleiotropic in nature, many of their effects may be explained by improving endothelial function. The regular consumption of cacao products high in flavonoid content has also been demonstrated to provide beneficial cardiovascular effects. Natural cacao products are rich in monomeric and polymeric forms of the flavonoids (−)-epicatechin (EPI) and catechin (60/40 ratio) and can contain up to 10% flavonoids by weight. The consumption of cacao products can ameliorate
hypertension in animal models and in humans. This effect has been ascribed to the ability of cacao flavonoids to increase NO levels via eNOS activation. The vascular effects of cacao can be reproduced by EPI. EPI induces the relaxation of preconstricted aortic rings and stimulates NO production via eNOS activation.

Studies in animals have demonstrated a critical role for NO in mediating cardioprotection. It has been demonstrated that EPI pretreatment of rats limits infarct size in the setting of myocardial ischemia-reperfusion injury or with a permanent coronary occlusion. In humans, the sustained consumption of chocolate is associated with a decrease in mortality postmyocardial infarction. The above described effects of EPI may be accounted for by the acute or chronic effects on eNOS. However, the signaling pathways by which the acute administration of EPI induces eNOS activation in endothelial cells are unclear.

In this study, we identified the intracellular signaling pathways by which EPI acts on eNOS to stimulate NO production using cultures of human coronary artery endothelial cells (HCAECs). Our results suggest the participation of multiple pathways and the likely presence of a unique cell surface acceptor-effector for EPI.

### Methods

#### Cell Culture

HCAECs were obtained from Cell Applications Inc using 3 different lots. The company data sheet indicated that the cells were obtained from 14-, 40-, and 60-year-old normal male donors in apparent good health.

Cells were maintained in a humidified atmosphere at 37°C with 5% CO₂ and 95% O₂ in HCAEC growth medium. Sixteen hours before the experiments, HCAECs were washed with phenol red–free Hanks salt solution and kept in phenol red–free M-199 supplemented with 200 mmol/L L-glutamine and 1% antibiotic mix. Treatments were typically applied to confluent cell cultures.

#### NO Measurements

NO levels were measured using a fluorometer (FLx800, Bio-Tek Instruments Inc) at excitation and emission wavelengths of 360 and 430 nm, respectively. EPI was diluted in water (which served as vehicle for the control cells). EPI-induced NO time- and dose-response curves were generated. For time-response curves, cells were treated with 1 µmol/L EPI, and culture medium samples were collected at 0, 5, 10, 20, 30, 60, 120, 180, and 240 minutes. For concentration-response curves, cells were treated with EPI (0.1 nmol/L to 100 µmol/L) for 10 minutes. Data are expressed as mean±SD (n=3).

Other methods are given in the supplemental material, available online at http://hyper.ahajournals.org.

### Results

#### Time Course of EPI-Induced NO Production

A time course for EPI-induced NO production was performed using an arbitrarily selected concentration of 1 µmol/L.
Results are shown in Figure 1A. Increases in NO were detected as early as 5 minutes after treatment, with a maximal effect occurring at 10 to 30 minutes. Given the comparable levels attained at these time points, all subsequent NO production experiments were carried out using 10 minutes of incubation. We performed EPI concentration-response curves on NO production. Results indicate that a maximal effect was obtained with 1 µmol/L EPI (Figure 1B). Subsequent experiments used this concentration and a 10-minute time frame.

eNOS Activation by EPI
We examined the effects of EPI on eNOS activity by immunoblots, comparing the relative amount of phosphorylation at the activation residues Ser1177 and Ser633 and at the inactivation residue Thr495. Total eNOS levels were used to normalize phospho-eNOS values. eNOS activity was measured under basal conditions (control) and with EPI. Bradykinin (BK) (1 µmol/L), a known eNOS activator, was used as a positive control. Untreated cells have basal levels of phosphorylation of Ser1177, Ser633, and Thr495 (Figure 2). As can be observed, a relatively higher phosphorylation status of Thr495 exists. In contrast, with EPI or BK treatment, a significant change in relative levels of the phosphorylation of Ser1177 and Ser633 and dephosphorylation of Thr495 was induced.

