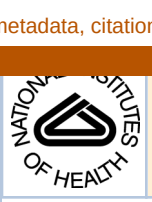

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PECAM-1 regulates eNOS activity and localization through STAT3-dependent NOSTRIN expression

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Abstract

Objective—Nitric oxide (NO) produced by the endothelial NO synthase (eNOS) is an important regulator of cardiovascular physiology and pathology. 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 goal of the current study is 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 NOSTRIN expression in the absence of PECAM-1. Furthermore, we demonstrate that blunted STAT3 activation in the absence of PECAM-1 results in decreased NOSTRIN expression via direct binding of STAT3 to the NOSTRIN promoter.

Conclusions—Taken together, our results reveal an elegant mechanism of eNOS regulation by PECAM-1 through STAT3-mediated transcriptional control of NOSTRIN.

Keywords

endothelial cell; eNOS - PECAM-1 - STAT3; NOSTRIN

The production of NO is critical for cardiovascular homeostasis as 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 nitric oxide synthase (eNOS or NOS3) enzyme. eNOS function is critical, as genetic deletion of eNOS results in increased blood pressure[2, 3], impaired angiogenesis, abnormal vascular remodeling, and accelerated atherosclerosis[4].

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Disclosures

None.

Numerous stimuli promote the activation of eNOS through phosphorylation of serine residue 1179, leading to NO production. In this regard, it has been shown that the cell adhesion molecule 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 post-translational lipid modifications, phosphorylation, localization and protein-protein interactions[7–9]. Together, these regulatory mechanisms ensure proper responses to diverse stimuli. eNOS is basally repressed through its interaction with an integral membrane protein, caveolin-1, which inhibits NO production[10]. In addition, studies show that trafficking and proper subcellular localization of eNOS are also critical for regulation of its activity[10, 11]. There are three pools of eNOS located within the cell: (1) the perinuclear Golgi complex; (2) the plasma membrane (primarily in caveolae); and (3) a cytosolic compartment. It is increasingly appreciated that eNOS cantraffic between these compartments and that subcellular targeting can affect NO production in response to various stimuli[12, 13]. Recent studies have identified several eNOS trafficking proteins including NOSIP (eNOS interacting protein)[14] and NOSTRIN (eNOS traffic inducer)[12]. NOSTRIN is expressed in ECs both *in vitro* and *in vivo*[12, 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)physiologic consequences are not fully understood.

Here, 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 kindly provided by Dr P. Newman (Blood Research Institute, BloodCenter of Wisconsin, Milwaukee) and eNOS-GFP transgenic mice were kindly provided by Rini de Crom (Erasmus University Medical Center, Rotterdam, The Netherlands). All mice were bred in house and used in accordance with the guidelines of the National Institute 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 12–16 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-RC) were prepared as described[17]. HUVECs (Lonza) were grown in M199 media supplemented with EGM2 bullet kit. Cucurbitacin I was purchased from Tocris Bioscience (Ellsville, Missouri). Antibodies to phospho-Ser1179 eNOS and phospho-Tyr STAT3 were from Cell Signaling (Danvers, MA). Anti-GM130 was from BD Biosciences (Transduction, San Diego) and total STAT3 (Santa Cruz, CA) and total GAPDH from 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 OG buffer plus protease inhibitors[19]. Equivalent volumes of lysate were pre-cleared with Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology, Inc) for 1 hour at 4°C. Supernatants were then incubated with Protein A/G beads previously conjugated with anti-rabbit caveolin-1 (BD

Transduction). Complexes were washed three 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 then blocked in 1% BSA in PBS for 30 minutes at 37°C. Cells were stained for total eNOS (BD Transduction) and Alexa488-conjugated Giantin (Covance). Images were taken using either the Zeiss LSM5 Pascal Confocal microscope using a 63X oil lens. Co-localization was determined using ImageJ and the thresholded Mander's colocalization coefficient[20]. Aorta *en face* preparations we prepared as previously described[21]. *En face* preparations were evaluated with a Zeiss LSM5 Pascal microscope.

Cellular Fractionation

Confluent PE-RC and PE-KO cells were fractionated as previously described[22]. 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 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 then treated with either 100mmol/L L-Name, 1μmol/L ionomycin or L-Name plus ionomycin. Media NO levels were evaluated using the Griess reagent (NO Assay kit, R&D Systems). PECAM-1 $^{+/+}$ and $^{-/-}$ animals were anesthetized using intraperitoneal (IP) injections of ketamine (100mg/kg) and xylazine (15mg/kg) and injected with 100U heparin. Plasma was isolated for NO measurements, which were performed using a NO analyzer (Sievers Instruments, Boulder, CO) as previously described[23].

