Platelet-Endothelial Cell Adhesion Molecule-1 Regulates Endothelial NO Synthase Activity and Localization Through Signal Transducers and Activators of Transcription 3–Dependent NOSTRIN Expression

Margaret E. McCormick, Reema Goel, David Fulton, Stefanie Oess, Debra Newman, Ellie Tzima

Background—NO produced by the endothelial NO synthase (eNOS) is an important regulator of cardiovascular physiological and pathological features. eNOS is activated by numerous stimuli, and its activity is tightly regulated. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) has been implicated in regulating eNOS activity in response to shear stress. The current study was conducted to determine the role of PECAM-1 in the regulation of basal eNOS activity.

Methods and Results—We demonstrate that PECAM-1–knockout ECs have increased basal eNOS activity and NO production. Mechanistically, increased eNOS activity is associated with a decrease in the inhibitory interaction of eNOS with caveolin-1, impaired subcellular localization of eNOS, and decreased eNOS traffic inducer (NOSTRIN) expression in the absence of PECAM-1. Furthermore, we demonstrate that activation of blunted signal transducers and activators of transcription 3 (STAT3) in the absence of PECAM-1 results in decreased NOSTRIN expression via direct binding of the signal transducers and activators of transcription 3 to the NOSTRIN promoter.

Conclusion—Our results reveal an elegant mechanism of eNOS regulation by PECAM-1 through signal transducers and activators of transcription 3–mediated transcriptional control of NOSTRIN. (Arterioscler Thromb Vasc Biol. 2011;31:643-649.)

Key Words: endothelial function ■ NO ■ NO synthase ■ NOSTRIN ■ PECAM-1

The production of NO is critical for cardiovascular homeostasis because NO regulates many fundamental cellular processes, including regulation of vessel tone, cell proliferation, and angiogenesis.1 In the vascular endothelium, NO is synthesized from l-arginine by the constitutively expressed endothelial NO synthase (eNOS or NOS3) enzyme. eNOS function is critical because genetic deletion of eNOS results in increased blood pressure,2,3 impaired angiogenesis,4 abnormal vascular remodeling, and accelerated atherosclerosis.4 Numerous stimuli promote the activation of eNOS through phosphorylation of serine 1179, leading to NO production. Of note, platelet-endothelial cell adhesion molecule-1 (PECAM-1) regulates eNOS activation in vitro and in vivo, possibly via a direct interaction between PECAM-1 and eNOS.5,6 However, the specifics of this interaction are not known.

eNOS is regulated by multiple interdependent control mechanisms, including posttranslational lipid modifications, phosphorylation, localization, and protein-protein interactions.7–9 Together, these regulatory mechanisms ensure proper responses to diverse stimuli. eNOS is basically repressed through its interaction with an integral membrane protein, caveolin-1, that inhibits NO production.10 In addition, studies10,11 show that trafficking and proper subcellular localization of eNOS are also critical for regulation of its activity. There are 3 pools of eNOS located within the cell: (1) the perinuclear Golgi complex, (2) the plasma membrane (primarily in caveolae), and (3) a cytosolic compartment. eNOS can traffic between these compartments, and subcellular targeting can affect NO production in response to various stimuli.12,13 Recent studies have identified several eNOS trafficking proteins, including an eNOS-interacting protein (NOSIP)14 and eNOS traffic inducer (NOSTRIN).12 NOSTRIN is expressed in ECs both in vitro and in vivo12,15 and regulates eNOS trafficking and localization.12,15,16 Although proteins that influence localization have been identified, the mechanisms that control eNOS trafficking and the (patho)physiological consequences are not fully understood.

Herein, we investigate the role of PECAM-1 in the regulation of basal eNOS activity. We reveal an elegant mechanism of eNOS regulation through transcriptional control of NOSTRIN expression.
Methods

Animals
PECAM-1−/− C57BL/6 mice were provided by P. Newman, PhD, (Blood Research Institute, BloodCenter of Wisconsin, Milwaukee), and eNOS-green fluorescent protein–transgenic mice were provided by Kini de Crom, PhD, (Erasmus University Medical Center, Rotterdam, the Netherlands). All mice were bred in-house and used in accordance with the guidelines of the National Institutes of Health and the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Mice aged 12 to 16 weeks were used for all experiments.

