Pin1 Prolyl Isomerase Regulates Endothelial Nitric Oxide Synthase

Ling Ruan, Christina M. Torres, Jin Qian, Feng Chen, James D. Mintz, David W. Stepp, David Fulton, Richard C. Venema

Objective—The Pin1 prolyl isomerase acts in concert with proline-directed protein kinases to regulate function of protein substrates through isomerization of peptide bonds that link phosphoserine or phosphothreonine to proline. We sought to determine whether Pin1 interacts with endothelial nitric oxide synthase (eNOS) in endothelial cells in a manner that depends on proline-directed phosphorylation of the eNOS enzyme and whether this interaction influences basal or agonist-stimulated eNOS activity.

Methods and Results—Inhibitors of the extracellular-regulated kinase (ERK) 1/2 MAP kinases inhibit proline-directed phosphorylation of eNOS at serine 116 (Ser116) in bovine aortic endothelial cells (BAECs). Moreover, eNOS and Pin1 can be coimmunoprecipitated from BAECs only when Ser116 is phosphorylated. In addition, phosphomimetic Ser116Asp eNOS, but not wild-type eNOS, can be coimmunoprecipitated with Pin1 coexpressed in COS-7 cells. Inhibition of Pin1 in BAECs by juglone or by dominant negative Pin1 increases basal and agonist-stimulated NO release from the cells, whereas overexpression of wild-type Pin1 in BAECs suppresses basal and agonist-stimulated NO production. Overexpression of wild-type Pin1 in intact aortae also reduces agonist-induced relaxation of aortic rings.

Conclusion—Our results demonstrate a novel form of eNOS regulation in endothelial cells and blood vessels through Ser116 phosphorylation–dependent interaction of eNOS with Pin1. (Arterioscler Thromb Vasc Biol. 2011;31:392-398.)

Key Words: nitric oxide ■ nitric oxide synthase ■ signal transduction ■ vascular biology

Endothelial nitric oxide synthase (eNOS), through generation of the vasodilating and vasculoprotective molecule nitric oxide (NO), plays a key role in blood pressure control and in protection from atherosclerotic lesion formation. eNOS is regulated posttranslationally through the alternative mechanisms of reversible phosphorylation and protein-protein interactions.1–2 eNOS regulation by phosphorylation is complex, and to date, 7 specific sites of regulatory phosphorylation have been identified in bovine eNOS, at tyrosine 83 (Tyr83), serine 116 (Ser116), threonine 497 (Thr497), serine 617 (Ser617), serine 635 (Ser635), tyrosine 659 (Tyr659), and serine 1179 (Ser1179). Equivalent sites are found in human eNOS at Tyr81, Ser114, Thr495, Ser615, Ser633, Tyr657, and Ser1177. Among these various phosphorylation sites, Ser1179/Ser1177 has been particularly well documented as having an important role in positively modulating eNOS activity.2 The eNOS protein-protein interactome is also very complex, with many proteins that are known to interact with eNOS either directly or indirectly to influence eNOS activity or subcellular localization. Among these protein-protein interactions, one of the best-studied examples is that of caveolin-1. Caveolin-1 binds directly to eNOS and tonically inhibits its catalytic activity.3