RNA Isolation and Quantitative PCR

Total RNA was isolated from a confluent cell monolayer using the TRIzol reagent (Invitrogen) and 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.

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin immunoprecipitation was performed using the fast ChIP method described by Nelson et al[24] using 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

Band intensity of immunoblots was quantified using the ImageJ program. Each experimental group was analyzed using single factor analysis of variance in Excel (Microsoft). P-values were obtained by performing two-tailed Student's *t* test using Excel. Statistical significance was defined as $P<0.05$.

Results

PECAM-1 regulates basal eNOS activity

Previous studies suggest a requirement for PECAM-1 in shear-induced eNOS activation[10, 25, 26]. Our own results are in agreement with these observations (data not shown). Unexpectedly, our data revealed increased levels of phosphorylated eNOS in PECAM-1 knockout (PE-KO) cells compared to PECAM-1 expressing cells (PE-RC) (Figure 1A). PECAM-1 deletion, however, 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 to PE-RC cells. Stimulation with ionomycin increased NO production in both cell types, whereas L-NAME inhibited the production of NO. To determine if these observations are corroborated in whole animals, we measured NO levels from plasma. Indeed, plasma NO levels were higher in PECAM-$1^{-/-}$ compared to PECAM-$1^{+/+}$ animals (Figure 1C).

To further investigate the role of PECAM-1 in regulation of basal eNOS phosphorylation, we used siRNA to knockdown PECAM-1 expression in human umbilical vein endothelial cells (HUVECs). The degree of knockdown was assessed by Western blot (Supplemental Figure IA). Interestingly, we observed an increase in both basal eNOS phosphorylation (Supplemental Figure IB) and NO production (Supplemental Figure IC) in PECAM-1-siRNA infected ECs compared to non-specific siRNA control cells. Together, these data suggest that 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

We next wanted to address the mechanism(s) responsible for the increased basal eNOS activity in the absence of PECAM-1. To determine if PECAM-1 affects the interaction between eNOS and known regulatory proteins we performed co-immunoprecipitation assays. The caveolar scaffolding protein, caveolin-1, has been shown to regulate 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 if PECAM-1 affects the association of eNOS with caveolin-1, we performed co-immunoprecipitation assays. As shown in Figure 2, there was a decrease in basal eNOS-caveolin-1 association in the PE-KO cells compared to the PE-RC cells. Additionally, consistent with the immunoprecipitation data, immunofluorescence confocal analysis also 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 if 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 (PM) (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 (Mander's 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 $^{-/-}$ 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 utilized 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 5–30% sucrose interface (fractions 3–4), whereas soluble proteins and heavy membranes remain at the bottom of the gradient (fractions 9–11). In all gradients, the distribution 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 two distinct pools: light membranes highly enriched in caveolin-1 and heavy membranes enriched in GM130. In PE-KO cells, eNOS is also distributed into two pools. However, we noticed a significant redistribution of eNOS out of the Golgi-enriched fractions (fractions 10–11). These observations are consistent with the immunofluorescence data described above. Notably, PE-KO cells have increased eNOS activity and NO levels despite increased expression levels of the negative regulator caveolin-1 (not shown).

PECAM-1 mediates STAT3-dependent NOSTRIN expression in ECs

eNOS localization is a dynamic and well-coordinated process, mediated by a number of players including dynamin-2 and NOSTRIN[9]. To determine if 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 due to 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).

We next addressed the mechanism by which PECAM-1 affects NOSTRIN protein expression. It has previously been shown that the cytoplasmic tail of PECAM-1 is able to function as a scaffold for numerous signaling pathways, including signal transducers and activators of transcription (STAT) protein family members STAT5 and STAT3[31, 32]. Additionally, ECs isolated from PECAM-$1^{-/-}$ mice have reduced STAT3 phosphorylation[32]. We asked whether the reduced levels of active STAT3 in the PECAM-1 knockout might result in decreased NOSTRIN expression. To determine if STAT3 phosphorylation is reduced in cells that lack PECAM-1, we probed PE-KO cell extracts for total STAT3 and phospho-STAT3. Importantly, our data are in agreement with previous reports showing that phospho-STAT3 levels are lower in the absence of PECAM-1 (Figure 5A). To determine if STAT3 activity affects NOSTRIN expression, we treated the PECAM-1 expressing ECs with cucurbitacin, a selective inhibitor of STAT3/JAK 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 endothelial cells