Cell Culture and Reagents
PECAM-1–knockout (PE-KO) cells and cells reconstituted with murine full-length PECAM-1 (PE-RCs) were prepared as previously described.17 Human umbilical vein ECs (Lonza, Walkersville, MD) were grown in M199 media supplemented with an EGM2 bullet kit. Cucurbitacin I was purchased from Tocris Bioscience, Ellsville, MO. Antibodies to phosphorylated serine 1179 eNOS and phosphorylated tyrosine signal transducers and activators of transcription 3 (STAT3) were obtained from Cell Signaling, Danvers, MA; total STAT3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; and total GM130 were obtained from BD Transduction Laboratories, San Diego, CA; total GAPDH, Millipore, Temecula, CA.

Immunoprecipitation and Western Blotting
Cells were harvested in lysis buffer, as previously described.18 Lysates were assayed by Western blot. For immunoprecipitation, cells were harvested in octyl glucoside (OG) buffer plus protease inhibitors.19 Equivalent volumes of lysate were precleared with beads (Protein A/G Plus-Agarose beads; Santa Cruz Biotechnology, Inc) for 1 hour at 4°C. Supernatants were then incubated with beads previously conjugated with anti–rabbit caveolin-1 (BD Transduction Laboratories). Complexes were washed 3 times with OG buffer and then assayed by Western blot.

Immunofluorescence
PE-RC and PE-KO cells were fixed and permeabilized in 3.7% formaldehyde with 0.2% Triton X-100 for 30 minutes at 37°C and then blocked in 1% BSA in PBS for 30 minutes at 37°C. Cells were stained for total eNOS (BD Transduction Laboratories) and Alexa488-conjugated giantin (Covance, Princeton, NJ). Images were taken using a confocal microscope (Zeiss LSM5 Pascal) with a ×63 oil lens. Colocalization was determined using software (ImageJ) and the thresholded Manders colocalization coefficient.20 Aorta en face preparations were prepared as previously described.21 En face preparations were evaluated with a microscope (Zeiss LSM5 Pascal).

Cellular Fractionation
Confluent PE-RC and PE-KO cells were fractionated as previously described.22 The samples (25 μL) were run on 4% to 12% SDS-PAGE gels and then transferred to nitrocellulose membrane for Western blot analysis. Membranes were probed with total eNOS, GM130 as a Golgi marker, and caveolin-1 as the plasma membrane marker.

In Vitro and In Vivo NO Measurements
Cells were serum starved for 24 hours and then treated with either 100 mmol/L L-NAME, 1 μmol/L ionomycin, or 1-NAME plus ionomycin. Media NO levels were evaluated using a reagent (Griess, NO Assay kit; R&D Systems). PECAM-1−/− and PECAM-1−/+ animals were anesthetized using intraperitoneal injections of ketamine (100 mg/kg) and xylazine (15 mg/kg) and injected with 100 U of heparin. Plasma was isolated for NO measurements, which were performed using an NO analyzer (Sievers Instruments), as previously described.23

RNA Isolation and Quantitative PCR
Total RNA was isolated from a confluent cell monolayer using a reagent (TRIZol; Invitrogen), and first-strand cDNA was transcribed using random primers and transcriptase (SuperScript II Reverse Transcriptase; Invitrogen). A real-time quantitative PCR was performed using a mix (ABsolute SYBR Green ROX mix; Thermo Scientific). Relative levels of gene expression were normalized to mouse 18s expression using the comparative cycle threshold (Ct) method.

Chromatin Immunoprecipitation Assays
Chromatin immunoprecipitation was performed using the fast chromatin immunoprecipitation method described by Nelson et al24 with a rabbit anti–STAT3 K15 antibody (Santa Cruz Biotechnology, Inc). Quantitative PCR was performed using primers specific for the NOSTRIN promoter.

Quantification and Statistical Analysis
The band intensity of immunoblots was quantified using computer software (ImageJ). Each experimental group was analyzed using single-factor analysis of variance using a computer program (Excel; Microsoft). Probability values were obtained by performing a 2-tailed Student t test using the same program (Excel). Statistical significance was defined as P<0.05.