Regulation of protein function by reversible phosphorylation is generally thought to occur through direct effects of phosphorylation on the 3-dimensional conformation of the phosphorylated protein. However, there is an alternative mechanism by which phosphorylation can affect protein function. This mechanism involves phosphorylation-dependent conformational changes that are induced by the Pin1 prolyl isomerase.4 Pin1 catalyzes the cis to trans isomerization of peptide bonds that link phosphoserine or phosphothreonine to proline. Conformational changes induced by this mechanism are initiated by proline-directed phosphorylation of serines or threonines immediately preceding proline in substrate proteins by one of a large family of proline-directed protein kinases, which include the cyclin-dependent kinases, the mitogen-activated protein (MAP) kinases, and glycogen synthase kinase 3. Subsequent to proline-directed phosphorylation, Pin1 binds to the phosphoserine or phosphothreonine and catalyzes an isomerization reaction at the adjacent peptide bond that can have profound effects on protein conformation and hence on protein function. Pin1 (protein interacting with never in mitosis A) was first discovered and cloned in 1996.4 Pin1 was initially found to be
essential to regulation of mitosis. Subsequently, a large body of literature has been produced implicating Pin1 as also being important in cancer and in Alzheimer’s disease. For example, Pin1 is overexpressed in many human cancers, where it functions as a critical enzyme in multiple oncogenic pathways. In contrast, Pin1 is downregulated in degenerative neurons of Alzheimer’s disease patients, which contributes to age-dependent neurodegeneration. A recent report has shown that inducible nitric oxide synthase, which is not expressed in endothelial cells under basal conditions, is negatively regulated by Pin1 in endothelial cells after induction of inducible nitric oxide synthase by lipopolysaccharide and interferon-γ. It should be noted that the Pin1 referred to here is distinct from another similarly named protein, PIN, the small protein (89 amino acids) inhibitor of neuronal nitric oxide synthase that inhibits neuronal nitric oxide synthase activity by binding to this enzyme and preventing its dimerization. In the present study, we have investigated whether eNOS may be regulated in endothelial cells and in blood vessels by the Pin1 prolyl isomerase and whether such regulation occurs in a manner that depends on site-specific, proline-directed phosphorylation of the eNOS enzyme.

Methods

Cell Culture
Primary cultures of bovine aortic endothelial cells (BAECs) were purchased from VEC Technologies Inc and were used for experiments between passages 2 and 6.

Immunoprecipitation and Immunoblotting
Immunoprecipitation and immunoblotting are described in detail in the supplemental materials, available online at http://atvb.ahajournals.org.

Transfection of COS-7 Cells
COS-7 cells were transfected with various cDNA constructs cloned into the pcDNA3.1/V5-His A,B,C plasmid vector from Invitrogen. DNA-Lipofectamine 2000 complexes (Invitrogen) were added directly to the cells in culture medium according to the manufacturer’s instructions.

DNA Cloning of Pin1
Pin1 cDNA was cloned from human aortic total RNA (United States Biological) by reverse transcription–polymerase chain reaction using a Phusion RT-PCR Kit from New England Biolabs. Primers used are listed in the online supplement.

Site-Directed Mutagenesis
Site-directed mutagenesis of wild-type Pin1 cDNA was performed using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Primers used are listed in the online supplement. Cloning of wild-type bovine eNOS and mutagenesis of the wild-type sequence to produce Ser116Asp eNOS were described previously.

Construction, Purification, and Transduction by Adenoviruses
Adenoviruses were generated by the procedure of He et al as described in the online supplement.

Measurement of NO Release
NO release was determined by a chemiluminescence assay that measures nitrite levels in conditioned media. Media were deproteinized by ethanol precipitation, and samples containing nitrite were refluxed in glacial acetic acid containing 65 mmol/L sodium iodide.

Results

Identification of ERK 1/2 as Responsible for eNOS Phosphorylation at Ser116 in Endothelial Cells
Proline-directed phosphorylation, requiring a proline at the +1 position, is a prerequisite for Pin1 interaction with (and consequent Pin1-catalyzed isomerization of) Pin1 substrates. Of the 5 known serine/threonine phosphorylation sites in bovine eNOS, only Ser116 conforms to the proline at +1 requirement. Because it has been reported previously that the ERK 1/2 MAP kinase inhibits eNOS activity through phosphorylation of an unidentified site in the eNOS enzyme, we considered the possibility that ERK 1/2, which has an absolute requirement for proline in the +1 position, is responsible for Ser116 phosphorylation of eNOS in endothelial cells under basal conditions. BAECs were either not treated or treated with the selective MAP kinase kinase 1/2 (and hence ERK 1/2) inhibitor PD98059 (50 μmol/L for 1 hour).

Under these conditions, nitrite is quantitatively reduced to NO. The resultant NO was purged from the reaction cell with 100% nitrogen and directly quantified after reaction with ozone in an NO-specific chemiluminescence analyzer (Sievers, model 280i).

Transduction of Mouse Aortic Endothelium With Recombinant Adenoviruses and Determination of Vascular Reactivity of Transduced Vessels
Transduction of mouse aortae with adenoviruses and determination of vascular reactivity of transduced vessels were carried out essentially as reported previously and are described in detail in the online supplement.

