A Role for Endoglin in Coupling eNOS Activity and Regulating Vascular Tone Revealed in Hereditary Hemorrhagic Telangiectasia

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Abstract—Decreased endothelial NO synthase (eNOS)-derived NO bioavailability and impaired vasomotor control are crucial factors in cardiovascular disease pathogenesis. Hereditary hemorrhagic telangiectasia type 1 (HHT1) is a vascular disorder associated with ENDOGLIN (ENG) haploinsufficiency and characterized by venous dilatations, focal loss of capillaries, and arteriovenous malformations (AVMs). We report that resistance arteries from Eng<sup>−/−</sup> mice display an eNOS-dependent enhancement in endothelium-dependent dilatation and impairment in the myogenic response, despite reduced eNOS levels. We have found that eNOS is significantly reduced in endoglin-deficient endothelial cells because of decreased eNOS protein half-life. We demonstrate that endoglin can reside in caveolae and associate with eNOS, suggesting a stabilizing function of endoglin for eNOS. After Ca<sup>2+</sup>-induced activation, endoglin-deficient endothelial cells have reduced eNOS/Hsp90 association, produce less NO, and generate more eNOS-derived superoxide (O<sub>2</sub><sup>−</sup>), indicating that endoglin also facilitates eNOS/Hsp90 interactions and is an important regulator in the coupling of eNOS activity. Treatment with an O<sub>2</sub><sup>−</sup> scavenger reverses the vasomotor abnormalities in Eng<sup>−/−</sup> arteries, suggesting that uncoupled eNOS and resulting impaired myogenic response represent early events in HHT1 pathogenesis and that the use of antioxidants may provide a novel therapeutic modality. (Circ Res. 2005;96:684-692.)

Key Words: endothelium ■ superoxide ■ vascular disease ■ vascular tone ■ vasodilatation

Resistance arteries normally contract in response to increases in perfusion pressure. This reflex, referred to as the myogenic response (MR), plays an important role in the local regulation of vascular tone and blood flow. By limiting the transmission of excessive hemodynamic forces to downstream capillaries and venules, the arterial MR also preserves the structural integrity of the microcirculation. Although the MR is an intrinsic property of vascular smooth muscle, it is modulated by endothelium-derived vasoactive factors, most notably NO. In the vasculature, endothelial NO synthase (eNOS) produces NO in response to humoral and mechanical stimuli via location-specific and dynamic interactions with its various allosteric regulators, including caveolin-1 (Cav-1) and Hsp90. Impaired vasomotor control attributable to decreased eNOS-derived NO bioavailability is suggested to be a crucial factor in cardiovascular disease pathogenesis. Moreover, a mathematical model of microcirculatory hemodynamics predicts that a loss of vasomotor control may cause arteriovenous malformations (AVMs).

Hereditary hemorrhagic telangiectasia type 1 (HHT1) is a vascular disorder characterized by AVMs. This autosomal dominant disorder is associated with endoglin (ENG) haploinsufficiency and vascular abnormalities ranging from venous dilatations to focal loss of capillaries leading to AVMs. Endoglin (Eng) is a 180-kD glycoprotein expressed on endothelial cells, acting as an ancillary receptor for several transforming growth factor (TGF)-β superfamily ligands and modulating TGF-β1 and TGF-β3 responses. Eng-null mice die at mid-gestation from severe cardiovascular defects, whereas Eng<sup>−/−</sup> mice can develop vascular abnormalities characteristic of HHT1. The earliest detectable lesions in HHT1 are focally dilated post-capillary venules, which enlarge and eventually connect directly with dilated arterioles. We hypothesized that these early lesions may be secondary to an endothelium-dependent impairment of the MR in resistance arteries predisposing downstream vessels to excessive hemodynamic stress. Impaired arterial MR could thus contribute to venular dilatation and capillary loss and represent an early event in the pathogenesis of HHT1.