Analysis of the NOSTRIN promoter revealed several putative STAT3 binding sites (Supplemental Table I). 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]. We next investigated the possibility that STAT3 directly binds to the NOSTRIN promoter to modulate mRNA expression by chromatin immunoprecipitation (ChIP) 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. ChIP 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 a number of 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 as well as PECAM-$1^{-/-}$ mice. An attractive hypothesis to explain 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[6], while other studies have shown just the opposite[5]. Our own results suggest only a weak basal association of eNOS and PECAM-1 in static ECs (data not shown). In addition, ultra-structural and biochemical analyses suggest that eNOS resides within caveolae[10, 35], whereas PECAM-1 is found at a membrane network just below the plasmalemma at the cell borders that is distinct from caveolae[36]. Furthermore, studies in HUVECs have shown no colocalization between PECAM-1 and caveolin-1 and that these two proteins do not co-migrate on sucrose gels[36]. 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 towards 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[12]. Conversely, decreased NOSTRIN expression also influences eNOS subcellular localization and may contribute to the increased NO levels observed in the PECAM-1 knockout. Interestingly, overexpression of the eNOS-binding partner, caveolin-1, leads to accelerated atherosclerosis formation in mice, partially through reduced NO production[37]; while persistent eNOS activation secondary to caveolin-1 deficiency induces pulmonary hypertension[38]. Thus, tight regulation of eNOS regulatory protein levels, including NOSTRIN and caveolin-1 expression, is required for proper eNOS function.

Here, 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 are the Src-family kinases, as Src-mediated STAT activation has been previously reported[39, 40]. Following activation, STAT3 dimerizes and translocates into

the nucleus where it modulates 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 due to other undetermined mechanisms. Of note, it has been reported that eNOS, caveolin-1 and NOSTRIN form a ternary complex to facilitate eNOS translocation[16]. Additionally, our data are consistent with the hypothesis that NOSTRIN might serve to stabilize the inhibitory effect of caveolin-1 on eNOS[16].

The signaling pathway identified here relates to the regulation of basal eNOS activity via PECAM-1. However, PECAM-1 is also known to regulate eNOS activation in response to the physiologic stimulus of shear stress[5, 6, 25, 41]. 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). It is worth noting here that PECAM-1 is also required for flow-induced activation of Akt and Src, two important upstream mediators of eNOS activity[25, 41]. The role of shear stress in NOSTRIN-mediated eNOS regulation is currently under investigation.

Blood flow and the NO signaling pathway are both known modulators of cardiovascular development and physiology. Two recent studies have provided compelling evidence for an evolutionarily conserved, shear-stress- and NO-mediated pathway that also regulates hematopoiesis[42–44]. PECAM-1is thought to be involved in flow mechanosensing, based on *in vitro* and *in vivo* experiments showing PECAM-1-dependentactivation of flow-mediated intracellular signaling pathways and vascular remodeling[6, 45, 46]. Interestingly, PECAM-1 is required for NO-mediated dilation in response to shear stress in isolated skeletal muscle arterioles[41] as well as in the mouse coronary circulation[47], 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[48]. We now reveal a sophisticated dual mode of eNOS regulation by PECAM-1; while 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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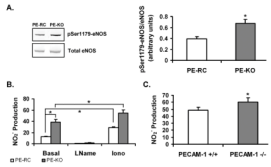

Figure 1.

Basal eNOS phosphorylation and NO production in the PECAM-1 KO. A, PE-KO cells have increased basal peNOS compared to PE-RC (n=6, *P<0.005). B, PE-RC and PE-KO cells were treated with 1mmol/L L-Name or 1μmol/L ionomycin and NO levels measured (n=3, *P<0.01 vs PE-RC basal). C, Plasma levels of NO from PECAM-1 +/+ and −/− mice were assayed as described. (n=7 mice;*P<0.05 vs PECAM-1 +/+).