Statistical Analysis
All data are representative of at least 3 separate experiments and are reported as mean±SE. Overall differences were analyzed by 2-way ANOVA and by repeated-measures ANOVA. Differences were considered significant at \( P<0.05 \).

Figure 1. Effects of ERK 1/2 inhibition on basal phosphorylation of eNOS at Ser116 in BAECs. BAECs were either not treated or treated with PD98059 (50 μmol/L for 1 hour) (A) or U0126 (10 μmol/L for 1 hour) (B). Cells were lysed, and lysates were immunoblotted (IB) with phospho-Ser116-specific and non–phospho-specific anti-eNOS antibodies. Similar results were obtained in at least 3 separate experiments.
Figure 2. Phosphorylation-dependent association of eNOS and Pin1 in BAECs. A, BAEC lysates were immunoprecipitated (IP) with anti-Pin1 antibody and immunoblotted (IB) with anti-eNOS antibody. B, BAEC lysates were immunoprecipitated with anti-eNOS antibody and immunoblotted with anti-Pin1 antibody. C, BAECs were either not pretreated or pretreated with PD98059 (50 μmol/L for 1 hour) before immunoprecipitation with anti-eNOS antibody and immunoblotting with either anti-Pin1 or anti-eNOS antibodies. Results are representative of at least 3 different experiments.

Figure 3. Phosphorylation-dependent association of eNOS and Pin1 following ectopic expression of Pin1 with either WT eNOS or phosphomimetic (Ser116Asp) eNOS. COS-7 cells were transfected with either WT eNOS or Ser116Asp with and without cotransfection with V5-tagged Pin1. A, cell lysates were immunoprecipitated (IP) with anti-V5 antibody and immunoblotted (IB) with anti-eNOS antibody. B, cell lysates were immunoprecipitated with anti-V5 antibody and immunoblotted with anti-V5 antibody. Similar results were obtained in 3 experiments.

endothelial cells, we lysed BAECs and subjected the lysates to immunoprecipitation with anti-eNOS antibody and anti-Pin1 antibodies. Anti-eNOS immunoprecipitates were then immunoblotted with anti-Pin1 antibody, and anti-Pin1 immunoprecipitates were immunoblotted with anti-eNOS antibody. As shown in Figure 2A, eNOS (130 kDa) was specifically coimmunoprecipitated from endothelial cell lysates by the anti-Pin1 antibody. In addition, Pin1 (18 kDa) was specifically coimmunoprecipitated by the anti-eNOS antibody (Figure 2B). Importantly, we also determined whether blockade of Ser116 phosphorylation of eNOS in BAECs by PD98059 is associated with reduced complex formation of eNOS and Pin1. BAECs were either not pretreated or pretreated with PD98059 (50 μmol/L for 1 hour) before immunoprecipitation from lysates of eNOS with anti-eNOS antibody. Immunoprecipitated proteins were then immunoblotted with anti-eNOS and anti-Pin1 antibodies. As shown in Figure 2C, coimmunoprecipitation of the 2 proteins was clearly detectable from untreated cells but was significantly reduced from cells in which Ser116 phosphorylation was blocked by PD98059 treatment. Densitometric and statistical analysis showed an 81 ± 5% decrease (mean ± SE, n = 3, P < 0.05) in complex formation of eNOS and Pin1 in PD98059-treated cells.

The phosphorylation dependence of eNOS interactions with Pin1 was further examined using a different experimental model system. COS-7 cells, which ordinarily express no endogenous eNOS, were transfected with either wild-type bovine eNOS (WT eNOS) or a phospho-Ser116-mimetic form of bovine eNOS (Ser116Asp eNOS) in which Ser116 was changed to an aspartate by site-directed mutagenesis. In addition, full-length human Pin1 cDNA was cloned from human aortic total RNA using reverse transcription–polymerase chain reaction. Pin1 cDNA was then used to cotransfect eNOS-transfected cells. Pin1 was expressed as a Pin1/V5 fusion protein to allow immunoprecipitation of the protein with anti-V5 antibody. Cells were lysed, Pin1/V5 protein was immunoprecipitated with anti-V5 antibody, and immunoprecipitates were immunoblotted with anti-eNOS antibody. Figure 3A shows that although the phosphomimetic Ser116Asp eNOS was bound by the Pin1/V5 fusion protein in these experiments, WT eNOS was clearly not bound. In addition, we also confirmed that no endogenous Ser116 phosphorylation of WT eNOS (as occurs in BAECs and promotes Pin1 binding) occurs in WT eNOS-transfected COS-7 cells. Furthermore, we confirmed that equal amounts of Pin1/V5 were immunoprecipitated from each cotransfection condition (Figure 3B). Thus, it appears that Pin1 interactions with eNOS are dramatically increased by mimicking phosphorylation of Ser116, likely because of an increased binding affinity of the phosphomimetic eNOS protein for Pin1.