We report that resistance arteries from Eng<sup>−/−</sup> mice display an eNOS-dependent impairment in the regulation of local vascular tone, despite reduced eNOS levels and NO produc-
tion. We identify Eng as an essential component of the eNOS activation pathway, where it promotes coupling of eNOS activity by facilitating its association with Hsp90. Eng-deficient cells display reduced eNOS/Hsp90 association and uncoupled eNOS activity evidenced by increased eNOS-derived O$_2^•$. Treatment of Eng$^{-/-}$ resistance arteries with an O$_2^•$ scavenger restores vasomotor function, suggesting that uncoupled eNOS and impaired MR may be early events in HHT1 pathogenesis.

**Materials and Methods**

**Vascular Responses in Eng$^{-/-}$ Resistance Arteries**

Mice were generated in the animal facility of the Hospital for Sick Children, and wild-type female C57BL/6 mice used for backcrosses were purchased from the Jackson Laboratory. Eng$^{-/-}$ and Eng$^{+/+}$ mice (8- to 12-week-old N6-N14 C57BL/6 backcrosses) were anesthetized with ketamine/xylazine (100/10-mg/kg intraperitoneal).

Using a perfusion myograph (Living Systems), isolated second-order mesenteric arteries (200 μm) were subjected to increases in perfusion pressure (PP) in physiological salt solution and changes in internal vessel diameters were recorded as described.16 MR at a given PP was determined by MR$_{PP}$=1−(PD$_{PP}$−AD$_{PP}$)/PD$_{baseline}$×100, where AD$_{PP}$ and PD$_{PP}$ are the active and passive vessel diameters, respectively, at a given PP, and PD$_{baseline}$ is the passive vessel diameter at 100 mm Hg. Endothelial removal was accomplished by air injection.17 NOS activity was inhibited with L-NAME (10−4 mol/L) and diameters were recorded in 10% of the baseline diameter in L-NAME pretreated endothelium-intact vessels from both groups of mice. O$_2^•$ scavenged with 1 mmol/L Tiron (Sigma-Aldrich). Dilatation to acetylcholine (ACh) (5×10−5 mol/L) was absent in endothelium-denuded vessels and reached a maximum of ∼10% of the baseline diameter in l-NAME pretreated endothelium-intact vessels from both groups of mice. O$_2^•$ scavenged with 1 mmol/L Tiron (Sigma-Aldrich). All protocols were in accordance with the Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee of the Hospital for Sick Children.

Pressurized mesenteric arteries (60 mm Hg) were preconstricted with phenylephrine (PE: 10−6 mol/L) and diameters were recorded in response to cumulative doses of ACh (10−10 to 5×10−7 mol/L) to assess endothelium-dependent relaxation. Smooth muscle contractility was measured by responses to cumulative doses of PE (10−9 to 5×10−3 mol/L). Endothelium-independent relaxation was analyzed by recording changes in vessel diameter to cumulative doses of sodium nitroprusside (10−8 to 10−3 mol/L) in PE/l-NAME preconstricted endothelium-intact arteries. Responses were calculated by 1−(AD$_{baseline}$−AD$_{stabilized}$/PD$_{baseline}$)×100, where AD$_{stabilized}$ is the active diameter at a given agonist dose.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) from control newborns and from those with characterized ENG mutations were prepared and grown on gelatinized plates as described.17 Murine Eng$^{+/+}$, Eng$^{-/-}$, and Eng$^{+/+}$ embryonic endothelial cells were generated by Dr S. Liebner and Dr E. Dejana from E8.5 embryos and grown as described.18

**Metabolic Labeling, Pulse-Chase, and Western Blotting**

HUVECs and murine endothelial cells were labeled with Trans [35S] Label (MP Biochemicals) and Triton X-100 extracts were immunoblotted with pAb to Eng (Dr C. Bernabeu) and immunoprecipitated for Eng.

**Immunofluorescence Staining**

HUVECs were grown on gelatinized glass slides, fixed in 2% paraformaldehyde, and permeabilized with PBS (0.5% BSA, 0.1% Triton X-100). Cells were simultaneously labeled with mouse mAb SN06 (Dr B.K. Seon) to Eng and rabbit pAb to eNOS, incubated with FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit IgG (Cedarlane), and visualized by confocal microscopy (Carl Zeiss).