Role of Phosphatidylinositol 3-Kinase in EPI-Induced eNOS Activation
eNOS phosphorylation can occur via the phosphatidylinositol 3-kinase (PI3K) pathway. Two downstream effectors of PI3K are protein kinase cAMP-dependent (PKA) and Akt; when activated, they modulate eNOS activity through the selective phosphorylation of activation residues. PKA regulates Ser1177 and Ser633, whereas Akt phosphorylates only Ser1177. Wortmannin (WORT)- and SH5-treated cells demonstrate less EPI-induced Akt phosphorylation, but no effect was seen with the PKA-inhibitor H89 (Figure 3A). In turn, the EPI-induced phosphorylation of eNOS Ser1177 and Ser633 was partially blocked with WORT and H89 preincubation. Phosphorylation of eNOS Ser1177 was diminished, but Ser633 phosphorylation did not decrease with the use of SH5, as Akt does not phosphorylate this residue (Figure 3B). We also explored the effect of EPI on NO production in the presence of the inhibitors. Results demonstrate that EPI-induced NO synthesis was partially blocked when WORT, SH5, and H89 were used (Figure 3C).

Role of Caveolin and Calmodulin in EPI-Induced eNOS Activation
Given the reported role of caveolin-1 (Cav-1) and calmodulin-dependent kinase I (CaMKI) on eNOS signal transduction and dephosphorylation on Thr495. Pretreatment with WORT and H89 led to a decrease in eNOS phosphorylation on Ser1177 and Ser633. SH5 treatment decreased Ser1177 phosphorylation. The effects of WORT, H89, and SH-5 on EPI-induced NO synthesis. Control values were arbitrarily set to zero. Statistical analysis shows no difference between WORT and H-89. SH-5 effects were significantly different vs H89. Data are expressed as mean± SD (n=3). *P<0.05.

Figure 3. PI3K signaling pathway mediates EPI-induced eNOS activation. HCAECs were treated with WORT or H89 or SH-5 (see text for details) and stimulated with EPI (1 µmol/L) for 10 minutes. Western blots were probed with specific antibodies against Akt, phosphorylated (p-)Akt, eNOS, p-eNOS Ser1177, Ser633, and Thr495; NO was measured as stated in the text. A, EPI-induced AKT phosphorylation; this effect was blocked by WORT and SH5 pretreatment. Normalized control densitometric levels were arbitrarily set to 100 in all cases. B, eNOS activity was measured by its phosphorylation on Ser1177 and Ser633 and dephosphorylation of Thr495. Pretreatment with WORT and H89 led to a decrease in eNOS phosphorylation on Ser1177 and Ser633. SH5 treatment decreased Ser1177 phosphorylation. C, The effects of WORT, H89, and SH-5 on EPI-induced NO synthesis. Control values were arbitrarily set to zero. Statistical analysis shows no difference between WORT and H-89. SH-5 effects were significantly different vs H89. Data are expressed as mean± SD (n=3). *P<0.05.
tion, we analyzed the interaction of eNOS with these proteins under EPI treatment. Untreated and EPI-treated cells were immunoprecipitated (IP) using anti-Cav-1 or anti-calmodulin 1 (CaMI) antibodies, and the IP phase, as well as the supernatant (SN), were used for immunoblots of total eNOS protein and for the phosphorylation levels of Ser1177, Ser633, and Thr495. Results are summarized in Figure 4A and 4B. Control cells demonstrated an “inactive” eNOS protein bound to Cav-1, as evidenced by the appearance of the eNOS protein in the IP phase and the relatively low phosphorylation levels of Ser1177 and Ser633 versus Thr495. The SN showed negligible amounts of eNOS. With EPI treatment, only Cav-1 was observed in the IP phase, suggesting the uncoupling of eNOS. The presence of activated eNOS was seen in the SN phase, with increased phosphorylation of Ser1177 and Ser633 and low levels of Thr495 (Figure 4A and 4B). Results from immunoprecipitation with anti-CaMI antibodies demonstrate that under basal conditions, the IP phase contains only the CaMI protein. With EPI treatment, immunoprecipitation with anti-CaMI demonstrates the coupling of activated eNOS to CaMI (Figure 4C and 4D).

Role of CaMKII in EPI-Induced eNOS Activation
We determined whether EPI could also stimulate calcium-dependent eNOS-related intracellular signaling pathways. Experiments were initially performed to determine whether EPI treatment induces the accumulation of inositol trisphosphate (IP3) in cells. The results are summarized in Figure 5 and demonstrate that 10 minutes after treatment there was a significant accumulation of IP3 in cells. Bradykinin (1 μmol/L) was used as a positive control. The EPI- and BK-induced increase in IP3 was blocked with the preincubation with U73122.