NO Production by Endothelial Cells is Negatively Modulated by Pin1

The effects of eNOS-Pin1 interactions in modulation of eNOS activity in cultured endothelial cells were determined using 3 different approaches. In the first, full-length human Pin1 cDNA was used to prepare and purify a wild-type Pin1 adenovirus for overexpression of the protein in BAECs. Endothelial cells were transduced either with a negative control β-galactosidase (β-gal) adenovirus or with an adeno-
virus expressing Pin1. After 48 hours of virus infection, the amount of basal NO release was measured by quantifying nitrite in conditioned media using an NO-specific chemiluminescence analyzer as described previously. As shown in Figure 4A, the amount of NO released from Pin1-overexpressing cells during 48 hours of virus infection was significantly reduced (≈70%) compared with that from control cells. Media were also changed, and cells were treated with 1 μmol/L bradykinin (BK) for 30 minutes. BK-stimulated NO release was also significantly decreased (≈90%) by Pin1 transduction, suggesting that Pin1 effects on eNOS activity are not rapidly reversible by agonist stimulation (Figure 4B). Cells were also lysed, and eNOS-Pin1 association was assessed by immunoblotting of anti-eNOS immunoprecipitates with anti-Pin1 antibody. Pin1 binding to eNOS was confirmed to be dramatically increased in Pin1-overexpressing cells (Figure 4C). In an additional set of experiments, Pin1 overexpression produced by adenoviral infection was carried out in the absence and presence of PD98059 (50 μmol/L). As shown in Figure 4D, blocking Ser116 phosphorylation by PD98059 almost completely prevented the inhibitory effect of Pin1 overexpression to suppress the amount of NO released from the cells, demonstrating clearly the phosphorylation dependence of the Pin1 effect.

