Mechanisms of activation of eNOS by 20-HETE and VEGF in bovine pulmonary artery endothelial cells

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NITRIC OXIDE (NO) is a major endothelium-derived relaxing factor (24) and is generated by the enzyme eNOS. Early experimentation revealed that calcium as well as calmodulin (CaM) play an important role in activation of eNOS (9, 11). Since this time, considerable effort has been made to understand the molecular basis for regulation of eNOS [see review by Fulton et al. (23)]. NO plays a vital role in a number of cardiovascular-related functions. Besides being a potent vasorelaxant, it is also angiogenic. In addition, it regulates vascular remodeling and platelet aggregation (29). Indeed, the potent angiogenic factor VEGF is able to activate eNOS (35, 36). On the basis of several studies, Fontana et al. (20) proposed a molecular model to explain growth factor-stimulated eNOS activation. Steps in this pathway include 1) activation of PLC-y and phosphotyrosinol 3-kinase (PI3-kinase/Akt pathways by VEGF, 2) displacement of caveolin (an inhibitor of eNOS mentioned above) from eNOS by calcium-activated CaM, 3) interaction of Hsp90 with eNOS, and 4) phosphorylation of eNOS by Akt at serine 1179 (serine 1179 is located in bovine eNOS corresponding to serine 1177 in the human enzyme), which is stabilized by Hsp90. This action leads to enhanced electron flux from the reductase to the oxygenase domain of the functional eNOS dimer to become available for conversion of arginine to citrulline and NO.

Our group (40) recently observed that the pulmonary vasodilator lipid 20-HETE increased release of NO from bovine pulmonary artery endothelial cells (BPAECs). This response was calcium dependent and mediated via activation of eNOS. In addition, we determined that the angiogenic factor VEGF dilated small bovine pulmonary arteries and this response was attenuated by cytochrome P-450 inhibitors that block synthesis of endogenous 20-HETE as well as eNOS inhibitors (31). We therefore examined the impact of 20-HETE and VEGF on steps in the molecular signaling pathways associated with activation of eNOS, including phosphorylation of serine 1179, phosphorylation of Akt, and association of eNOS with Hsp90. Our results demonstrate that 20-HETE initiates many of the same responses as VEGF in BPAECs in culture. Because inhibition of cytochrome P-450 family 4 (CYP4) blocks VEGF-induced phosphorylation of eNOS, activation of CYP4 appears to mediate, at least in part, VEGF-associated activation of eNOS in BPAECs. This is also the first report demonstrating that 20-HETE stimulates phosphorylation of eNOS and that the lipid as well as VEGF do not enhance binding of Hsp90 with eNOS in BPAECs.

MATERIALS AND METHODS

Cell culture. BPAECs were obtained from two independent sources and used before they reached passage 8. They were either purchased...
from VEC Technologies (Rensselaer, NY) or isolated in the laboratory as described below. All cells were cultured in RPMI 1640 media (12–702F; Bio Whittaker, Walkersville, MD) supplemented with 10% FBS, and antibiotics (A5955; Sigma, St. Louis, MO). Bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies and maintained under the same conditions as BPAECs.

**Primary endothelial cell culture.** Primary cultures of BPAECs were prepared as previously described (42). Pulmonary arteries (0.5 to 2.0 mm in diameter) were microdissected from lungs of animals obtained at a local abattoir, slit open along their lengths, and washed with PBS to remove blood. The vessels were placed with the lumen side down onto 100-mm tissue culture dishes. After they adhered for 5 min, the arteries were covered with media (RPMI containing 20% FBS) and allowed to grow for 3 days in a tissue culture incubator. Tissue pieces were then lifted out of the medium, and adherent endothelial cells were allowed to proliferate. The cells were trypsinized, diluted, and plated in a 96-well dish with one or two cells/well to select for a pure population of cells based on characteristic cobblestone appearance. Selected cell populations were pooled and probed with the endothelial cell marker platelet endothelial cell adhesion molecule 1 (PECAM-1) and α-smooth muscle actin. Populations in which >90% cells that stained positive for PECAM-1 but negative for α-smooth muscle actin were used for further experimentation.

