Independent Regulation of Endothelial Nitric Oxide Synthase by Src and Protein Kinase A in Mouse Aorta Endothelial Cells

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Endothelial nitric oxide synthase (eNOS) plays a critical role in vascular biology and pathophysiology. Its activity is regulated by multiple mechanisms such as calcium/calmodulin, protein-protein interactions, sub-cellular locations and phosphorylation at various sites. Phosphorylation of eNOS-Ser1177 (based on mouse sequence) has been identified as an important mechanism of eNOS activation. However, signaling pathway leading to it phosphorylation remains controversial. The regulation of eNOS-Ser1177 phosphorylation by Src and protein kinase A (PKA) was investigated in the present study using cultured mouse aorta endothelial cells. Expression of a constitutively active Src mutant in the cells enhanced phosphorylation of eNOS and protein kinase B (Akt). The Src-stimulated phosphorylation was not attenuated by the expression of a dominant negative PKA regulatory subunit. Neither activation nor inhibition of PKA activity had any significant effect on tyrosine phosphorylation of eNOS. For example, recent studies have proposed that non-receptor protein kinase Src regulates phosphorylation of eNOS-Ser1177 through activating phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in response to fluid shear stress. Heat shock protein 90, a GTP-binding protein dynamin-2, a voltage-dependent anion channel porin and NOS-interacting protein (NOSIP) have also been identified to regulate eNOS activity by protein-protein interactions. Post-translational modification of eNOS has also been recognized as a critical mechanism controlling eNOS activity. eNOS is subjected to myristoylation and palmitoylation which target the enzyme to the plasma membrane or Golgi. The sub-cellular locations of eNOS has been found to be critical for the enzyme activation through signal transduction.

The importance of eNOS phosphorylation has only been recognized recently. At least five specific phosphorylation sites on eNOS have been recognized; Ser114, Thr495, Ser615, Ser633 and Ser1177, numbering based on mouse eNOS sequence. Although the evidence supporting the importance of these phosphorylation sites on eNOS has been growing, there is significant controversy regarding the protein kinases and phosphatases that regulate phosphorylation of each site. For example, recent studies have proposed that non-receptor protein kinase Src regulates eNOS-Ser1177 phosphorylation by activating phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in response to fluid shear stress. However, earlier studies have questioned whether Akt was responsible for eNOS activation by shear stress. Instead, PI3K/protein kinase A (PKA) pathway was proposed to be a more plausible mechanism. Therefore questions remain if these two pathways regulate eNOS independently.

Cross-talk between PKA and Src signaling has also been noticed. COOH-terminal Src kinase, which negatively
regulates Src activity by phosphorylating at Tyr527, has in
turn shown to be activated by PKA-dependent phosphorylation
at Ser364.\(^{20}\) PKA directly phosphorylates Src at Ser17
although the resulting effect on Src activity is controversial.\(^{27,20}\)
Therefore, in the present study, the association of Src and
PKA in eNOS regulation was examined by expressing Src
and PKA mutants in cultured mouse aorta endothelial cells
(MAECs).

Materials and Methods

Isolation and Culture of MAECs. MAECs were isolated
from pathogen-free male C57BL/6 mice as described
previously.\(^{20}\) The cells were plated on 0.5% gelatin-coated
100-mm plates (Falcon) and maintained at 37°C and 5% CO\(_2\)
in a growth medium [Dulbecco’s modified Eagle’s medium
(DMEM) containing 20% fetal bovine serum, 100 µg/mL
endothelial cell growth supplement (Sigma), and 1% penicillin/
streptomycin]. Cells used in this study were between passages
4 and 10.