The second approach that was used to define the role of Pin1 in regulation of eNOS in cultured endothelial cells was to inhibit endogenous Pin1 activity with a dominant negative form of Pin1. Pin1 is phosphorylated on serine 16, and a Ser16Ala Pin1 mutant that is refractory to phosphorylation has been shown to function as a dominant negative mutant. In addition, a phosphomimetic Ser16Glu Pin1 mutant has also been shown to exhibit a reduced binding capacity for various Pin1 substrates. To confirm that phosphomimetic Ser16Glu Pin1 also displays reduced binding to phospho-Ser116-eNOS, we cotransfected COS-7 cells with either wild-type Pin1 and Ser116Asp eNOS or with Ser16Glu Pin1 and Ser116Asp eNOS before assessing Pin1-eNOS binding by coimmunoprecipitation. Immunoblotting of lysates showed equivalent levels of expression of Ser116Asp eNOS and the 2 forms of Pin1 in these experiments. Lysates were also immunoprecipitated with anti-eNOS and anti-Pin1 antibodies. As shown in Supplemental Figure I, phosphomimetic Pin1 showed a markedly reduced degree of coimmunoprecipitation with eNOS as compared with wild-type Pin1 when equal amounts of the 2 forms of Pin1 were immunoprecipitated. Next, we prepared and purified a Ser16Ala dominant negative Pin1 adenovirus. BAECs were transduced with either an adenovirus expressing β-gal or adenovirus expressing Pin1. After 48 hours, the amount of basal NO release was measured by quantifying nitrite accumulation in conditioned media using the NO analyzer (mean±SE, n=6, *P<0.05 versus control). B, 48 hours after transduction with either the adenovirus expressing β-gal or the adenovirus expressing Pin1, medium was changed, and cells were treated with 1 μmol/L BK for 30 minutes. Following BK treatment, nitrite in conditioned media was quantified with the NO analyzer (mean±SE, n=6, *P<0.05 versus control). C, At the end of the experiment, cells were lysed, and lysates were subjected to immunoprecipitation (IP) with anti-eNOS antibody and immunoblotting (IB) with anti-Pin1 and anti-eNOS antibodies. Results shown are representative of 3 experiments. D, BAECs were transduced with either β-gal or Pin1 adenoviruses in the presence and absence of 50 μmol/L PD98059. After 48 hours, basal NO release was measured by quantifying nitrite accumulation in conditioned media using the NO analyzer (mean±SE, n=6, *P<0.05 versus control).
unaffected.\(^{19,20}\) BAECs were treated without or with juglone (1 \(\mu\)mol/L for 1 hour). Juglone was dissolved in dimethyl sulfoxide and applied to the cells as a 1000-fold concentrated solution. An equal volume of vehicle solution was applied to control cells. Basal NO release during the treatment time was determined, and as shown in Figure 5D, inhibition of Pin1 activity by juglone, like inhibition by the Ser16Ala dominant negative Pin1, also resulted in a significant increase in NO production. The requirement of eNOS phosphorylation at Ser116 for inhibition of eNOS by Pin1 in endothelial cells was also investigated using a nonphosphorylatable dominant negative Ser16Ala form of eNOS. Adenoviruses that express wild-type and Ser16Ala forms of eNOS were prepared, and BAECs were infected with \(\beta\)-gal (negative control), WT eNOS, and Ser16Ala eNOS adenoviruses. Immunoblotting of cell lysates after 48 hours of virus infection showed an approximately 2-fold increase in total eNOS expression in both the WT eNOS and Ser16Ala eNOS adenovirus transduction conditions compared with that of the negative control (Supplemental Figure IIB). After 48 hours of infection, conditioned medium was also analyzed for NO release by measuring nitrite accumulation. As shown in Supplemental Figure IIA, whereas WT eNOS produced an approximately 2-fold higher level of NO release relative to the control, the Ser116Ala dominant negative eNOS produced a statistically significant higher level of NO release (\(\sim\)3-fold over control), presumably because of the lack of Ser116 phosphorylation of Ser16Ala eNOS with subsequent loss of phospho-Ser116-dependent Pin1 binding to eNOS.

**Vascular Reactivity of Intact Blood Vessels Is Negatively Modulated by Pin1**

To determine the role of Pin1 in modulation of vascular reactivity of intact blood vessels, we carried out experiments in which Pin1 was overexpressed in aortae of imprinting control region mice. Mice were anesthetized and exsanguinated, followed by perfusion with saline. The thoracic aorta of the mice were then infused with adenoviruses expressing either Pin1 or \(\beta\)-gal as a negative control. The virus-filled vessels were incubated in situ for 2 hours and then incubated in vitro overnight. Aortic rings were then prepared for isometric force recording in a multimyograph apparatus. Rings were preconstricted with 10\(^{-5}\) mol/L serotonin, and dose-response curves to acetylcholine and sodium nitroprusside were constructed. Rings demonstrated similar preconstriction force to serotonin with either Pin1 or \(\beta\)-gal overexpression (2.3\(\pm\)0.2 g versus 2.2\(\pm\)0.2 g, \(P<0.05\) versus control). As shown in Figure 6A, rings from aortae of mice infected with the Pin1 adenovirus had significantly reduced relaxant responses to acetylcholine (\(\sim\)30% reduction in maximal relaxation) compared with rings from \(\beta\)-gal-infected control mice, indicating an important role for Pin1 in negatively modulating vascular reactivity. No differences were observed, however, in relaxant responses to sodium nitroprusside demonstrating that the differences observed for acetylcholine-induced relaxation were endothelium dependent (Figure 6B).

