Shear Stress Stimulates Phosphorylation of Endothelial Nitric-oxide Synthase at Ser\textsuperscript{1179} by Akt-independent Mechanisms

ROLE OF PROTEIN KINASE A \textsuperscript{a}

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Recently, we have shown that shear stress stimulates NO\textsuperscript{\textsuperscript{1}} production by the protein kinase B/Akt (Akt)-dependent mechanisms in bovine aortic endothelial cells (BAEC) (Go, Y. M., Boo, Y. C., Park, H., Maland, M. C., Patel, R., Pritchard, K. A., Jr., Fujio, Y., Walsh, K., Darley-Usmar, V., and Jo, H. (2001) J. Appl. Physiol. 91, 1574–1581). Akt has been believed to regulate shear-dependent production of NO by directly phosphorylating endothelial nitric-oxide synthase (eNOS) at the Ser\textsuperscript{1179} residue (eNOS-S\textsuperscript{1179}), but a critical evaluation using specific inhibitors or dominant negative mutants (Akt\textsuperscript{AA} or Akt\textsuperscript{AAA}) has not been reported. In addition, other kinases, including protein kinase A (PKA) and AMP kinase have also shown to phosphorylate eNOS-S\textsuperscript{1179}. Here, we show that shear-dependent phosphorylation of eNOS-S\textsuperscript{1179} is mediated by an Akt-independent, but a PKA-dependent, mechanism. Expression of Akt\textsuperscript{AA} or Akt\textsuperscript{AAA} in BAEC by using recombinant adenoviral constructs inhibited phosphorylation of eNOS-S\textsuperscript{1179} if cells were stimulated by vascular endothelial growth factor (VEGF), but not by shear stress. As shown before, expression of Akt\textsuperscript{AA} inhibited shear-dependent NO production, suggesting that Akt is still an important regulator in NO production. Further studies showed that a selective inhibitor of PKA, H89, inhibited shear-dependent phosphorylation of eNOS-S\textsuperscript{1179} and NO\textsuperscript{\textsuperscript{2}} production. In contrast, H89 did not inhibit phosphorylation of eNOS-S\textsuperscript{1179} induced by expressing a constitutively active Akt mutant (Akt\textsuperscript{MYR}) in BAEC, showing that the inhibitor did not affect the Akt pathway. 8-Bromo-cAMP alone phosphorylated eNOS-S\textsuperscript{1179} within 5 min without activating Akt, in an H89-sensitive manner. Collectively, these results demonstrate that shear stimulates phosphorylation of eNOS-S\textsuperscript{1179} in a PKA-dependent, but Akt-independent manner, whereas the NO production is regulated by the mechanisms dependent on both PKA and Akt. A coordinated interaction between Akt and PKA may be an important mechanism by which eNOS activity is regulated in response to physiological stimuli such as shear stress.

Endothelial cells are constantly subjected to shear stress, the dragging force generated by blood flow. Shear stress triggers a variety of biochemical and physical changes in cell structure and function. For example, shear stress regulates vascular tone and diameter, inflammatory responses, hemostasis, and vessel wall remodeling (1). Laminar shear stress has been shown to play anti-atherogenic roles by inhibiting some of the key pro-atherogenic events, including apoptosis of endothelial cells and binding of monocytes to endothelium (2–5). Although the exact mechanisms by which laminar shear stress prevents atherosclerosis are not known, NO produced from endothelium seems to play essential roles by mediating many effects of laminar shear stress: vessel relaxation, inhibition of apoptosis, and monocyte adhesion triggered by the pro-atherogenic factors (2, 4–6).

Although it is well known that exposure of endothelial cells to shear stress stimulates production of NO from endothelial nitric-oxide synthase (eNOS)\textsuperscript{3} both in cultured cells and in intact vessels (7, 8), the molecular mechanisms by which shear stress regulates NO\textsuperscript{\textsuperscript{4}} production have not been clearly elucidated. eNOS is known as a Ca\textsuperscript{2+}-calmodulin (CaM)-dependent form of NO (9). Indeed, most humoral ligands, including bradykinin, acetylcholine, and ATP, stimulate NO production by raising the level of intracellular Ca\textsuperscript{2+}, which forms Ca\textsuperscript{2+}-CaM complex (9). In the basal state, the majority of eNOS appears to be bound to caveolin-1 with its enzyme activity repressed in caveolae (10, 11). This tonic inhibition of eNOS can be released by displacing caveolin-1 with Ca\textsuperscript{2+}-CaM in response to Ca\textsuperscript{2+}-mobilizing agonists (10). Unlike Ca\textsuperscript{2+}-mobilizing hormones, however, shear stress stimulates production of NO from eNOS by a mechanism that does not require a maintained intracellular Ca\textsuperscript{2+} level or CaM (8, 12, 13). Other potential mechanisms that could mediate the acute, shear-dependent activation of eNOS include phosphorylation, acylation, and translocation of the enzyme as well as its interaction with other molecules such as heat shock protein 90 (14–19).