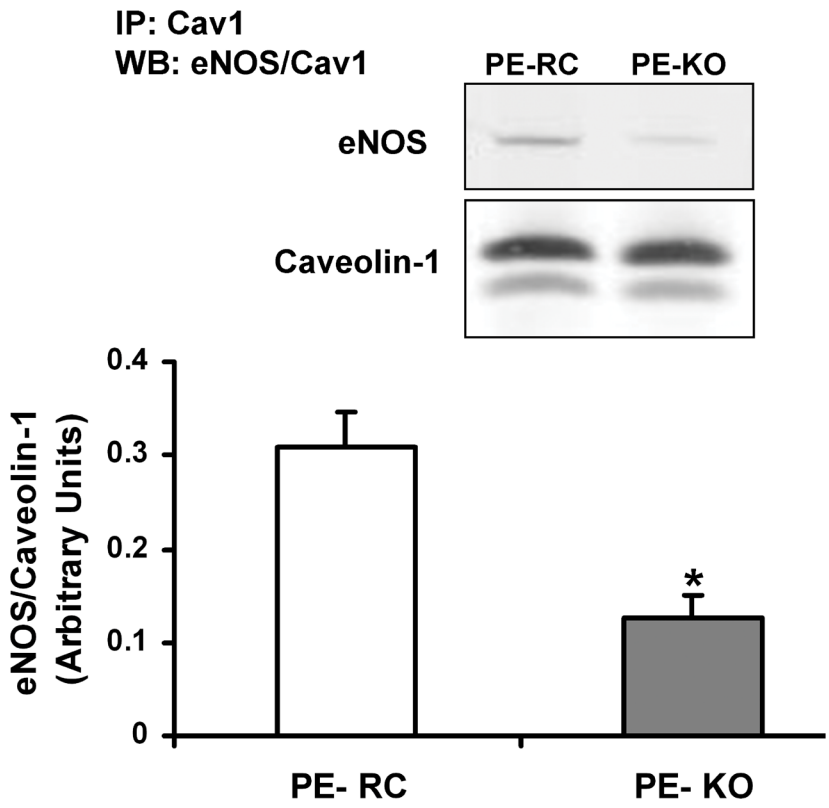

Figure 2.

Differential association of the negative eNOS-binding partner, caveolin-1, in the absence of PECAM-1. PE-RC and PE-KO cells were immunoprecipitated using an antibody against Cav1. Western blots were performed for total eNOS and Cav1. Quantitation is shown on the right (n=6; *P<0.001 vs PE-RC).

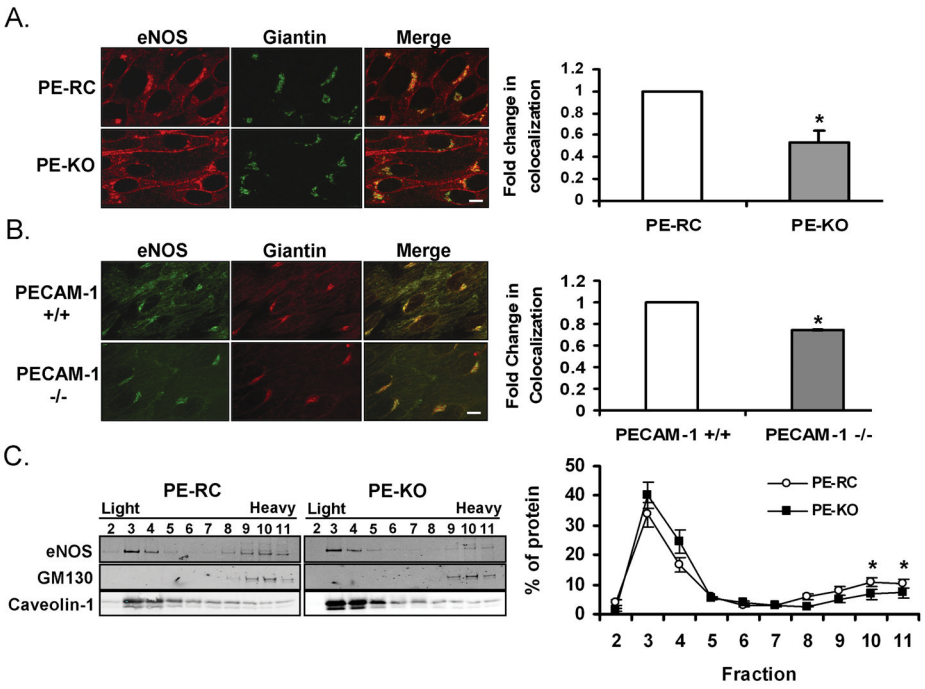

Figure 3.

PECAM-1 regulates eNOS subcellular localization. A, PE-RC and PE-KO cells stained for total eNOS (*red*) and Giantin (*green*). Colocalization was determined using the thresholded Mander's coefficient (n=4, 25 cells/experiment; *P<0.05). B, *En face* aortas from eNOS-GFP/PECAM-1 +/+ or −/− mice stained for Giantin (*red*) (50 cells/genotype; *P<0.05). C, Subcellular fractionation of PE-RC and PE-KO cells. Fractions were immunoblotted for total eNOS, GM130 and Cav-1 (n=3, *P<0.05). Bar = 5μm.