**Discussion**

The results of the present study identify the ERK 1/2 MAP kinase as being responsible for phosphorylation of eNOS at Ser116 in endothelial cells under basal conditions. Ser116 phosphorylation has been shown previously by Kou et al to be reduced by the protein kinase C (PKC) inhibitor calphostin C, implicating PKC as a mediator of this specific phosphorylation reaction.\(^{21}\) However, Shaw and colleagues\(^{22,23}\) have recently shown that the AGC kinases (protein kinase A, protein kinase G, and PKC), as well as the calmodulin-dependent protein kinases, cannot phosphorylate serines or threonines in protein substrates containing a proline at the \(P+1\) position. Proline at \(P+1\) is thus a “veto residue” that precludes phosphorylation by AGC and calmodulin-
Figure 6. Effects of Pin1 overexpression on vasodilation in serotonin-preconstricted aortic rings of imprinting control region mice. A, Aortae were transduced with either β-gal or Pin1 adenovirus, aortic rings were prepared, and preconstricted with serotonin. Dose-response curves of relaxation to acetylcholine were then determined (mean±SE, n=3, *P<0.05 versus β-gal control). B, aortae were transduced with either β-gal or Pin1 adenoviruses, aortic rings were prepared, and preconstricted with serotonin. Dose-response curves of relaxation to sodium nitroprusside were then determined (mean±SE, n=3, P=not significant versus β-gal control).

dependent protein kinases. This feature of proline-directed phosphorylation provides very tight control in preventing reciprocal substrate specificity between proline-directed protein kinases and AGC/calmodulin-dependent protein kinases. Because Ser116 in the eNOS amino acid sequence immediately precedes Pro117, it is reasonable to expect that Ser116 would be a site of proline-directed phosphorylation by a kinase such as ERK 1/2 rather than a site of PKC or other AGC/calmodulin-dependent protein kinase phosphorylation. It is thus likely that the reduced Ser116 phosphorylation due to PKC inhibition by calphostin C that was reported previously might be explained by an indirect effect of calphostin C, such as blockade of PKC phosphorylation of Raf-1. This reaction is known to activate the Raf-1/Map kinase kinase 1/2/ERK 1/2 cascade, and its inhibition might contribute to ERK 1/2 inhibition.

The role of ERK 1/2 MAP kinases in eNOS regulation and the role of Ser116 phosphorylation in eNOS regulation have been subject to alternative explanations in the scientific literature. In various studies, ERK 1/2 inhibition in endothelial cells has been shown to either attenuate, not change, or enhance eNOS activity. The latter study, whose conclusions are most consistent with our own, showed using coimmunoprecipitation that eNOS exists in a protein-protein complex with ERK 1/2 in endothelial cells and that immunoprecipitated eNOS can be phosphorylated by ERK 2 in the eNOS interactome in vitro, resulting in a reduction in eNOS enzyme activity. No specific ERK 1/2 phosphorylation site in eNOS was identified in this study. However, at the time the previous study was carried out, Ser1179 was the only specific eNOS phosphorylation site yet known. Until recently, some controversy has also existed about whether phosphorylation of eNOS at Ser116 is stimulatory or inhibitory in nature. For example, phosphorylation of Ser116 in endothelial cells in response to increased fluid shear stress, a stimulus known to substantially increase eNOS activity, has been demonstrated by mass spectrometry. This would tend to suggest that phosphorylation may be stimulatory in nature. An additional study has reported that fluid shear stress, vascular endothelial growth factor, and 8-bromo cAMP have no effect on the phosphorylation status of Ser116 in endothelial cells. However, studies by Kou et al and from our own laboratory have provided convincing evidence that phosphorylation of Ser116 in eNOS is in fact inhibitory in nature and that phosphorylation of this site significantly reduces eNOS activity. Furthermore, Kou et al have shown that agonist stimulation of endothelial cells by vascular endothelial growth factor induces calcineurin-mediated Ser116 dephosphorylation of eNOS rather than Ser116 phosphorylation. In our own studies, we have confirmed this effect of vascular endothelial growth factor and have also found that many other eNOS-activating agonists, including BK, thapsigargin, ATP, and angiotensin, promote calcineurin-mediated dephosphorylation of eNOS at Ser116 (unpublished observations).