**Cell stimulation.** The cells were cultured in T-75 flasks and seeded onto 100-mm tissue culture dishes, which were maintained in a tissue culture water-jacketed incubator under 95% air-5% CO2 at 37°C until they attained 80% confluence. They were washed three times with PBS (Sigma), and growth was arrested by incubation in RPMI 1640 containing 0.1% BSA (low endotoxin and fatty acid-free BSA; Sigma) for 4 h. The cells were treated with either VEGF (8.3 nM of recombinant human VEGF obtained from the National Cancer Institute BRB Repository, Rockville, MD), 20-HETE (1 μM synthesized in the laboratory of Dr. J. R. Falck, University of Southern Texas, Dallas, TX), or vehicle (PBS for VEGF and ethanol for 20-HETE) for 10 min unless otherwise mentioned, at 37°C.

**Western analysis.** After stimulation, the cells were chilled on ice and washed three times with cold PBS (Sigma). They were resuspended by scraping in the presence of 0.5 ml RIPA buffer (20-188; Upstate, Lake Placid, NY) supplemented with phosphatase inhibitors (P2850; Sigma), protease inhibitors (1 836 170; Roche, Mannheim, Germany), 2 mM Na3VO4, and 2 mM NaF. The mixture was kept on ice for 15 min, after which lysates were centrifuged for 10 min at 20,000 g and the supernatants were used for determining protein concentration via the BioRad protein assay kit. Equal amounts of protein (50 μg/lane) were boiled for 5 min in Laemmli sample buffer (161-0737; BioRad, Hercules, CA) supplemented with β-mercaptoethanol, resolved on a 10% Tris-HCl SDS polyacrylamide gel (BioRad) and transferred to nitrocellulose membranes as described previously (30, 31). The blots were developed with appropriate antibodies as mentioned for each experiment in RESULTS: phospho-eNOS (serine 1177: 07-428; Cell Signaling, Beverly, MA), eNOS (9D10; Zymed, South San Francisco, CA, or Santa Cruz Biotech, Santa Cruz, CA), phospho-Akt (serine 473: 9271; Cell Signaling, Akt (9272; Cell Signaling), or CYP4 (299230; Daiichi Pure Chemicals, Tokyo, Japan). This step was followed by reaction with matched secondary anti-mouse or anti-rabbit antibodies (1:3,000 dilution) conjugated to horseradish peroxidase. Specific antibody binding was developed by use of ECL Plus detection reagent (RPN 2133; Amersham Biosciences, Piscataway, NJ). Blots were first probed with a phospho-specific antibody (peNOS or pAkt) and then stripped and reprobed with the corresponding antibody (eNOS or Akt, respectively). The blots were scanned with an Alpha Image 220 Analysis System (Alpha Innotech), and the relative densities of the peNOS bands for the same blot were compared with eNOS to determine peNOS-to-eNOS ratios. Each experiment was repeated at least four times using separate batches of cells to ensure reproducibility.

**Immunoprecipitation.** Cell stimulation and lysis were carried out as described above. For each pull-down experiment, an aliquot of protein (500 μg) was preclari ed by rotating end-to-end in a nutator with 40 μ1 of a 50% slurry of protein A-Sepharose (P-3391; Sigma) in 0–5 ml PBS. The pellets were resuspended in Laemmli sample buffer containing β-mercaptoethanol and processed similarly to that described in Western analysis (above). The blots were carefully cut in two at migration distance corresponding to ~100 kDa (kleidoscope markers from BioRad). The portion closer to the point of application of the samples was developed with the eNOS antibody, whereas the lower half was treated with anti-Hsp90 (Transduction Laboratories, Lexington, KY). The relative intensities of specific bands for eNOS and Hsp90 were measured. Values obtained under the same experimental conditions for each protein from the same blot were compared with computed ratios of Hsp90 to eNOS.