Results

PECAM-1 Regulates Basal eNOS Activity
Previous studies10,25,26 suggest a requirement for PECAM-1 in shear-induced eNOS activation. Our own results are in agreement with these observations (data not shown). Unexpectedly, our data revealed increased levels of phosphorylated eNOS in PE-KO cells compared with PE-RC cells (Figure 1A). However, PECAM-1 deletion does not affect total eNOS expression.25 To evaluate the functional consequences of increased basal eNOS phosphorylation in the PE-KO cells, we measured NO production. As shown in Figure 1B, there is a 3-fold increase in basal NO production in PE-KO cells compared with PE-RC cells. Stimulation with ionomycin increased NO production in both cell types, whereas L-NAME inhibited the production of NO. To determine whether these observations are corroborated in whole animals, we measured NO levels from plasma. Indeed, plasma NO levels were higher in PECAM-1−/− compared with PECAM-1−/+ animals (Figure 1C).

To further investigate the role of PECAM-1 in regulation of basal eNOS phosphorylation, we used small-interfering RNA to knock down PECAM-1 expression in human umbilical vein ECs. The degree of knockdown was assessed by Western blot (supplemental Figure IA; available online at http://atvb.ahajournals.org). Interestingly, we observed an increase in both basal eNOS phosphorylation (supplemental Figure IB) and NO production (supplemental Figure IC) in PECAM-1–small-interfering RNA–infected ECs compared with nonspecific small-interfering RNA control cells. Together, these data suggest that the absence of PECAM-1 results in higher basal eNOS activity and NO production.

Differential Association of eNOS and Caveolin-1 in the Absence of PECAM-1
Next, we wanted to address the mechanism(s) responsible for the increased basal eNOS activity in the absence of PECAM-1. To determine whether PECAM-1 affects the
interaction between eNOS and known regulatory proteins, we performed coimmunoprecipitation assays. The caveolar scaffolding protein, caveolin-1, regulates eNOS activity, primarily as an inhibitor of basal enzyme function.10,19,27 Caveolin-1 is able to bind eNOS and block the calmodulin-binding site important for enzyme activation.28 To determine whether PECAM-1 affects the association of eNOS with caveolin-1, we performed coimmunoprecipitation assays. As shown in Figure 2, there was a decrease in basal eNOS–caveolin-1 association in the PE-KO cells compared with the PE-RC cells. In addition, consistent with the immunoprecipitation data, immunofluorescence confocal analysis revealed a slight decrease in the colocalization of eNOS and caveolin-1 in the absence of PECAM-1 (data not shown). These data suggest that PECAM-1 may regulate basal eNOS activity by promoting the association of eNOS with caveolin-1.

**Role of PECAM-1 in eNOS Localization/Trafficking**

Subcellular localization of eNOS influences its activation.11,13,23,29 To determine whether PECAM-1 influences eNOS localization, we performed double-labeling immunofluorescence confocal microscopy in PE-RC and PE-KO cells. Cells were stained for total eNOS and giantin, a membrane-inserted component of the cis– and medial Golgi complex. In PECAM-1–expressing cells, eNOS is found at both the perinuclear/Golgi complex and the plasma membrane (Figure 3A), consistent with the localization described in blood vessels in vivo.30 In contrast, in cells that lack PECAM-1, eNOS is redistributed away from the perinuclear region (Figure 3A). Indeed, quantitative colocalization analysis (Manders colocalization coefficient) showed decreased colocalization of eNOS with giantin in the absence of PECAM-1 (Figure 3A). Together, these data suggest that PECAM-1 influences the subcellular localization of eNOS.

To further support our in vitro findings, we isolated aortas from PECAM-1+/+ and PECAM-1−/− mice expressing an eNOS-GFP fusion protein. The aortas were stained en face for giantin and visualized using confocal microscopy (Figure 3B). Similar to the PE-KO cells, quantitative colocalization analysis revealed a significant redistribution of eNOS away from the Golgi complex in the PECAM-1−/− aortas.

As a complementary approach, we used sodium carbonate extraction of cells, followed by a discontinuous sucrose gradient. In this procedure, cholesterol-rich microdomains, including lipid rafts and caveolae, float as buoyant membranes at the 5% to 30% sucrose interface (fractions 3 to 4), whereas soluble proteins and heavy membranes remain at the bottom of the gradient (fractions 9 to 11). In all gradients, the distributions of caveolin-1 and GM130, a Golgi marker, were examined to confirm adequate separation of the fractions (Figure 3C). In PECAM-1–expressing ECs, eNOS distributed primarily into 2 distinct pools: light membranes highly enriched in caveolin-1 and heavy membranes enriched in GM130. In PE-KO cells, eNOS is also distributed into 2 pools. However, we noticed a significant redistribution of eNOS out of the Golgi-enriched fractions (fractions 10 to 11). These observations are consistent with the immunofluorescence data previously described. Notably, PE-KO cells have increased eNOS activity and NO levels despite increased expression levels of the negative regulator caveolin-1 (data not shown).
PECAM-1 Mediates STAT3-Dependent NOSTRIN Expression in ECs