Recent evidence suggested that the phosphorylation of eNOS at Ser\textsuperscript{1179} (based on the bovine eNOS sequence and equivalent

\textsuperscript{3}The abbreviations used are: eNOS, endothelial nitric-oxide synthase; Akt\textsuperscript{MYR}, a constitutively active Akt mutant; Akt\textsuperscript{AA} and Akt\textsuperscript{AAA}, dominant negative Akt mutants; BAEC, bovine aortic endothelial cells; CaM, calmodulin; ERK, extracellular signal regulated kinase; HA, hemagglutinin; PI3K, phosphoinositide-3-kinase; PKA, protein kinase A; PKG, protein kinase G; DMEM, Dulbecco's modified Eagle’s medium; VEGF, vascular endothelial growth factor; m.o.i., multiplicity of infection; 8-Br-cAMP, 8-bromo-cAMP; PDK1, phosphoinositide-dependent kinase-1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
to human eNOS-S1177 by a sequential activation of phosphoinositide-3-kinase (PI3K) and protein kinase B/Akt (Akt) pathway is the underlying mechanism by which shear stress stimulates NO\(^+\) production in a Ca\(^{2+}\)-CaM-insensitive manner (14, 20). In the cases of vascular endothelial growth factor (VEGF), sphingosine 1-phosphate, and estrogen, there is strong evidence supporting PI3K activation of Akt, which in turn is responsible for regulating the phosphorylation and activation of eNOS. However, published reports (14, 19, 20) have not provided direct evidence that Akt is indeed the protein kinase directly responsible for phosphorylation of eNOS-S1179 and its subsequent activation in response to shear stress. For example, it has not been reported whether expression of a dominant negative Akt constructs such as Akt\(^{AA}\) or Akt\(^{AAA}\) can block the shear-dependent phosphorylation of eNOS-S1179 and NO\(^+\) production in endothelial cells (19, 20).

In addition, it is becoming increasingly clear that Akt is not the only protein kinase that can phosphorylate eNOS-S1179. Other protein kinases, including protein kinase A (PKA), protein kinase G (PKG), and AMP kinase have also been shown to phosphorylate eNOS-S1179 (24–27). It may be possible that eNOS-S1179 can be phosphorylated by different protein kinases depending upon each given stimuli.

Here, we examined whether Akt regulates phosphorylation of eNOS-S1179 in response to shear stress in bovine aortic endothelial cells (BAEC) by using adenoviral constructs expressing a constitutively active form (Akt\(^{Myr}\)) or dominant negative forms of Akt mutants (Akt\(^{AA}\) and Akt\(^{AAA}\)). Because our initial results indicated that shear stimulates phosphorylation of eNOS-S1179 in an Akt-independent manner, we examined other protein kinases to characterize their role in the phosphorylation. Our results demonstrate that, unlike VEGF, shear stress stimulates phosphorylation of eNOS-S1179 by an Akt-independent, but PKA-dependent, manner.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—BAEC harvested from descending thoracic aortas were maintained (37 °C, 5% CO\(_2\)) in a growth medium ( Dulbecco’s minimum Eagle’s medium (DMEM) containing 1-glucose (Invitrogen) and 20% fetal bovine serum (Atlanta Biologica) without antibiotics (28)). BAEC used in this study were between passages 5 and 10. Unless specified otherwise, 2 million cells were seeded in 100-mm tissue culture dishes (Falcon) and grown to confluency in the growth medium before exposure to shear stress.