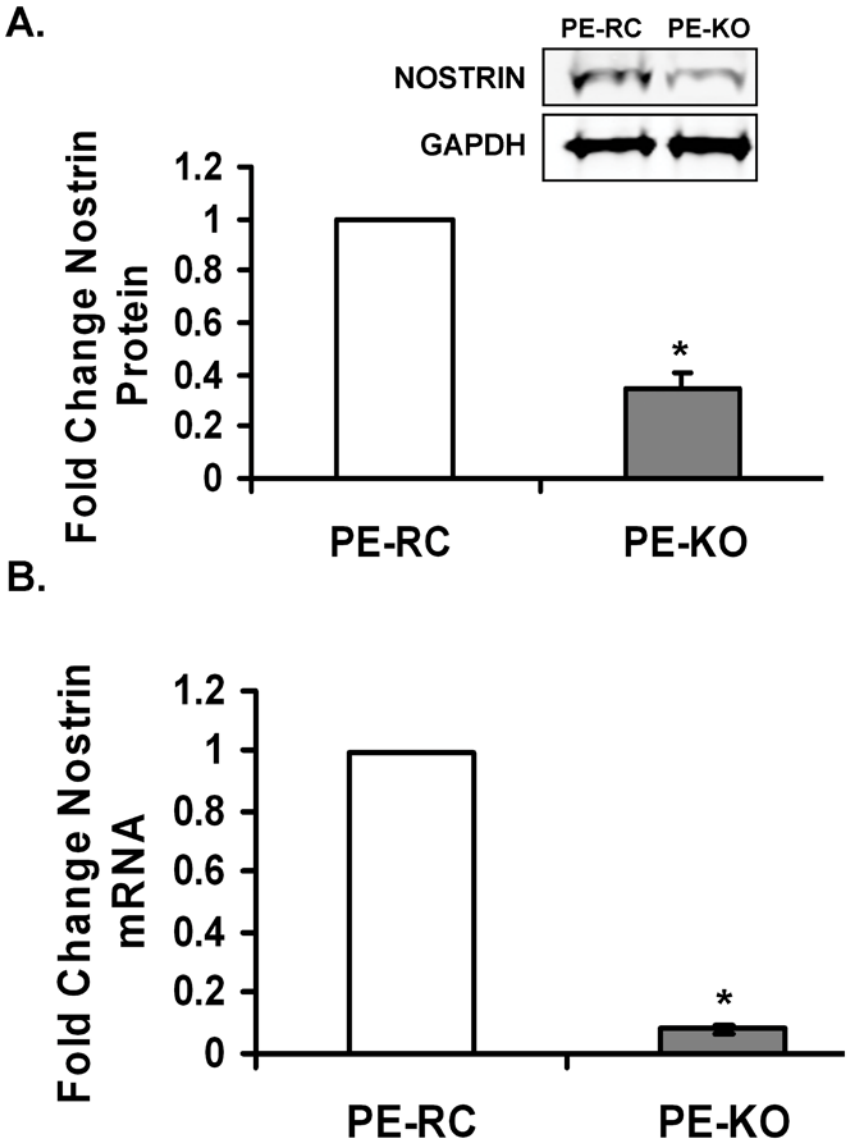

Figure 4.

Reduced NOSTRIN expression in ECs lacking PECAM-1 A, Levels of NOSTRIN protein were measured in PE-RC and PE-KO cells by Western Blot. GAPDH was measured for loading (n=4, *P<0.001 vs PE-RC). B, Real-time PCR analysis revealed decreased NOSTRIN mRNA levels in PE-KO cells (n=5, *P<0.001 vs PE-RC).