eNOS localization is a dynamic and well-coordinated process, mediated by several players, including dynamin-2 and NOSTRIN.9 To determine whether PECAM-1 mediates eNOS localization through NOSTRIN, we measured NOSTRIN protein expression in PE-RC and PE-KO cells. Unexpectedly, we observed a significant decrease in NOSTRIN protein expression in PE-KO cells (Figure 4A). Reduced NOSTRIN expression in PE-KO cells could be because of either increased protein degradation or a reduction in mRNA levels. To distinguish between these possible mechanisms, we first measured ubiquitination of NOSTRIN but did not observe a difference between the PE-RC and PE-KO cells (data not shown). Next, we used quantitative real-time PCR to determine the effect of PECAM-1 deletion on NOSTRIN mRNA levels. Our data demonstrate that NOSTRIN mRNA is significantly decreased in the absence of PECAM-1 (Figure 4B).

Next, we addressed the mechanism by which PECAM-1 affects NOSTRIN protein expression. Previously, it was shown that the cytoplasmic tail of PECAM-1 is able to function as a scaffold for numerous signaling pathways, including STAT protein family members STAT5 and STAT3.31,32 In addition, ECs isolated from PECAM-1−/− mice have reduced STAT3 phosphorylation.32 We asked whether the reduced levels of active STAT3 in the PE-KO cells might result in decreased NOSTRIN expression. To determine whether STAT3 phosphorylation is reduced in cells that lack PECAM-1, we probed PE-KO cell extracts for total STAT3 and phosphorylated STAT3. More important, our data are in agreement with previous reports showing that phosphorylated STAT3 levels are lower in the absence of PECAM-1 (Figure 5A). To determine whether STAT3 activity affects NOSTRIN expression, we treated the PECAM-1-expressing ECs with cucurbitacin, a selective inhibitor of STAT3/janus kinase signaling, and assayed for changes in NOSTRIN expression by Western blot and quantitative real-time PCR. Interestingly, cucurbitacin treatment results in a dose-dependent decrease in both NOSTRIN protein expression (Figure 5B) and mRNA levels (Figure 5C). These results suggest that PECAM-1 mediates STAT3-induced NOSTRIN expression, which, in turn, regulates eNOS localization.

 Binding of STAT3 to the NOSTRIN Promoter in ECs

Analysis of the NOSTRIN promoter revealed several putative STAT3 binding sites (supplemental Table). The STAT family members bind to a conserved sequence of TT and AA duplicates typically separated by 5 bases. In addition to binding TT(N5)AA nanomers, STAT3 is also able to bind octomers and decamers.33,34 Next, we investigated the possibility that STAT3 directly binds to the NOSTRIN promoter to...
modulate mRNA expression by chromatin immunoprecipitation assays with STAT3 antibodies. Immunoprecipitation of the chromatin lysates was followed by PCR with NOSTRIN promoter primers; NOSTRIN exon 10 primers served as a control. Chromatin immunoprecipitation assays show that STAT3 binds to the NOSTRIN promoter region but not the NOSTRIN coding region (Figure 5D). Together, these data suggest that STAT3 may regulate NOSTRIN mRNA levels by specifically binding to the NOSTRIN promoter.

Discussion

Understanding the mechanisms that regulate NO production in the endothelium can provide important insight into processes that initiate endothelial dysfunction and lead to the development of atherosclerosis. NO production is dynamically regulated by several humoral and mechanical factors. In this study, we investigated PECAM-1 regulation of basal eNOS activity. An unexpected finding of this study was the increased basal eNOS activity and NO production in PE-KO cells and PECAM-1−/− mice. An attractive hypothesis for explaining the basal regulation of eNOS by PECAM-1 is their reported physical association. However, reports of this interaction have been highly conflicting. One group showed that shear stress induces a transient increase in the association of PECAM-1 and eNOS, whereas other studies have shown just the opposite. Our own results suggest only a weak basal association of eNOS and PECAM-1 in static ECs (data not shown). In addition, ultrastructural and biochemical analyses suggest that eNOS resides within caveolae, whereas PECAM-1 is found at a membrane network just below the plasmalemma at the cell border that is distinct from caveolae. Furthermore, studies in human umbilical vein ECs have shown no colocalization between PECAM-1 and caveolin-1 and that these 2 proteins do not comigrate on sucrose gels. The lack of physical association between eNOS and PECAM-1 led us to investigate differences in eNOS protein interactions and localization as possible mechanisms of regulation.