Expression Vectors. PKA-Cqr and PKA-Rab constructs
were kind gifts from Dr. G. Stanley McKnight, University of
Washington. PKA-Cqr is a constitutively active mutant of
mouse PKA catalytic subunit C\(_{\alpha}\) which contains double
mutations (His87Gln and Trp196Arg),\(^{30}\) and PKA-Rab is a
dominant negative mutant of PKA regulatory subunit RI\(_{\alpha}\)
which has mutations of Gly200Glu, Gly324Asp and Arg332His.\(^{31}\)
The entire coding sequences of PKA-Cqr and Rab were sub-
cloned into a bi-cistronic vector, pAdTrack (a kind gift from
Dr. Bert Vogelstein, Johns Hopkins University) which also
expresses green fluorescent protein. The pSGT vector
expressing constitutively active chicken c-Src-Y527F has
been described in detail elsewhere.\(^{20}\)

Antibodies. The following primary antibodies were used
for Western blotting: polyclonal antibodies for phosphor-Akt
(Ser473), phosphor-eNOS (Ser1177), Phospho-Src (Tyr416
and Tyr527) from Cell Signaling Technology, polyclonal
antibodies for total Akt, Src, and actin from Santa Cruz
Biotechnology, and monoclonal antibodies for total eNOS,
PKA-CA\(_{\alpha}\), PKA-RI\(_{\alpha}\), and phosphor-Cav-1 (Tyr14) from BD
Transduction Laboratories.

Shear Stress Treatment. A confluent MAEC monolayer
grown on a 100-mm dish was exposed to non-pulsatile,
 laminar shear stress in a low serum-containing medium A
with or without treatments for specified periods. The conditioned
media were collected and treated with nitrate reductase to
reduce nitrate into nitrite. The total nitrite was
then quantified by converting it to NO in acidic potassium
iodide solution where NO was continuously monitored by an
NO-sensor.

Western blotting. Cells were washed in ice-cold phosphate
buffered saline and treated in lysis buffer (10 mM Tris-HCl,
ph 7.6, 1 mM sodium vanadate and 1% SDS). The lysate was
further homogenized by repeated aspiration through a 25-
gauge needle. Protein content was measured by a Bio-Rad DC
assay. Aliquots of cell lysates (20 µg protein per well) were
resolved on a 10% SDS-PAGE gel and proteins were transferred
onto a polyvinylidene difluoride membrane (Millipore). The
membrane was incubated with a primary antibody
overnight at 4°C, then with a secondary antibody conjugated
with alkaline phosphatase for 1 h at room temperature. The
signal was detected by a chemiluminescence method.

Statistical Analysis. Statistical analysis was performed by
Student’s t-test. The values were considered to be statistically
significant at \(p < 0.05\) based on at least three independent
experiments.

Results

eNOS phosphorylation in MAECs. MAECs were examined
for eNOS phosphorylation at Ser1177 in response to vascular
endothelial growth factor (VEGF), eAMP, and fluid shear
stress. As shown in Fig. 1, all the stimuli tested increased
eNOS phosphorylation in a time-dependent manner whereas
total eNOS protein level remained unchanged. The results
were comparable to those observed previously in bovine aorta
endothelial cells.\(^{20}\)

Active Src stimulates eNOS. The regulation of Src activity
occurs at Tyr416 and Tyr527 (based on chicken c-Src);
Phosphorylation of Tyr416 in the activation loop of the kinase
domain activates the enzyme while the Tyr527 phosphorylation
at C-terminus leads to its inactivation.\(^{30}\) The dephosphorylation
of Tyr527 leads to the stimulation of Src catalytic activity.
Thus substitution of Tyr527 by another amino acid residue
constitutively activates c-Src as in the case of c-Src-Y527F
mutant.\(^{30}\)

To examine the role of Src in eNOS activation, MAECs
were transfected with a vector expressing a constitutively
active Src mutant (c-Src-Y527F), and control cells were
transfected with an empty vector. The transfected cells were

incubated in the presence and absence of nitric oxide synthase inhibitor, nitro-L-arginine methyl ester (L-NAME) for 20 h, and NO production was investigated. As shown in Fig. 2A, protein expression of c-Src-Y527F stimulated NO production which was inhibited by L-NAME.