**Ring tension studies.** For details, see the companion article in this issue (31). In brief, cow lungs were obtained from a local abattoir. Pulmonary arteries were microdissected from these tissues, and rings ~0.3–1 mm in diameter were prepared in ice-cold PSS. Rings were mounted on tungsten wires connected to a force displacement transducer (model FT03E, Grass Instruments) for measuring isometric tension. The apparatus was immersed in pH-adjusted, oxygenated PSS solution (95% O2-5% CO2) at 37°C. Tension data were relayed to transducers to a signal amplifier, which were acquired and analyzed with CODAS software (DataQ Instruments). Rings were preloaded...
with 0.3–0.5 g of passive tension and then equilibrated for an additional 30 min before the studies began. Tensions were continuously recorded in the presence of experimental reagents such as VEGF or vehicle. Vessels were pretreated with vehicle, the PI3-kinase-Akt inhibitor wortmannin (27), or the tyrosine kinase inhibitor, which blocks association of Hsp90 to eNOS radicicol (14, 39) and then challenged with VEGF (10 nM) in some experiments. Viability of all rings was confirmed by measuring the contractile response to the addition of 60 mM KCl in the bath. Data from rings that did not show at least twofold increase in tension to KCl were eliminated from the analysis. All experimental results have been averaged after pooling data from multiple rings dissected from three or more animals. Separate sets of rings were used for vehicle controls and those pretreated with wortmannin before addition of VEGF or 20-HETE.

Statistical analysis. Pooled data from each experiment were used to calculate the means ± SE for control (vehicle treated) or experimental (treated with 20-HETE or VEGF) samples. The data were tested for significance by a Student’s t-test (for unpaired samples) or Mann-Whitney’s rank sum test for three or more groups with SigmaStat software (Jandel). Experiments with \( P < 0.05 \) were considered significant.

RESULTS

Effect of 20-HETE and VEGF on phosphorylation of serine 1179 on eNOS in BPAECs. Treatment with 20-HETE increased the ratio of peNOS to eNOS in BPAECs compared with vehicle-treated cells (Fig. 1, A and B; \( P = 0.002, n = 6 \)). VEGF also stimulated phosphorylation of eNOS in BPAECs (Fig. 2, A and B; \( P = 0.029, n = 4 \)) within 10 min. Hence, both 20-HETE and VEGF stimulate eNOS phosphorylation of serine 1179 in early passages of primarily cultured pulmonary artery endothelial cells, supporting the hypothesis that relaxation of pulmonary arteries to both agents is attributable at least in part to eNOS stimulation associated with phosphorylation at serine 1179.

To test the role of CYP4 in VEGF-induced activation of eNOS, we pretreated cells with the CYP4 inhibitor of DDMS and then determined the capacity of VEGF to induce phosphorylation of eNOS. In contrast to cells pretreated with vehicle (ethanol), BPAECs treated with 10 \( \mu M \) DDMS (dissolved in ethanol) did not demonstrate a significant increase in phosphorylation of eNOS at serine 1179 \( (n = 4; \text{Fig. 2, A and B}) \) after treatment with VEGF. These data support the hypothesis that CYP4 contributes to VEGF-induced phosphorylation of eNOS in BPAECs.

VEGF and 20-HETE induce phosphorylation of Akt. Phosphorylation of eNOS at serine 1179 is reported to be mediated by Akt. This enzyme is present in the cytosol and recruited to the membrane after stimulation with different agonists. Activation often includes phosphorylation at serine 473. We measured the phosphorylation of Akt after stimulation with 20-HETE.
HETE or VEGF for 10 min each. As shown in Fig. 3, A and B, 20-HETE (1 μM) modestly but significantly increased phosphorylation of Akt at serine 473 (P = 0.029, n = 4). Like 20-HETE, VEGF (8.3 nM) also increased Akt phosphorylation (Fig. 4, A and B; P < 0.001, n = 7).