It is well recognized that correct subcellular targeting of eNOS is critical for proper regulation of its activity and NO bioavailability; thus, tight control of eNOS targeting to different compartments appears to be essential. In this regard, our data point toward a requirement for tightly regulated levels of NOSTRIN expression within ECs. Overexpression of NOSTRIN can promote the translocation of eNOS from the plasma membrane to intracellular vesicles, with a concomitant reduction in eNOS enzyme activity. Conversely, decreased NOSTRIN expression also influences eNOS subcellular localization and may contribute to the increased NO levels observed in the PE-KO cells. Interestingly, overexpression of the eNOS-binding partner, caveolin-1, leads to accelerated atherosclerosis formation in mice, partially through reduced NO production; persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension. Thus, tight regulation of eNOS regulatory protein levels, including NOSTRIN and caveolin-1 expression, is required for proper eNOS function.

Herein, we present exciting data to support a novel mechanism of eNOS regulation by PECAM-1. Our current working model is summarized in Figure 6. The cytoplasmic tail of
PECAM-1 acts as a scaffold for STAT3 and mediates its activation. A possible candidate for the activation of STAT3 is the Src family kinases because Src-mediated STAT activation has been previously reported. After activation, STAT3 dimerizes and translocates into the nucleus, where it modulates the expression of gene targets, including NOSTRIN. Once expressed, NOSTRIN facilitates eNOS trafficking and its correct subcellular localization. It is possible that the reduced NOSTRIN levels account for the difference in eNOS–caveolin-1 association; however, this could be because of other undetermined mechanisms. Interestingly, eNOS, caveolin-1, and NOSTRIN form a ternary complex to facilitate eNOS translocation. In addition, our data are consistent with the hypothesis that NOSTRIN might serve to stabilize the inhibitory effect of caveolin-1 on eNOS.

The signaling pathway identified herein relates to the regulation of basal eNOS activity via PECAM-1. However, PECAM-1 is known to regulate eNOS activation in response to the physiological stimulus of shear stress. PE-KO cells are unable to activate eNOS in response to shear stress, yet they activate eNOS in response to ionomycin, indicating a specific requirement for PECAM-1 in flow-induced eNOS activation (Figure 1B and supplemental Figure II). PECAM-1 is also required for flow-induced activation of Akt and Src, 2 important upstream mediators of eNOS activity. The role of shear stress in NOSTRIN-mediated eNOS regulation is being investigated.

Blood flow and the NO signaling pathway are both known modulators of cardiovascular development and physiological features. Recent studies have provided compelling evidence for an evolutionarily conserved shear stress– and NO-mediated pathway that also regulates hematopoiesis. PECAM-1 is thought to be involved in flow mechanosensing, based on in vitro and in vivo experiments showing PECAM-1–dependent activation of flow-mediated intracellular signaling pathways and vascular remodeling. Interestingly, PECAM-1 is required for NO-mediated dilation in response to shear stress in isolated skeletal muscle arterioles and in the mouse coronary circulation, thus underscoring the importance of both PECAM-1 and NO in flow-mediated remodeling. Previous studies have also identified the importance of eNOS in flow-mediated remodeling. We reveal a sophisticated dual mode of eNOS regulation by PECAM-1. Although PECAM-1–/- ECs are unable to activate eNOS in response to shear stress, their basal eNOS activity and NO levels are, paradoxically, increased through STAT3-mediated transcriptional control of NOSTRIN.

Acknowledgments

We thank Bob Bagnall, PhD, for help with quantitative colocalization analysis; Tina van Italie, PhD, for cell fractionation experiments; Zhongming Chen, PhD, for help with vessel isolation; and Tzima laboratory colleagues for critical reading of the manuscript.