The cell lysates were subjected to Western blotting to examine expression and phosphorylation of eNOS (Fig. 2B). A robust expression of c-Src-Y527F was evident as compared with actin. As expected, the expressed c-Src-Y527F underwent auto-phosphorylation at Tyr416 leading to the enzyme activation. The activity of Src was further verified by the phosphorylation of Cav-1 at Tyr14, a known endogenous substrate of Src.

The expression of active Src enhanced eNOS phosphorylation markedly without affecting the expression of eNOS. These results indicate that Src signaling is sufficient to stimulate eNOS phosphorylation and activation.

**PKA-dependent eNOS regulation does not involve Src signaling.** It has been shown that PKA activation leads to the phosphorylation and activation of eNOS. Therefore, involvement of Src activity in PKA-dependent eNOS regulation was examined utilizing a constitutively active PKA catalytic subunit mutant (PKA-Cqr) and a dominant negative PKA regulatory subunit mutant (PKA-Rab).

PKA holoenzyme is composed of two catalytic (C) subunits, and two regulatory (R) subunits. The enzyme activity is repressed when C subunits are bound to the inhibitory R subunits. When intracellular cAMP level increases, cAMP binds to R subunits changing their conformation. This allows the C subunits to be released from the R subunits and become catalytically active. PKA-Cqr has mutations at the binding sites for R subunits and thus is resistant to inhibition by R subunits. It is thus constitutively active even at the basal level of intracellular cAMP. On the other hand, cAMP cannot bind to PKA-Rab because this subunit has mutations at cAMP binding sites. The subunit tends to bind to C subunits even if the intracellular cAMP level is elevated. PKA-Rab mutant thus inhibits PKA activity in a dominant negative manner.

When MAECs were transfected with these constructs, the expression of PKA-Cqr and Rab was clearly detected by antibodies for C and R subunits, respectively, as shown in Fig. 3. The actin blot served as a control. Expression of PKA-Cqr stimulated NO production and eNOS phosphorylation at Ser1177 without affecting the total eNOS protein level. However, neither PKA-Cqr nor Rab expression affected phosphorylation status of Src at Tyr416 and Tyr527. Src-Tyr527 was highly phosphorylated while Tyr416 was minimally phosphorylated, indicating Src activity was repressed. Thus it is implicated that PKA may regulate eNOS without affecting Src signaling.

**Src regulates eNOS by an Akt-dependent but PKA-independent mechanism.** Next, it was questioned whether Src signaling regulates eNOS involving a PKA-dependent mechanism. To test this, c-Src-Y527F construct or an empty pSGT vector was co-transfected with either PKA-Rab or an empty pAdTrack vector in MAECs. As shown in Fig. 4, NO production and eNOS phosphorylation stimulated by Src were
not inhibited by co-expressed PKA-Rab. This result implicates that Src regulates eNOS phosphorylation and activation by a PKA-independent mechanism. As expected, active Src stimulated Akt phosphorylation that was not affected by PKA-Rab expression, supporting a role of Akt in Src-stimulated eNOS activation (Fig. 4).

**Discussion**

NO production from endothelial cells is stimulated by fluid shear stress, cyclic stretching and a variety of humoral factors ranging from growth factors and peptide hormones. The mechanisms by which NO production is regulated are quite variable and eNOS activity is regulated by Ca\(^{2+}\)-dependent or -independent mechanisms or both in response to a certain stimulus.\(^{37}\)

Serine/threonine phosphorylation of eNOS has been noticed as one of the major "Ca\(^{2+}\)-independent mechanism" for eNOS activation for the past several years.\(^{21}\) eNOS has multiple phosphorylation sites, and each site appears to play a unique role in the regulation of eNOS. eNOS-Ser1177 has been most extensively studied and its phosphorylation is known to lead to enzyme activation. The site was initially found as an Akt phosphorylation site,\(^{17,38}\) but later studies showed that many other protein kinases such as protein kinase A and G,\(^{39}\) AMP-activated kinase,\(^{40}\) and CaM kinase II\(^{41}\) also regulated the phosphorylation of this site. Therefore, it is currently viewed that eNOS-Ser1177 phosphorylation is regulated by multiple mechanisms involving different protein kinase(s) at specific cellular circumstances.