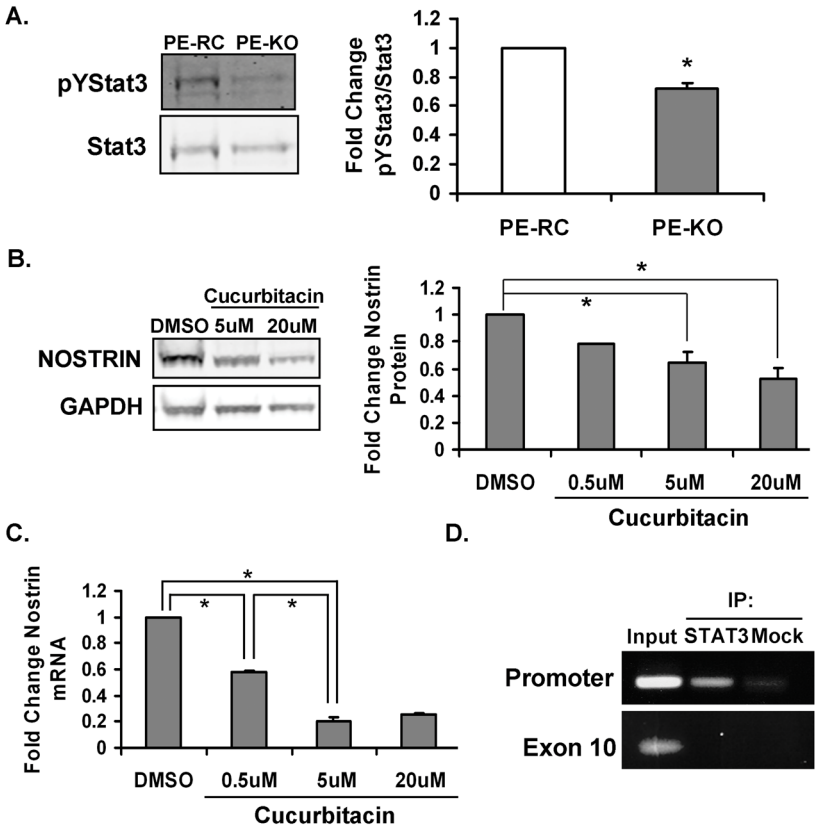

Figure 5.

PECAM-1 regulates NOSTRIN in a STAT3-dependent manner. A, Western blot for phosphorylated STAT3 in PE-RC and PE-KO cells. Quantitation shown on right (n=3, P<0.01 vs PE-RC). B, PE-RC and PE-KO cells treated for 24 hours with 0.5, 5 and 20μmol/L cucurbitacin then lysed and NOSTRIN protein expression determined by Western Blot. Values normalized to GAPDH (n=3, *P<0.05). C, Real-time PCR analysis of NOSTRIN mRNA expression in PE-RC cells treated for 12hrs with cucurbitacin. (n=3, *P<0.001). D. ChIP on PE-RC cells for STAT3 binding to NOSTRIN promoter region.

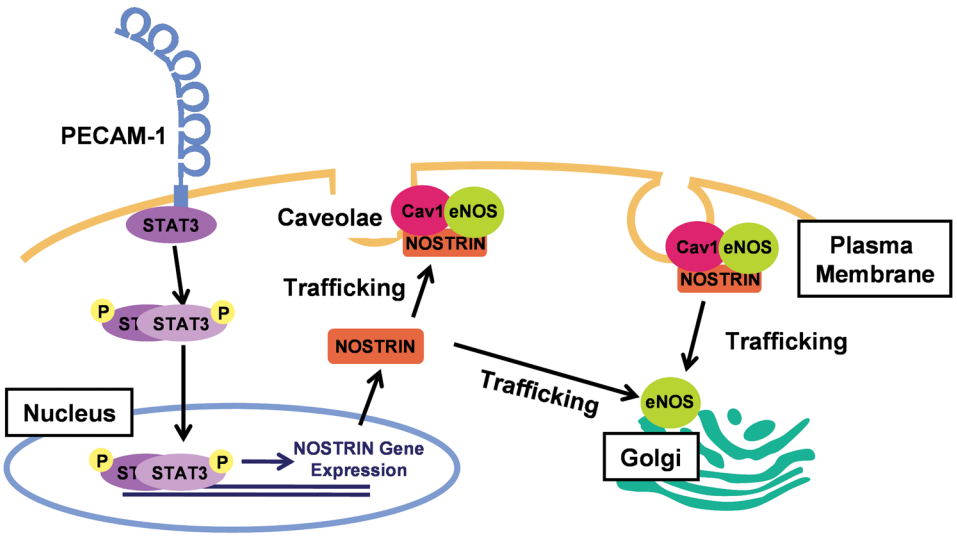

Figure 6.

Model of PECAM-1-mediated NOSTRIN expression and eNOS trafficking. The cytoplasmic tail of PECAM-1 acts as a scaffold for STAT3 binding. Following activation, STAT3 translocates to the nucleus where it regulates NOSTRIN mRNA expression. NOSTRIN protein is then able to bind eNOS and regulate its trafficking and localization with the cell.