**Adenoviral Infections**—BAEC were infected with recombinant adenovirus at 50 multiplicity of infection. Cells were infected with adenovirus in serum-free DMEM for 1 h and then incubated for 48 h in a growth medium before the treatment. Three different recombinant adenoviral constructs (Ad-Akt\(^{Myr}\), Ad-Akt\(^{AA}\), and Ad-Akt\(^{AAA}\)) were used to overexpress Akt mutants. Akt\(^{AA}\) is a constitutively active Akt mutant generated by fusing a myristoylation signal to its amino terminus (29). Akt\(^{AAA}\) is an Akt mutant generated by substituting Ala at two major regulatory phosphorylation sites (Thr\(^{308}\) and Ser\(^{473}\)) (29). Akt\(^{AAA}\) is another Akt mutant in which the phosphate transfer residue in catalytic site (Lys\(^{179}\)), in addition to Thr\(^{308}\) and Ser\(^{473}\), was replaced by Ala (22). Both Akt\(^{AA}\) and Akt\(^{AAA}\) have been shown to inhibit Akt specifically in dominant negative manners (22, 29). Recombinant adenovirus encoding β-galactosidase (Ad-β-gal) was used as a control. Infection efficiency of BAEC with recombinant adenovirus at 50 multiplicity of infection (m.o.i.) was close to 100% as determined by immunohistochemical staining of β-galactosidase as described previously (28).

**Shear Stress Studies**—A confluent BAEC monolayer grown in a 100-mm tissue culture dish was exposed to laminar shear stress by rotating Krebs-Ringer carbonate buffer (25 mM NaHCO\(_3\), pH 7.4, 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2PO\(_4\), and 11 mM glucose) with a cone in a 5% CO\(_2\) incubator at 37 °C. To measure accumulation of NO in the medium, a 1–ml sample was collected, replaced with fresh medium, and kept dark on ice until nitrite assay. After shear exposure, cells were washed with ice-cold phosphate-buffered saline and scraped in lysis buffer to measure the amount of protein and subsequent Western blot analysis. A fluorescence assay using 2,3-diaminonaphthol was used to measure nitrite, because it accounts for more than 90% of total NO metabolite accumulating in the medium in response to shear stress (31–33).

**Statistical Analysis**—Statistical analysis was performed by the Student’s test. The significance, p < 0.05, based on at least three or more independent experiments, was considered to be statistically significant.

**RESULTS**

**Shear Stress Stimulates Phosphorylation of eNOS-S1179 and Akt-Thr\(^{308}\)/Ser\(^{473}\)**—We first confirmed that shear stress stimulates phosphorylation of Akt and eNOS. As shown previously (14), exposure of BAEC to an arterial level of laminar shear stress (15 dyn/cm\(^2\)) stimulated the phosphorylation of eNOS-S1179 in a time-dependent manner (Fig. 1A). The phosphorylation of eNOS-S1179 was apparent as early as 2 min after shear onset and reached a maximum by 30 min. During this time period the amount of eNOS did not change as determined by Western blot using an antibody detecting total eNOS.

Shear stress also stimulated phosphorylation of Akt at the two key regulatory sites (Thr\(^{308}\) and Ser\(^{473}\)) as determined by Western blots using antibodies specific for each phosphorylated site (Fig. 1B). The time courses of shear-dependent phosphorylations of Akt at both Thr\(^{308}\) and Ser\(^{473}\) residues were essentially identical. In addition, the time course of Akt phosphorylation was very similar to that of eNOS phosphorylation.

**PKA-dependent Phosphorylation of eNOS-Ser\(^{1179}\) by Shear Stress**—To examine whether the shear-dependent phosphorylation of eNOS and Akt are regulated by PKA-dependent mechanisms, BAEC were pretreated for 30 min with the PKA inhibitor wortmannin, and then exposed to shear stress for up to 30 min. Treatment of the cells with wortmannin completely blocked shear-dependent phosphorylation of eNOS-S1179 (Fig. 2A). As shown previously (14, 31), wortmannin also blocked shear-dependent phosphorylation of Akt-Thr\(^{308}\) and Ser\(^{473}\) (Fig. 2B). In contrast, wortmannin did not block shear-dependent phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (Fig. 2C). It was noteworthy that wortmannin completely blocked shear-induced phosphorylation of Akt (Fig. 2B), whereas it did not inhibit the basal phosphorylation of eNOS-S1179 (Fig. 2A). The results shown in Figs. 1 and 2 clearly demonstrate that shear stress stimulates phosphorylation of eNOS-S1179 and Akt-Thr\(^{308}/Ser\(^{473}\) by a PKA-dependent mechanism, confirming previous reports (14, 31).
Expression of a Constitutively Active Akt Mutant (AktMyr) Induces Phosphorylation of eNOS-S1179