**Functional implications of VEGF or 20-HETE-induced phosphorylation of Akt.** To test the functional implications of Akt phosphorylation on VEGF- and 20-HETE-associated decreases in pulmonary artery tone, we treated pulmonary arteries with vehicle or the Akt inhibitor wortmannin (5 × 10^-7 M final concentration) before addition of either of these agents to the bath. Wortmannin treatment blocked relaxation to both 20-HETE and VEGF (Fig. 5, A and B), supporting a role for PI3-kinase/Akt in stimulated release of NO by both of these compounds.

**Interaction of Hsp90 with eNOS.** A third well-defined molecular event for activation and stabilization of functional eNOS is increased association with the chaperone Hsp90. Stimulation with 20-HETE (Fig. 6, A and B) or VEGF (Fig. 7, A and B) did not increase interaction of the protein partners Hsp90 with eNOS. In fact, 20-HETE modestly decreased protein association to 76% of that bound by stimulation with vehicle (P = 0.006; n = 9). Because this result was different from the action of VEGF on other cell types (25), including aortic endothelial cells, we repeated the experiment with primary isolates of BAECs. We observed consistent increases in binding of eNOS to Hsp90 after stimulation of BAECs with VEGF (8.3 μM) in a subconfluent population of BAECs (Fig. 8, A and B; P = 0.015; n = 5).

**Functional implications of inhibition of Hsp90 association with eNOS.** To determine whether Hsp90 association with eNOS contributed to VEGF- or 20-HETE-associated relaxation of pulmonary arteries, we treated pulmonary arteries with radicicol (20 μM final concentration). Pretreatment with this agent had no impact on 20-HETE-evoked relaxation of pulmonary arteries (Fig. 9A), consistent with the observation that 20-HETE did not increase Hsp90 association with eNOS. Radicicol treatment partially but incompletely blocked VEGF-stimulated relaxation of pulmonary arteries (Fig. 9B)

**DISCUSSION**

We demonstrated that VEGF-induced dilation of bovine pulmonary arteries was mediated by CYP4/20-HETE (31). This conclusion was based on observations that treatment with two mechanistically distinct inhibitors of CYP4 blocked VEGF-induced relaxation of small pulmonary arteries. We also reported increased production of NO by application of 20-HETE to BAECs (40) and VEGF-induced generation of NO in BAECs, which is attenuated by CYP4 inhibition (31). In this report, we examined the action of both VEGF and 20-HETE on three known molecular events that activate eNOS (23): 1) phosphorylation at serine 1179, 2) phosphorylation of the eNOS protein partner Akt, which can lead to phosphorylation of eNOS at serine 1179, and 3) association of Hsp90.
with eNOS (25). In addition, inhibition of CYP4 with DDMS also blocked VEGF-stimulated phosphorylation of eNOS. Our results demonstrate that two pulmonary vasodilators, VEGF and 20-HETE, increase the ratio of peNOS (serine 1179)/eNOS as well as pAkt (serine 473)/Akt. However, neither of these agonists increases the interaction of the chaperone protein Hsp90 with eNOS in BPAECs. Because all three signaling events seen after VEGF-mediated stimulation of bovine pulmonary arteries are similar to those measured using 20-HETE, these results support our hypothesis that VEGF-induced dilatation of bovine pulmonary arteries is mediated in part by CYP4/20-HETE.