To further characterize the EPI-induced calcium-dependent activation of intracellular signaling pathways, we examined the extent of phosphorylation/activation on the IP3 receptor (IP3R), CaMKII, and CaMI using the inhibitors U73122 and KN-93, which block the activation of phospholipase C and CaMKII, respectively. As shown in Figure 6A, EPI treatment induced higher levels of IP3R phosphorylation versus controls. EPI-induced phosphorylation of IP3R was partially blocked with the preincubation of cells with U73122 or KN-93. To evaluate EPI effects on downstream effectors of the CaMI signaling pathway, we evaluated the phosphorylation/activation of CaMKII and CaMI. As expected, the levels of phosphorylation were higher in EPI-treated versus control cells. The addition of each of the inhibitors led to decreased levels of phosphorylation of CaMKII (Figure 6B) and CaMI (Figure 6C).

We also assessed eNOS activity by measuring the phosphorylation of Ser1177, Ser633, Thr495, and total eNOS in the absence or presence of U73122 and KN-93 (Figure 6D). Preincubation with U73122 induced a decrease in the phosphorylation of Ser1177 and Ser633. Interestingly, Thr495 remained partially phosphorylated. The same effect was observed after incubation with the inhibitor KN-93, where Ser1177, Ser633, and Thr495 were all phosphorylated (Figure 6D). Similarly, the synthesis of NO was diminished after

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**Figure 4.** EPI-induced eNOS decoupling from the caveola. HCAECs were treated with EPI (1 μmol/L) for 10 minutes, lysed and IP with anti-Cav-1 or -CaMI antibodies. Both the IP and SN phases were analyzed for phosphorylation of Ser1177, Ser633, and Thr495, as well as Cav-1 or CaMI and eNOS by immunoblots. A, Control HCAECs demonstrated inactive eNOS associated with Cav-1. EPI-treated HCAECs showed only Cav-1 in the IP and activated eNOS in the SN phase. B, Control HCAECs demonstrated no association between the active or inactive forms of eNOS and CaMI, and the SN phase demonstrated the inactive form of eNOS. Data are expressed as mean ± SD (n = 3).
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In a study performed in rats, the administration of protective actions in animals and humans. For example, in a trol. BK (1 expressed as mean/P/H11006 3). *

Data are decreased with the preincubation with U73122. The EPI effect on NO production. In fact, coincubation with stimulation of cells with catechin induced only catechin displaced the EPI-induced concentration-dependent EPI effect on NO production. In fact, coincubation with treatment. This time-frame of NO stimulation is well within stimulation of NO occurred within 10 to 30 minutes after Figure 7 summarizes the effects of 1 mol/L catechin (a structural stereoisomer of EPI) in comparison with EPI. The stimulation of cells with catechin induced only ≈25% of the EPI effect on NO production. In fact, coincubation with catechin displaced the EPI-induced concentration-dependent increases in NO curves to the right (data not shown).

**Catechin Effects on NO Production**

Figure 7 summarizes the effects of 1 mol/L catechin (a structural stereoisomer of EPI) in comparison with EPI. The stimulation of cells with catechin induced only ≈25% of the EPI effect on NO production. In fact, coincubation with catechin displaced the EPI-induced concentration-dependent increases in NO curves to the right (data not shown).

**Discussion**

In an effort to identify intracellular signaling pathways involved in eNOS activation induced by EPI and gather information relevant to vascular and cardioprotective effects, we pursued experiments using human coronary artery endothelial cell cultures. Time-course and dose-response experiments were performed to determine the peak time and concentration at which maximal EPI stimulation would be exerted. Peak stimulation of NO occurred within 10 to 30 minutes after treatment. This time-frame of NO stimulation is well within those reported for physiological stimulators of eNOS, such as BK or estrogens. Concentrations of EPI as low as 0.1 nmol/L stimulated NO production with a maximal effect observed at 1 μmol/L. The amount of NO stimulation observed at 1 μmol/L, although significant (≈100% increase over control), was only 7–5% of that observed for BK at similar concentrations.

We believe that the in vitro effects generated with 1 μmol/L EPI correlate to reported vasodilatory and cardioprotective actions in animals and humans. For example, in a study performed in rats, the administration of 0.5 g/kg cocoa powder or EPI at 1 mg/kg (by gavage) yielded ≈1 μmol/L plasma levels that peaked at ≈1 hour after consumption. The 1 μmol/L concentration of EPI is also equivalent to peak blood levels observed at 2 hours in humans who consume flavanol-rich cocoa-containing products. Recently, we reported on the cardioprotective effects of 1 mg/kg per day EPI in rats subjected to myocardial ischemia reperfusion injury. Observations from these studies suggest that the dose of EPI used is within the range of concentrations that can be readily achieved in animals and humans.