**Lipid Raft Isolation**

Eng$^{+/+}$ and Eng$^{-/-}$ endothelial cells were harvested in 0.5 mol/L sodium carbonate (pH 11) and subjected to detergent-free sucrose gradient centrifugation as described.19 Fractions were immunoblotted for Cav-1, Eng, and eNOS. eNOS immunoprecipitates of Cav-1-containing and Eng-containing fractions (5 and 6) were immunoblotted for Eng.

**eNOS Activity Assay**

HUVECs were detached with PBS/EDTA (1 mmol/L) and homogenized in 10 mmol/L Tris-HCl pH 7.4, [H]-l-arginine to [H]-l-citrulline conversion was measured with 1 mmol/L CaCl$_2$, with or without l-NAME (1 mmol/L) using a NOS assay kit (Calbiochem). Murine Eng$^{+/+}$, Eng$^{-/-}$, and Eng$^{+/+}$ cells were cultured in 6-well gelatinized plates, pre-incubated with or without l-NAME (1 mmol/L)
L), and NOS activity was assayed in intact cells in the absence/presence of ionomycin (1 μmol/L) after 45 minutes as described.  

Hsp90/eNOS Association
HUVECs or murine endothelial cells were serum-starved for 3 hours and stimulated with vehicle or ionomycin (1 μmol/L) for 10 minutes. Extracts were precleared with protein G and equal protein concentrations were immunoprecipitated with mAb to eNOS. eNOS immunoprecipitates were immunoblotted for Hsp90 and total eNOS levels.

NOS-Dependent O\textsubscript{2}^{-} Production
O\textsubscript{2}^{-} generation was measured in murine Eng\textsuperscript{+/+}, Eng\textsuperscript{+/−}, and Eng\textsuperscript{−/−} endothelial cells by monitoring ferricytochrome c reduction at 550 nm in the presence and absence of ionomycin (1 µmol/L) and/or L-NAME (1 mmol/L) and/or superoxide dismutase (SOD 1000 U/mL; Sigma) as described. NOS-dependent O\textsubscript{2}^{-} generation was determined by the level of L-NAME inhibitable cytochrome c reduction.

Systemic Blood Pressure and Plasma NO\textsubscript{x}
Eng\textsuperscript{+/+} and Eng\textsuperscript{−/−} mice were anesthetized with ketamine/xylazine (100/10-mg/kg intraperitoneal). The common carotid artery was isolated and cannulated with a Millar Mikro-tip pressure transducer and individual pressure tracings were recorded as described. In a separate set of experiments, mice were euthanized and blood was collected. Plasma NO\textsubscript{x} levels were quantified using NO analyzer (Sievers Instrument Inc) as described.

Statistical Analysis
Comparisons between groups were performed by one- or two-way ANOVA and significant overall differences were evaluated post hoc using the Bonferroni procedure. Results are expressed as the mean±SEM, with P<0.05 representing significance.

Results
Enhanced Endothelium-Dependent and NOS-Dependent Dilatation Impairs the MR in Eng\textsuperscript{−/−} Resistance Arteries
Compared with Eng\textsuperscript{+/+}, Eng\textsuperscript{−/−} mesenteric arteries displayed a significant decrease in MR over perfusion pressures from 80 to 140 mm Hg (Figure 1A). The loss of this differential response after endothelial denudation suggested that Eng\textsuperscript{−/−} vessels have normal pressure-induced smooth muscle contractile function and an endothelium-dependent impairment in their MR (Figure 1B). L-NAME abolished differences between Eng\textsuperscript{−/−} and Eng\textsuperscript{+/+} arteries (Figure 1C), suggesting that the impaired MR in untreated Eng\textsuperscript{−/−} endothelium-intact
vessels is NOS-dependent. All arteries were devoid of overt structural abnormalities and displayed identical active (at 60 mm Hg) and passive pressure–diameter relationships.