To test the functional implications of Akt phosphorylation and association of Hsp90 with eNOS on VEGF- and 20-HETE-evoked relaxations, we treated pulmonary arteries with wortmannin and radicicol, respectively, before addition of these mediators to pulmonary artery rings. Wortmannin effectively blocked relaxations to both 20-HETE and VEGF, suggesting that the PI3-kinase-Akt pathway contributes to stimulated NO release by these agents (27). Consistent with the lack of ability of 20-HETE to increase association of Hsp90 with eNOS, relaxation to this lipid was unaffected in pulmonary arteries treated with radicicol. Similarly VEGF had no effect on association of Hsp90 in BPAECs, whereas treatment with radicicol partially blocked relaxation to this peptide. We believe that inhibition of VEGF-induced relaxation in pulmonary arteries treated with radicicol is likely attributable to the well-described effects of this drug on other pathways, including tyrosine kinases, K-ras, v-Src, and others (14, 27). Although VEGF and 20-HETE share some pathways in activation of eNOS and pulmonary vascular relaxation, they clearly have distinct effects on some endpoints as well, given the divergent response of pulmonary arteries pretreated with radicicol to these two activators.

Phosphorylation of eNOS at serine 1179/1177 activates the enzyme [reviewed by Fulton et al. (23) and Boo and Jo (6)]. Phosphorylation has been reported at a number of other amino acid locations, some activating and some inactivating eNOS (23). Phosphorylation at serine 1179 can be catalyzed by at least five kinases (7), Akt (17, 18, 22, 26, 33, 34), protein kinase A (8, 12, 34), protein kinase G (12), AMP-activated protein kinase (15), and CaM kinase II (19). The best-documented kinase of these is Akt or PKB, which is activated by the PI3-kinase pathway. When PI3-kinase is inhibited by the pharmacological agents wortmannin or LY-298004, agonists like VEGF or insulin do not generate increased release of NO in human umbilical vein endothelial cells (HUVECs) and other endothelial cells (37, 41). The kinase Akt is recruited from the cytoplasm to the plasma membrane after activation of PI3-kinase by growth factors via interaction with the pleckstrin homology domain of the enzyme. Akt is then phosphorylated at threonine 308 and serine 473 by 3-phosphoinositide-dependent protein kinase-1 (1–4). Phosphorylated Akt has been reported to mediate increase of phosphoserine 1179. Therefore, our observations (Figs. 4 and 5) of enhanced phosphorylation of Akt after treatments of BPAECs with VEGF as well as 20-HETE are consistent with this pathway affording one of the mechanisms by which these agonists phosphorylate and acti-

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Fig. 6. Effect of 20-HETE on interaction of eNOS with Hsp90 in BPAECs. A: eNOS protein was immunoprecipitated (IP) from lysates of BPAECs treated with vehicle or 20-HETE (1 μM) for 10 min. Immunoprecipitated proteins were electrophoresed and examined by Western analysis for Hsp90 that is bound to eNOS (top). Bottom: corresponding intensities of bands immunoblotted for eNOS in the same gel. B: average relative intensities of Hsp90 signal from both lanes on the same blot were compared with corresponding values for eNOS. Because all three signaling events seen after VEGF-mediated stimulation of bovine pulmonary arteries are similar to those measured using 20-HETE, these results support our hypothesis that VEGF-induced dilatation of bovine pulmonary arteries is mediated in part by CYP4/20-HETE.

Fig. 7. Effect of VEGF on interaction of eNOS with Hsp90 in BPAECs. A: eNOS protein was immunoprecipitated (IP) from lysates of BPAECs treated with vehicle or VEGF (8.3 nM) for 10 min. Immunoprecipitated proteins were electrophoresed and examined by Western analysis for Hsp90 that binds to eNOS (top). Bottom: corresponding intensities of bands immunoblotted (IB) for eNOS. B: average relative intensities of Hsp90 signal from both lanes on the same blot were compared with corresponding values for eNOS (plotted on y-axis). Graph depicts no difference in the relative amounts of protein partner Hsp90 interacting with eNOS after repeated independent treatments with vehicle vs. VEGF (n = 8; P = 0.105, indicating no statistical difference between the 2 bars).
vate eNOS. Importantly, inhibition of both VEGF- and 20-HETE-evoked decreases in pulmonary artery tone by the Akt inhibitor wortmannin supports a functional role for this signaling pathway to stimulate NO release and relaxation in these vessels.