Sources of Funding

This study was supported in part by grants T32 HL069768 (Dr McCormick) and HL088632 (Dr Tzima) from the National Institutes of Health; and predoctoral fellowship 4290007 (M. McCormick) from the American Heart Association. Dr Tzima is an Ellison Medical Foundation New Scholar.

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2011;31:643-649; originally published online December 23, 2010;
doi: 10.1161/ATVBAHA.110.216200
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

Cellular Fractionation

Cells were washed twice with cold PBS then scraped into 2 ml of PBS and centrifuged at 1000 rpm for 3 minutes to pellet cells. The PBS was removed and the cells were resuspended in 750 μl of 500mmol/L sodium carbonate, pH 11. The cells were dounce-homogenized (25 strokes) on ice and sonicated at 50% power with 5 10-second bursts. The homogenate was adjusted to 42.5% sucrose using a 58% sucrose solution prepared in MBS (25mmol/L MES, pH 6.5, 0.15mol/L NaCl). 500 μl of homogenate was placed at the bottom of an ultracentrifuge tube then 1 ml of 30% sucrose was added followed by 600ul of 5% sucrose in MBS. The samples were centrifuged at 48,000 rpm for 3 hours at 4°C in using the SW50 Rotor (Beckman). Aliquots of 200ul were taken starting from the top (for a total of 12 samples) and added to 50ul 10X LSB. The samples (25ul) were run on 4-12% SDS-PAGE gels then transferred to nitrocellulose membrane for Western blot analysis. Membranes were probed with total eNOS, GM130 as Golgi marker and caveolin-1 as the plasma membrane marker.

En Face Staining

Aortas were perfusion-fixed and dissected out under a dissection microscope. The aorta was trimmed of fat and excess tissue then cut longitudinally and permeabilized for 3 hours in 0.2% Tx100. Tissue was blocked in PBT (1% BSA, 0.1% Tween-20 in PBS) overnight at 4°C. Tissues were then placed on slides with the endothelium facing up, covered with Vectashield plus DAPI and mounted. Samples were incubated for 120 minutes at room temperature in Giantin antibody (1:50, Covance), then incubated for 60 minutes in secondary antibody at room temperature.

RNA Isolation and Quantitative PCR

Total RNA was isolated from a confluent cell monolayer using the TRIzol reagent (Invitrogen) and DNase treatment was performed using DNA-free (Ambion). First-strand cDNA was transcribed using random
primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using ABsolute SYBR Green ROX mix (Thermo Scientific). Relative levels of gene expression were normalized to mouse 18s expression using the comparative Ct method. Primer sequences are listed in Supplemental Table I.

**Chromatin Immunoprecipitation**

Chromatin-containing lysates were incubated with rabbit anti-STAT3 K15 (Santa Cruz Biotechnology, Inc) for 30 minutes at 4°C in an ultrasonicator bath. DNA-protein complexes were precipitated using Protein A/G agarose beads and treated with proteinase K. DNA samples were extracted using 10% wt/vol Chelex (Bio-Rad) and PCR amplified using primers specific for the NOSTRIN promoter (sequences listed in Supplemental Table I).
### Supplemental Table I. PCR and ChIP Primers

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<td>18s Rev</td>
<td>5’-CCTGCTGCCTTCCTTGGA-3’</td>
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<td>NOSTRIN Exon 10 Fwd</td>
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<td>NOSTRIN Exon 10 Rev</td>
<td>5’-AGACTTCCGTCGCTCTTTGTCC-3’</td>
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<td>NOSTRIN Promoter Fwd</td>
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<td>NOSTRIN Promoter Rev</td>
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Supplemental Table II. Predicted STAT binding sites within the NOSTRIN promoter

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Supplemental Figure I. PECAM-1 knockdown in HUVECs. A, HUVECs were treated with lentivirus expressing PECAM-1 siRNA (PECO2) or a non-specific control (NSC). Levels of knockdown were assayed by Western blot for PECAM-1 and tubulin. B, Lentivirus-treated HUVECs were assayed for pSer1179eNOS by Western blot (n=3; P<0.05 vs HUVEC NSC). C, NO levels in PECAM-1 knockdown HUVECS was assessed by DAF-2t levels.
**Supplemental Figure II.** PECAM-1 is not required for ionomycin-induced eNOS activation. PE-RC and PE-KO cells were stimulated with 1μM ionomycin and eNOS activation was assayed by Western blot. A Representative blot is shown (n=3, *P<0.01 vs PE-RC 0 min).