Our previously published and unpublished results suggest that transient Ser116 dephosphorylation may contribute to the agonist-stimulated eNOS activation process in the same way that agonist-induced Thr497 dephosphorylation appears to also contribute to this process. The results of the current study implicate Ser116 phosphorylation/dephosphorylation as having a second important role in eNOS regulation that is tonic in nature rather than transient in nature, as in the case of agonist activation associated with Ser116 dephosphorylation. Constitutive Ser116 phosphorylation in endothelial cells and blood vessels under basal conditions promotes Pin1 interaction with eNOS and suppression of eNOS activity in a manner that is analogous to the tonic suppression of eNOS activity that is produced by eNOS interaction with caveolin-1. This phosphorylation-dependent interaction of Pin1 with eNOS would be expected to induce a conformational change in the eNOS enzyme. Such a conformational change could alter eNOS catalytic activity directly or could affect eNOS activity indirectly by making the enzyme more or less susceptible to phosphorylation/dephosphorylation or to proteolytic degradation. Pin1-induced conformational changes could also affect eNOS activity indirectly by altering its interaction with other protein members of the eNOS interactome. Whether direct or indirect, however, it is clear that Ser116 phosphorylation–dependent interaction of eNOS with Pin1 serves to suppress eNOS activity under basal and agonist-stimulated conditions in endothelial cells and in intact blood vessels. It is possible that there are additional effects of eNOS phosphorylation at Ser116 that are not mediated by Pin1. Obtaining definitive evidence for this will likely require future investigations of purified recombinant eNOS before and after phosphorylation at Ser116, with and without preincubation with purified Pin1. Future studies using various small animal models of cardiovascular disease may also reveal whether altered Pin1 regulation of eNOS has a role in endothelial dysfunction in vascular disease. Deregulation of Pin1 has been clearly shown to contribute to the disease phenotypes of cancer and Alzheimer’s disease. Whether
deregulation of Pin1 is similarly associated with a cardiovascular disease phenotype remains to be determined.

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Disclosures
None.

References
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Supplemental Material

Materials
Anti-eNOS antibody was obtained from BD Biosciences and anti-phospho-S116 eNOS antibody was obtained from Upstate-Cell Signalling Solutions. Anti-Pin1 antibody was purchased from EMD and anti-V5 antibody was purchased from Invitrogen. PD98059, U0126, juglone, and bradykinin were purchased from Sigma-Aldrich.

Immunoprecipitation and Immunoblotting
COS-7 cells or BAECs were lysed in ice-cold lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaF, 15 mmol/L Na4P2O7, 1 mmol/L Na3VO4, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 g to remove insoluble material and were precleared by incubation with 50 μL of protein A/G-agarose for 2 h at 4°C with rocking. Agarose beads were pelleted by centrifugation at 1000 g. For immunoprecipitation, precleared lysates were incubated with either anti-eNOS (10 μL), anti-Pin1 (30 μL), or anti-V5 (3 μL) antibodies overnight at 4°C with rocking. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in SDS sample buffer. Immunoprecipitated proteins or cell lysates were immunoblotted with either anti-eNOS antibody (1:1,000 dilution), anti-P-S116 eNOS antibody (1:1,000 dilution), anti-V5 antibody (1:1,000 dilution), or anti-Pin1 antibody (1:200 dilution). Bound antibody was visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce Chemical) after incubation of the blot with peroxidase-conjugated secondary antibody for 1 h.

Primers Used for DNA Cloning of Pin1
Primers used were the following: sense, 5’-ATGGCGGAGCAGGAGAAGCTGC-3’, and antisense, 5’-TCACTCAGTGCGGAGGATGATG-3’. Cloned sequences were confirmed by automated DNA sequencing at Retrogen.

Primers Used for Site-Directed Mutagenesis
Primers used to create the mutations were as follows: S16A Pin1, sense, 5’-AGCGATGGCCGGAGCCGAGCTGAGCCGAGTG-3’, anti-sense, 5’-GCTGCGGAGCCATGCGCTTCTCCAGCCGAGAGG-3’, S16E Pin1, sense, 5’-AGCGATGGAGCGCGAGCGCTGAGCCGAGTG-3’, and antisense, 5’-GCTGCGCTCCATGCGCTTCTCCAGCCGAGAGG-3’. Sequences were confirmed by automated DNA sequencing at Retrogen.