Involvement of protein tyrosine kinase in eNOS regulation by Ca\(^{2+}\)-independent mechanism has been noticed in earlier studies; treatment of endothelial cells with tyrosine kinase inhibitors has been shown to inhibit NO production in response to shear stress.\(^{42,43}\) However, in other studies, tyrosine phosphatase inhibitor was shown to stimulate tyrosine phosphorylation of eNOS which was associated with a decrease in the enzyme activity due to interaction with Cav-1. Fleming et al. showed that treatment of endothelial cells with a tyrosine phosphatase inhibitor, phenylarsine oxide,
activates eNOS in a Ca\(^{2+}\)-independent manner.\(^6\) Although the Ca\(^{2+}\)-independent NO production was highly sensitive to tyrosine kinase inhibitors, eNOS was shown to be rapidly dephosphorylated after stimulation with phenylarsine oxide. These observations have suggested that protein tyrosine kinases are unlikely to phosphorylate eNOS directly. Instead, certain tyrosine kinase(s) such as Src may regulate eNOS activity indirectly by controlling the signaling pathway leading to eNOS phosphorylation.

Evidence for the involvement of Src in eNOS regulation has become available recently. It has been shown that Src regulates Ser1177 phosphorylation in response to various stimuli such as hydrogen peroxide,\(^4\) estrogen,\(^5\) sphingosine 1-phosphate and VEGF,\(^9\) and fluid shear stress.\(^2\) Src is considered to regulate eNOS by activating Akt in a PI3K-dependent mechanism, although it is also known to activate Akt by direct tyrosine phosphorylation.\(^6\) The present study demonstrated that stimulation of Src signaling alone is sufficient to induce eNOS activation by an Akt-dependent mechanism, independently of PKA. Supporting this, over-expression of a constitutively active Src mutant appeared to induce eNOS-Ser1177 phosphorylation (Fig. 2) and Akt phosphorylation (Fig. 4).

Direct effect of Src on PKA activity was not examined in the present study because PKA has been implicated to be a putative upstream regulator, rather than a regulatory target of Src. Instead, the effect of PKA inhibition on the Src-stimulated eNOS activation was investigated. As shown in Fig. 4, Src-stimulated phosphorylation of eNOS was not affected by PKA inhibition with a dominant negative mutant. Therefore, although it is yet unclear whether Src regulates PKA directly, PKA is not thought to play a critical role in eNOS activation by Src. Furthermore, the present study did not support the role of PKA in Src regulation, because the activation or inhibition of PKA activity had no significant effect on tyrosine phosphorylation of the activation and inactivation site in Src (Fig. 3). Based on these results, it is very likely that Src and PKA regulate eNOS independently, although additional studies using various activators or inhibitors of PKA would be necessary for a more conclusive validation.

As reported in a previous study, shear stress also stimulates eNOS-Ser1177 phosphorylation by a mechanism which is PI3K and PKA-dependent, but independent of Akt.\(^2\) Then, how is PKA activated by PI3K-dependent mechanism without involving Src or related tyrosine kinases? Class I PI3Ks expressed in endothelial cells are regulated by protein tyrosine kinase-dependent mechanism (PI3K\(\alpha\), \(\beta\) and \(\delta\)), or G protein-dependent mechanism (PI3K\(\gamma\), and \(\beta\)\(^\gamma\)). PI3K\(\gamma\), which is uniquely activated by a G protein-dependent and Src-independent mechanism, has been reported to be stimulated in endothelial cells exposed to fluid shear stress.\(^2\) Then shear stress-stimulated eNOS phosphorylation might be regulated by either G protein/PI3K\(\gamma\)/PKA pathway or Src/PI3Ks/Akt pathway. Further studies are imperative to delineate the signaling pathway regulating eNOS activation by shear stress.

In conclusion, this study implicates that Src/Akt pathway and PKA signaling may regulate eNOS phosphorylation independently. The existence of multiple mechanisms for eNOS phosphorylation should be beneficial to generate adequate NO production under various physiological conditions.

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**References**