Because eNOS is a central mediator of endothelium-dependent dilatation and is stimulated by ACh, we assessed ACh-induced relaxation in endothelium-intact PE preconstricted resistance arteries. Compared with Eng+/+, Eng+/− arteries displayed enhanced ACh-dependent dilatation (Figure 1D), supporting an eNOS-dependent abnormality in Eng−/− arterial vasomotor function. Endothelium-intact arteries in both groups displayed identical dose-responses to PE and sodium nitroprusside (Figure 1E and 1F), suggesting that Eng−/− resistance arteries exhibit no inherent defects in vascular smooth muscle contractility and endothelium-independent vasodilatation, respectively.

Decreased eNOS Levels in Eng-Deficient Vessels and Endothelial Cells

Immunoblots of Eng−/− liver extracts showed significantly reduced Eng expression to 55±2% of control. To test whether increased eNOS expression may account for the observed endothelium-dependent and NOS-dependent alterations in vasomotor function, we measured eNOS levels in Eng−/− vessels. Paradoxically, Eng−/− mesenteric vessel extracts showed significantly reduced eNOS protein levels to 68±3% of control (Figure 2A). iNOS and nNOS were not detectable in cultured endothelial cells, nor were differences in nNOS levels observed in intact vessels of Eng+/+ and Eng−/− mice (data not shown). These data indicate that NOS-dependent vasomotor abnormalities in intact Eng−/− resistance arteries are mediated by eNOS, despite its reduced levels.

To ascertain whether the reduction in eNOS levels in Eng−/− vessels is independent of neurohumoral and hemodynamic stimuli, we performed metabolic labeling of HUVECs derived from newborns with an ENG mutation. Specific immunoprecipitation revealed that Eng and eNOS levels were significantly reduced to 45±7% and 51±12%, respectively, of the levels in control HUVECs (Figure 2B). HUVECs from 6 newborns with different ENG mutations showed similar reductions in Eng and eNOS levels. Moreover, immunoblots of Eng+/+, Eng+/−, and Eng−/− murine endothelial cells expressing 100%, 65±9%, and 0% Eng levels displayed relative eNOS levels of 100%, 52±2%, and 46±4%, respectively (Figure 2C). These data suggest that partial or total loss of Eng in murine endothelial cells is associated with a 50% decrease in eNOS levels.

Eng Increases eNOS Protein Stability

To test whether reduced eNOS levels in Eng-deficient cells are attributable to a decrease in eNOS protein stability, we performed pulse-chase experiments in the presence of cycloheximide. The half-life of eNOS protein was reduced by 4-fold from 9.1 hours in Eng+/+ to 2.3 hours in Eng−/− endothelial cells (Figure 2D).

Eng Colocalizes With eNOS in Cav-1–Enriched Lipid Rafts

To further elucidate the functional relationship between Eng and eNOS, we characterized their distribution in endothelial cells. Immunofluorescence labeling of HUVECs revealed endoglin predominantly expressed at the plasma membrane with intense staining at focal points, and in several cases at the perinuclear region (Figure 4A). A similar pattern was seen for eNOS (Figure 4B), and image superimposition showed colocalization in both areas (Figure 4C). HUVECs harboring ENG mutations showed a similar pattern but with reduced Eng and eNOS staining (data not shown). To determine whether Eng/eNOS colocalization is attributable to a biochemical association, we tested whether these proteins coimmunoprecipitate. Because Cav-1 and Hsp90 are allosteric...
Because eNOS is targeted to caveolae and other lipid rafts,24 we investigated whether Eng also resides in caveolar lipid rafts. Sucrose gradient centrifugation of mouse endothelial cell extracts revealed Eng in Cav-1–containing fractions, whereas eNOS was partially associated with these fractions (Figure 4J to 4L). Immunoblots of eNOS immunoprecipitates from Cav-1–enriched and Eng-enriched fractions (5 and 6) revealed Eng, suggesting that the eNOS/Eng complex occurs in caveolae and in reduced amounts in Eng−/− cells (Figure 4M).