Construction, Purification, and Transduction of BAECs by Adenoviruses
Coding sequences were subcloned into a pAd-Track-CMV shuttle vector. The pAd-Track-CMV constructs were then linearized with Pme I and then cotransformed into E. Coli BJ5183 cells together with the adenoviral backbone plasmid, pAdEasy-1. Recombinants were selected by kanamycin resistance and verified by restriction enzyme digestion. The confirmed recombinants were then transfected into the adenoviral packaging HEK293 cell line. Viral production was monitored over 7-10 days by visualization of green fluorescent protein expression. Virus was harvested and purified by banding on a cesium chloride gradient. The purified virus was dialyzed and stored at -80°C. BAECs were infected with various MOIs of adenoviruses to determine a titer that gave maximal expression without significant cell death. In subsequent experiments, cells were infected for 24 h at an optimal MOI.
Measurement of NO Release
Media were deproteinized by ethanol precipitation and samples containing nitrite were refluxed in glacial acetic acid containing 65 mmol/L sodium iodide. Under these conditions, nitrite is quantitatively reduced to NO. The resultant NO was purged from the reaction cell with 100% nitrogen and directly quantified after reaction with ozone in an NO-specific chemiluminescence analyzer (Sievers, Model 280i).

Transduction of Mouse Aortic Endothelium with Recombinant Adenoviruses and Determination of Vascular Reactivity of Transduced Vessels
ICR mice were anesthetized and exsanguinated by transection of the abdominal aorta and perfusion of saline through the left ventricle. Heart and lungs were removed to expose the thoracic aorta. The aorta was then infused with high titer adenoviruses (1x10^7 pfu/μL) expressing either β-gal or wild-type human Pin1 and each end of the aorta was tied off with sutures. The virus-filled vessels were then incubated in situ at 37°C for 2 h. Aortae were then dissected free from the surrounding tissue and rinsed with saline and then incubated overnight in tissue culture medium at 37°C with 95%O₂/5%CO₂. We have demonstrated previously that efficient gene transfer primarily into the endothelium is accomplished by this method as confirmed by staining for β-gal. After overnight incubation, aortae were cut into 4 rings of equal diameter and mounted with 1 g of resting tension on a wire myograph and allowed to equilibrate for 1 h. After equilibration, rings were preconstricted with 10^-5 M serotonin then relaxed with serial doses of acetylcholine or sodium nitroprusside (10^-9-10^-4 M) to assess endothelium-dependent and –independent NO-mediated vasodilation, respectively. Rings that constricted less than 300 mg of force were excluded from analysis to rule out artifactual data from injured tissue. Baseline force was subtracted from the measured value at each dose and normalized as percent of preconstricted value with no dilation being expressed as 0% and complete reversal of constriction defined as 100%.

Figure Legends for Supplemental Data

Supplemental Figure I. Effect of S116E mutation on binding of Pin1 to phospho-S116-mimetic eNOS. COS-7 cells were cotransfected with S116D eNOS and either wild-type (WT) or S116E forms of Pin1. Cells were lysed and lysates were immunoprecipitated (IP) with anti-Pin1 antibody. Immunoprecipitated proteins were then immunoblotted (IB) with anti-eNOS or anti-Pin1 antibodies. Similar results were obtained in two separate experiments.

Supplemental Figure II. Increased NO production in endothelial cells by dominant negative S116A eNOS compared to NO production by wild-type eNOS. A, BAECs were transduced by adenoviruses expressing either β-gal, wild-type (WT) eNOS, or S116A eNOS. After 48 h, basal NO was measured by quantifying nitrite accumulation in conditioned medium using the NO analyzer (means ± S.E., n=3, *P<0.05 vs. β-gal, **P<0.05 vs. WT eNOS). B, 48 h after adenovirus infection, cell lysates were immunoblotted with anti-eNOS antibody.
Supplemental Figure I

IP: anti-Pin1

IB: anti-eNOS

IB: anti-Pin1

WT-Pin1  S16E-Pin1
Supplemental Figure II

A.

![Bar chart showing NO₂ accumulation (pmol/well) for β-Gal, WT-eNOS, and S116A-eNOS. The chart includes error bars and asterisks indicating statistical significance.]

B.

IB: anti-eNOS

![Image showing immunoblot for β-Gal, WT-eNOS, and S116A-eNOS with corresponding bands.]