Results indicate that the EPI-induced synthesis of NO was dependent on the activation of eNOS, as observed by increases in the phosphorylation of Ser1177 and Ser633 and the dephosphorylation of the Thr495 residues. These results are consistent with known effects of physiological NO dependent vasodilators (such as BK or estrogens) on the phosphorylation/dephosphorylation of eNOS residues, leading to NO production in endothelial cells. In an effort to identify intracellular pathways involved in the EPI-induced effects, we examined the involvement of the PI3K pathway, which has been described as importantly involved in the physiological modulation of eNOS activation and NO production. The kinases Akt and PKA are of particular importance as they are known members of the PI3K pathway and can phosphate eNOS. Akt is known to phosphorylate eNOS at Ser1177, whereas PKA phosphorylates eNOS Ser1177 and Ser633. By using specific Akt and PKA inhibitors, we identified a role for these kinases in EPI-induced eNOS activation. Inhibition of Akt with SH-5 diminishes NO synthesis by ≈25% with respect to maximum EPI-induced effects. Treatment with SH-5 prevents eNOS Ser1177 phosphorylation by ≈50%; however, Ser633 phosphorylation is not affected, which is in agreement with well-described Akt effects on eNOS phosphorylation. This result evidences the partial involvement of Akt on EPI-induced eNOS activation.

The inhibition of PKA decreases the activity of eNOS to ≈50% of EPI-induced maximal effects. In our experiments, we observed that the inhibition of PKA with H89 partially blocked the phosphorylation of eNOS Ser1177 and Ser633, which correlates with the magnitude of the decrease observed in NO production. Together, the results evidence the participation of the PI3K pathway in EPI-induced eNOS activation. Interestingly, a notable difference is observed in eNOS activation when EPI effects are contrasted with agents such as acetylcholine, BK, and black/red tea flavonoids. These compounds mainly activate eNOS via Akt, leading to the phosphorylation of Ser1177, whereas EPI-induced eNOS activation is mediated through both Ser1177 and Ser633 phosphorylation. The incomplete inhibition gained by blocking the PKA/Akt pathway suggests that EPI-induced eNOS activation also uses other complementary signaling pathways.

It is well established that when eNOS is inactive, it is bound to Cav-1 on the cytoplasmic side of the cellular membrane. Increases in intracellular Ca²⁺ activate CaM, which in turn couples to eNOS, leading to its release/solubilization. Our results indicate that EPI treatment stimulates the dissociation of eNOS from the cell membrane. As with other physiological vasodilators, these results suggest that EPI-induced eNOS activation is mediated via Ca²⁺/CaM/CaMII. To further test this hypothesis, we proceeded to determine the upstream involvement of phospholipase C, which is known to stimulate the release of Ca²⁺ from intracellular stores via IP₃ production. Treating cells with EPI...
induced significant increases in IP$_3$ production, as well as the phosphorylation of IP$_3$R in the endoplasmic reticulum. These 2 effects were blocked when the phospholipase C inhibitor U73122 was used. Thus, calcium-dependent kinases, such as CaMKII, can be activated that in turn activate eNOS.\(^{22}\)

Indeed, we further demonstrated this, because inhibiting CaMKII with KN93 induced a decrease in eNOS phosphorylation and activity, as well as the phosphorylation of IP$_3$R. On the basis of these results, we speculated that EPI exerts its effects via cell membrane–associated events. To evaluate the specificity of NO responses, we treated cells with a stereoisomer of EPI, catechin. Catechin has the same chemical composition as EPI but differs in the spatial orientation of 1 of its rings (Figure 7). The comparison of effects led to a notable difference in the levels of NO production with catechin, which, at the same concentrations, stimulated only $\approx$25% of maximal EPI effects. In fact, catechin displaced the EPI concentration–response curve to the right, suggesting an antagonist-like effect.

**Perspectives**

The results generated by this study highlight important differences in the activation of selected signaling pathways as induced by EPI versus those of known classical stimulators of vascular endothelium, such as BK (Figure 8). These differences suggest a distinct modulation of these pathways by EPI. On the basis of the results gathered and the observations...
noted above, it is reasonable to suggest the presence of an EPI cell membrane acceptor-effector, which harbors a “high” specificity given that the stereoisomer catechin only acts as a weak partial agonist or as an antagonist to EPI effects. It is very likely that this “acceptor-effector” is distinct from those described for other “cardioprotective” agents, such as resveratrol, as EPI does not compete for resveratrol receptor binding\(^{23,24}\) or for estrogen receptors, because the use of ICI182,780 (a selective estrogen receptor antagonist) did not block EPI effects on NO synthesis in our experiments (data not shown).