**Impaired eNOS/Hsp90 Association in Eng-Deficient Cells**

The absence of Ca2+-stimulated [3H]-L-citrulline production in Eng−/− cells (Figure 3C) suggested that Eng is necessary for the formation of the eNOS activation complex. During Ca2+-activation of eNOS, increased eNOS/Hsp90 interaction enhances and maintains NO production.3–5 To ascertain whether this Ca2+-induced eNOS/Hsp90 association is regulated by Eng, we immunoprecipitated eNOS from normal and Eng-deficient endothelial cell extracts and compared the Hsp90/eNOS ratios before and after ionomycin stimulation. In HUVECs harboring ENG mutations, ionomycin increased eNOS/Hsp90 association to a significantly lesser extent than in normal HUVECs (Figure 5A). In murine endothelial cells, basal Hsp90/eNOS association was significantly reduced in Eng−/− cells compared with Eng+/+ cells (Figure 5B). Ionomycin increased Hsp90/eNOS association in Eng+/+ cells, but not in Eng−/− cells, suggesting that Eng is necessary for eNOS/Hsp90 association during Ca2+-induced activation. Total Hsp90 levels were unchanged in HUVECs with ENG mutations and in Eng−/− cells (data not shown).

**Uncoupled eNOS Activity in Eng-Deficient Cells**

Failure of eNOS to couple oxygen to l-arginine metabolism results in increased eNOS-derived O2− and reduced NO release.25 Hsp90 mediates the balance of NO and O2− release from eNOS during Ca2+ activation, in which its association with eNOS favors NO production.23 We tested if reduced eNOS/Hsp90 association in Eng-deficient cells is accompanied by increased NOS-dependent O2− generation in ionomycin-stimulated endothelial cells with and without L-NAME. Ionomycin caused NOS-dependent O2− production in Eng-deficient cells (Figure 5C), suggesting that Eng modulates the ability of eNOS to generate O2−. This NOS-dependent O2− generation in Eng-deficient cells comprised 67% of the total SOD-sensitive portion of cytochrome c reduction.

**O2− Scavenger Reverses Vasomotor Abnormalities in Eng+/− Resistance Arteries**

Because Eng-deficient endothelial cells display eNOS-derived O2− production, we tested whether treatment of vessels with the O2− scavenger, Tiron, reverses the vasomotor abnormalities in intact Eng+/− vessels. Tiron enhanced MR magnitude and sensitivity in Eng+/−, but not in Eng+/+ vessels (Figure 6A). Furthermore, Tiron reduced ACh-induced endothelium-dependent dilation in Eng+/− vessels while having no significant effect on Eng+/+ vessels (Figure 6B). These data suggest that uncoupled eNOS activity in Eng+/− resistance arteries produces O2−, leading to the production of...
endothelium-derived hyperpolarizing factors (EDHFs) that enhance endothelium-dependent dilatation and impair MR.

Discussion

The uncoupling of eNOS activity is quickly emerging as an important mechanism in cardiovascular disease.26–28 The failure of eNOS to couple oxygen to l-arginine metabolism results in increased eNOS-derived O2•− at the expense of NO.25 This is observed during reductions in NOS substrate or cofactor levels25,29,30 and more recently as a consequence of reduced association of eNOS with Hsp90.22 In the current study of endothelial cells from humans and mice with HHT1, we have identified endoglin as another molecule that can influence the redox switch in eNOS catalysis and thus the regulation of vascular tone.

We have detected endoglin in endothelial caveolae and shown previously unrecognized associations with eNOS and its allosteric regulators, Cav-1 and Hsp90. The functional significance of Eng in the eNOS activation complex is apparent because endothelial cells lacking Eng also lose the capacity to generate NO in response to Ca2+ -dependent (ionomycin) eNOS activation. Instead, eNOS activity is uncoupled in Eng-deficient cells, as evidenced by severely reduced eNOS/Hsp90 association and increased eNOS-derived O2•−. We propose that Eng modulates the coupling of eNOS activity by acting as a scaffolding protein and bringing cytoplasmic Hsp90 into close proximity with caveolar eNOS (Figure 7).