It is well documented that VEGF increases association of Hsp90 and eNOS in endothelial cells from some vascular beds (25, 26). Stimulation of BAECs with VEGF in our hands also increased association of eNOS with protein partner Hsp90 over twofold (Fig. 8, A and B). However, neither VEGF (1–10 nM) nor 20-HETE (1 μM) enhanced eNOS-to-Hsp90 association in BPAECs with the same antibodies, conditions, buffers, immunoprecipitation protocols, and Western blotting techniques. In fact, 20-HETE decreased the association of eNOS and Hsp90 by ~24%, whereas VEGF had no significant effect. Most reports of activation of NO are associated with increased interaction of Hsp90 and eNOS (25), although NO can be generated in vitro in the absence of Hsp90 (20). The amount of NO released as assessed by chemiluminescent analysis (≈90 pmol of NO2/mg protein in unstimulated BPAECs) in the companion study (31) is similar to that generated in other cultured endothelial cells (20). Likewise, basal levels of Hsp90 interacting with eNOS in unstimulated BPAECs and BAECs does not seem to be very different (as determined by Western analysis). Because Hsp90 is abundant in most cells, it is not likely that access to this protein may be limiting. We speculate that there are alternate signaling pathways in BPAECs compared with BAECs to account for the differences in association of the two proteins after stimulation with VEGF. In addition to suggesting that Hsp90 association with eNOS is not a major mechanism of regulation of NO release in BPAECs, the difference in VEGF- and 20-HETE-associated relaxation of pulmonary arteries after treatment with the Hsp90 inhibitor radicicol supports the position that not all effects of VEGF on pulmonary vascular tone are mediated through 20-HETE.

One other example of increased eNOS activity that is not accompanied by increased binding of the protein partner Hsp90 is the initial burst of NO released by HUVECs in the first 30 min of exposure of cells to shear stress (7, 8). A number of mechanisms contribute to shear-induced activation of eNOS in HUVECs, including dissociation of eNOS from caveolin, increased association with Ca2+/CaM, G protein activation, increased phosphorylation of serine 617 by Akt, as well as increased phosphorylation of serine 1179 by protein kinase A to result in activation of eNOS, but not increased association of eNOS with Hsp90. This 30-min phase is followed by a long-lasting activation phase during which binding of Hsp90 is found to slightly increase until the reaction peaks at 60 min (7, 8). In our experiments, we also observed two molecular signaling events that activate eNOS (phosphorylation of eNOS and Akt), whereas its binding with Hsp90 does not seem to increase in BPAECs stimulated with VEGF or 20-HETE. Clearly, Hsp90 is associated with eNOS in BPAECs, but the
protein interaction is not increased after stimulation with either VEGF or 20-HETE within the 10-min time frame that we investigated. We cannot exclude a delayed effect of either 20-HETE or VEGF on this same endpoint.

In summary, this study describes evidence that VEGF-induced NO release is mediated by CYP4 enzymes that catalyze formation of the lipid 20-HETE. We also describe a set of molecular events that accompany activation of eNOS by vasoactive ligands in BPAECs, which include phosphorylation of Akt and eNOS but not increased association of Hsp90 with eNOS. It is also the first study to our knowledge that describes any molecular events for activation of eNOS by 20-HETE. The results support our observations that vasodilation by VEGF is partially mediated by activation of CYP4 in the bovine pulmonary arteries, through activation of eNOS, as evidenced by phosphorylation at serine 1179 and Akt phosphorylation. Further studies are required to enhance our understanding of this mechanism and to define steps in the pathway that lead to activation of CYP4 enzymes by stimulation of bovine pulmonary endothelium with VEGF.

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