Our results also suggest consideration of a more direct therapeutic approach to limit CVD using EPI, because commercial cacao products typically contain many other natural and added ingredients (EPI, catechin, quercetin, theobromine, sugar, flavorings, etc.) and usually have a high caloric content. The possible presence of a specific acceptor-effector for EPI is likely; however, there are many other mechanistic possibilities that will need to be explored, such as the following: (1) the interaction with receptors at allosteric sites, (2) the modulation of signaling pathways in a receptor-independent manner, and (3) the binding to intracellular receptors/molecules. The results reported here reflect only the acute effects of EPI; hence, chronic actions must be further explored, as they may also importantly account for vascular and cardioprotective effects. Thus, much more work is required to further explore these interesting possibilities.

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Disclosures
None.

References

Figure 7. NO measurements in HCAECs treated with EPI or catechin (CAT). NO production was measured in HCAECs treated with EPI or catechin (1 μmol/L). EPI induced an increase of ∼4-fold in NO vs catechin. Data are expressed as mean ± SD (n=3). *P<0.05.

Figure 8. EPI-induced eNOS activation in HCAEC. Schematic representation of signaling pathways involved in EPI-induced eNOS activation. BK receptor-activated pathways are included (double arrows) to demonstrate similarities. p-eNOS indicates phosphorylated eNOS; PLC, phospholipase C.
chocolate consumption on plasma oxidation status. *J Nutr.* 2000;130:2109S–2114S.


(-)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways
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(-)-Epicatechin activation of endothelial cell eNOS, NO and related signaling pathways. ONLINE SUPPLEMENT

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Running Title: (-)-Epicatechin, eNOS and nitric oxide

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METHODS

Cell Culture
HCAECs were obtained from Cell Applications, Inc. using three different lots. The company data sheet indicated that the cells were obtained from 14-, 40- and 60-year-old normal male donors in apparent good health. Cells were maintained in a humidified atmosphere at 37°C with 5% CO₂ and 95% O₂ in HCAEC growth medium. Sixteen hours prior to experiments, HCAECs were washed with phenol red free Hank’s salts solution and kept in phenol red free M-199 supplemented with 200 mmol/L of L-glutamine and 1% antibiotic mix. Treatments were typically applied to confluent cell cultures.

NO measurements
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Immunoblotting
Immunoblots were performed as previously described [1]. Immunoblots were developed using an ECL Plus detection kit (Amersham-GE). Band intensity was digitally quantified.

Immunoprecipitation
Cells were lysed with non-denaturing extraction buffer. Total protein was pre-cleared by adding 1 μg of normal rabbit IgG control and 20 μl protein G-agarose. Immunoprecipitation was carried out as previously described [1] using 3 μg of immunoprecipitating antibody (anti Cav-1 or anti CaMI).

Phosphoinositide hydrolysis
IP₃ levels were measured as previously described [2] using [³H]myoinositol labeling. Total [³H]IP₃ was determined by liquid scintillation spectrometry, and data were normalized to cellular protein content.

Ca²⁺/CaM/CaMKII and PI3K pathway inhibition
Previous to EPI treatment, cells were incubated with specific chemical inhibitors. The concentrations used for all compounds were higher than their reported IC₅₀. Phospholipase C (PLC) was inhibited with 10 μmol/L of U73122 (15 min) (IC₅₀ 2 μmol/L) [3,4]. CaMKII was inhibited with 0.5 μmol/L of KN-93 (15 min) (IC₅₀ 0.37 μmol/L) [5,6]. PI3K was inhibited with 100 nmol/L of WORT (10 min) (IC₅₀ 10 nmol/L) [7,8]. Akt was inhibited with 20 μmol/L of SH-5 (20 min) (IC₅₀ 5 μmol/L) [9,10], and PKA was inhibited with 10 μmol/L of H-89 (20 min) (IC₅₀ 135 nmol/L) [11,12].

Data analysis
A minimum of three experiments was performed (each in triplicate) unless otherwise noted. Statistical analysis was performed using t-test or ANOVA with a post-hoc analysis (Tukey) when appropriate. Significance was noted at P<0.05.
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