Our analyses of vasomotor responses in Eng+/− resistance arteries have unraveled abnormalities localized to the endothelium and associated with uncoupled eNOS activity. No intrinsic abnormalities in smooth muscle function were observed. Eng+/− arteries display exaggerated eNOS-dependent dilatation and impaired MR, despite reduced eNOS levels. The normalization of these vasomotor defects by an O2•− scavenger suggests that the product(s) of uncoupled eNOS activation are reactive oxygen species that exert a net hyperpolarizing or dilatory effect on the underlying smooth muscle and can be collectively referred to as EDHFs. In addition to recent evidence of O2•− directly inhibiting smooth muscle contraction in vitro,31 H2O2 generated by the dismutation of eNOS-derived O2•− by superoxide dismutase is also a known EDHF in mouse mesenteric arteries.32 Furthermore, because NO and O2•− can be produced in close proximity to one another in Eng-deficient endothelial cells, their combination can generate ONOO−,33 which may account for the eNOS-dependent changes in vasomotor function. In addition to their vasoactive properties, compounds such as O2•− or ONOO− can also oxidize the essential NOS cofactor, tetrahydrobiopterin (BH4), to dihydrobiopterin (BH2), thus effectively reducing BH4 levels and further uncoupling eNOS34 (Figure 7). Although Eng-deficient endothelial cells may have inherently reduced BH4 levels, our findings favor the view that this possibility would likely be the result of the oxidative microenvironment created by the uncoupling of eNOS activity because of its impaired interaction with Hsp90.

The finding that eNOS levels remain reduced in cultures of Eng-deficient human and murine cells in vitro indicates that eNOS levels in Eng+/− resistance arteries are independent of neurohumoral and hemodynamic stimuli. We have demonstrated that the stability of eNOS is significantly reduced in Eng-deficient endothelial cells, suggesting that the eNOS–Eng association described in this study may be necessary to maintain eNOS in a stable conformation and/or protect it...
from proteolysis. Decreased eNOS levels could also be related to a negative feedback regulation, whereby eNOS-derived O$_2^{-}$/H$^+$ and/or ONOO$^-$ interfere with eNOS expression. eNOS expression is increased in mice and endothelial cells treated with antioxidants.$^{35,36}$

During the preparation of this manuscript, reduced eNOS protein levels in Eng-deficient vessels were reported by Jerkic et al.$^{37}$ Although our data (Figure 2A) agree with this finding, we have observed increased endothelium-dependent vasodilatation in Eng$^{-/-}$ mice rather than the decreased vasodilatation suggested by them.$^{37}$ Because dilated vessels are characteristic of HHT1, we favor the view that endothelium-dependent dilatation is enhanced because of uncoupling of eNOS and the generation of eNOS-derived EDHFs after enzyme activation. Given our finding that an antioxidant reduced ACh-induced vasodilatation in Eng$^{-/-}$ arteries, the seemingly contradictory results between our report and that of Jerkic et al may reflect age, gender, dietary, environmental, and/or other experimental factors affecting the redox status of the arteries examined.

The prevailing notion on HHT1 pathogenesis posits that vascular abnormalities originate from inherently dilated post-capillary venules, which enlarge and eventually establish AVMs.$^{15}$ We propose that early lesions may instead be consequences of a preceding impairment in upstream arterial MR. Resistance arteries, with impaired MR caused by increased eNOS-derived O$_2^{-}$/H$^+$ and associated EDHFs, do not adequately contract in response to sudden increases in intraluminal pressure and would thus fail to limit the transmission of excessive and potentially damaging hydrostatic pressures to downstream structures. This could contribute to the genesis of early HHT-type vascular lesions such as capillary loss and venular dilatations. Our findings propose that sudden increases in perfusion pressure would not be well-tolerated in HHT1 patients and might explain the frequent epistaxis characteristic of this disease. They also suggest that additional stresses such as hypoxia and inflammation may further impair an already compromised vasoregulatory mechanism and contribute to disease manifestations. Although it remains to be proven that impaired arterial MR leads to the generation of AVMs, such an outcome is supported by a current mathematical model of microcirculatory hemodynamics predicting that a loss of vasomotor control may cause AVM.$^6$

Despite decreased eNOS levels and a diminished capacity to produce NO, Eng$^{-/-}$ mice appear to have a countervailing mechanism protecting them against increases in systemic blood pressure. Their enhanced endothelium-dependent dilatation and resulting defect in myogenic tone because of

![Figure 6. Treatment with Tiron reverses vasomotor abnormalities in Eng$^{-/-}$ resistance arteries. A, The MR of pressurized intact Eng$^{-/-}$ and Eng$^{-/-}$ mesenteric arteries in the absence (○, n=8) and presence (●, n=4) of Tiron. Tiron significantly enhanced the MR of intact Eng$^{-/-}$ vessels (ANOVA, **$P<0.01$ vs vehicle) and had no effect on that of control Eng$^{-/-}$ littermates. Passive diameters were unchanged in Eng$^{-/-}$ and Eng$^{-/-}$ arteries in the absence (○, n=8) and presence (●, n=4) of Tiron. B, Tiron significantly reduced ACh-induced endothelium-dependent dilatation in Eng$^{-/-}$ vessels (○, n=8) (ANOVA, **$P<0.01$ vs vehicle-treated Eng$^{-/-}$ vessels, ○ n=8) and had no significant effect in Eng$^{-/-}$ vessels.](http://circres.ahajournals.org/)

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**Figure 6.** Treatment with Tiron reverses vasomotor abnormalities in Eng$^{-/-}$ resistance arteries. A, The MR of pressurized intact Eng$^{-/-}$ and Eng$^{-/-}$ mesenteric arteries in the absence (○, n=8) and presence (●, n=4) of Tiron. Tiron significantly enhanced the MR of intact Eng$^{-/-}$ vessels (ANOVA, **$P<0.01$ vs vehicle) and had no effect on that of control Eng$^{-/-}$ littermates. Passive diameters were unchanged in Eng$^{-/-}$ and Eng$^{-/-}$ arteries in the absence (○, n=8) and presence (●, n=4) of Tiron. B, Tiron significantly reduced ACh-induced endothelium-dependent dilatation in Eng$^{-/-}$ vessels (○, n=8) (ANOVA, **$P<0.01$ vs vehicle-treated Eng$^{-/-}$ vessels, ○ n=8) and had no significant effect in Eng$^{-/-}$ vessels.
uncoupled eNOS activity and production of EDHFs may constitute the necessary mechanisms. In particular, increased $\text{ONOO}^-$ may be formed by the reaction of eNOS-derived $\text{O}_2^-$ with NO produced by endothelial cells, released from $\text{S}$-nitroso-hemoglobin,$^{38}$ or derived from nitrite reduction by deoxyhemoglobin.$^{39}$ This preferential production of $\text{ONOO}^-$ in the hypoxic microcirculation may contribute to the focal nature of HHT1 (Figure 7). As such, whereas Eng haploinsufficiency is associated with the development of vascular lesions, it may simultaneously offer protection against the development of systemic hypertension.

Until recently, Eng has been identified as an ancillary receptor of the TGF-$\beta$ signaling pathway. Our findings that Eng is an essential component of the eNOS activation complex, stabilizes eNOS protein, and facilitates the association of eNOS with Hsp90 indicate its crucial role in endothelial cell function, the regulation of local vascular tone, and early events in the pathogenesis of HHT1. Our current findings suggest that antioxidants may prevent or delay the development of vascular lesions in patients with HHT1, which may simultaneously offer protection against the development of systemic hypertension.

Figure 7. Model of eNOS activation in normal and HHT1 conditions. A pool of Eng resides in caveolae, where it acts as a molecular scaffold to bring eNOS and Hsp90 in close proximity to one another and facilitates their association during endothelial activation. Reduced eNOS/Hsp90 association in HHT1 leads to increased eNOS-derived $\text{O}_2^-$ and the formation of $\text{H}_2\text{O}_2$ (via superoxide dismutase [SOD]) and $\text{ONOO}^-$, which hyperpolarize smooth muscle and impair vascular contractions. Compounds such as $\text{O}_2^-$ or $\text{ONOO}^-$ can oxidize the essential NOS cofactor, tetrahydrobiopterin (BH$_4$), to dihydrobiopterin (BH$_2$), further uncoupling eNOS in HHT1. The preferential release of NO from $\text{S}$-nitroso-hemoglobin (SNO-Hb) in the microvasculature would favor the local production of $\text{ONOO}^-$ and may explain the focal nature of HHT1.

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