To address the issue whether Akt regulates phosphorylation of eNOS-S1179 in response to shear stress, we used three different recombinant adenoviral constructs to transiently express a constitutively active Akt mutant (AktMyr tagged with HA) or two dominant negative constructs (Akt AAA and Akt AA tagged with HA) in BAEC. First, BAEC were infected with Ad-Akt Myr (10 or 50 m.o.i.) or Ad-β-galactosidase (50 m.o.i.) as a control. The dose-dependent expression of Akt Myr was confirmed by Western blot with antibodies for HA and total Akt. Expression of Akt Myr also activated Akt as determined by Western blots with antibodies specific to phosphorylated forms of Akt-Ser473 and Akt-Thr308 (Fig. 3). As shown in Fig. 3, expression of Akt Myr alone was sufficient to stimulate phosphorylation of eNOS-S1179. In contrast, expression of β-galactosidase did not have any effect on phosphorylation of eNOS-S1179, showing the specific effect of Akt Myr as the kinase.

Dominant Negative Akt Mutants Inhibit Phosphorylation of eNOS-S1179 When Stimulated by VEGF but Not by Shear Stress—Here we directly examined whether Akt regulates shear-dependent NO production by phosphorylating eNOS-S1179 by infecting BAEC with dominant negative Akt mutants. First, we checked whether expression of Akt AAA inhibits shear-dependent NO production in BAEC. As shown previously (31), infection of BAEC with Ad-Akt AAA (50 m.o.i./cell) prevented production of NO stimulated by shear stress demonstrating the essential role of Akt in eNOS activation (Fig. 4). In contrast, when cells were infected with Ad-β-gal as a control, shear stress still stimulated NO production robustly (Fig. 4). This result dem-
Expression of a constitutively active Akt mutant (AktAA) in endothelial cells induces eNOS phosphorylation. BAEC were infected with Ad-AktAA at the indicated level. As a control, Ad-β-gal was used. Two days after the infection, cell lysates were prepared and analyzed by Western blot using antibodies specific for phosphorylated forms of eNOS-S1179 (pS-eNOS), Akt-Thr308 (pT-Akt), and Akt-Ser473 (pS-Akt). Antibodies detecting the total amount of each protein were used to re-probe the same membranes. Because Akt Myr was tagged with an HA epitope, an HA antibody was used to show expression of the transfected protein.

The above results showing that expression of dominant negative Akt mutant (AktAA or AktAAA) inhibits phosphorylation of eNOS-S1179 by VEGF but not by shear stress. A and B, 1 day after infecting BAEC with Ad-AktAA at indicated m.o.i., cells were exposed to shear stress (15 dyn/cm²) for 60 min or stimulated with 50 ng/ml VEGF for 5 min. C and D, cells were infected with Ad-AktAA or Ad-β-gal (50 m.o.i. each) 1 day before exposure to shear stress (15 dyn/cm²) for 60 min or 50 ng/ml VEGF for 5 min. Cell lysates were analyzed by Western blot with antibodies specific for phosphorylated form of eNOS-S1179 (pS-eNOS), total eNOS, total Akt, and HA epitope. The phosphorylated bands were quantified as in Fig. 1, and the bar graphs show means ± S.E. (n = 3).

Role of PKA and ERK Pathways in Shear-dependent Phosphorylation of eNOS-S1179. The above results showing that the shear-dependent phosphorylation of eNOS-S1179 is not mediated in an Akt-dependent manner prompted us to consider alternative mechanisms. In addition to Akt, several other protein kinases, including PKA, PKG, AMP kinase, and ERK pathways have been implicated in eNOS regulation (24–27, 34). We chose to use selective inhibitors of PKA and ERK pathways to examine whether they regulate shear-dependent phosphorylation of eNOS-S1179. First, BAEC were pretreated with 0–20 μM H89, a highly selective PKA inhibitor for 30 min, and then sheared for 30 min. As shown in Fig. 6A, H89 inhibited shear stress-dependent phosphorylation eNOS-S1179 in a dose-dependent manner with a maximum inhibitory effect observed at 10 μM.

This result could be derived from two different possible mechanisms. One possibility is that PKA is an upstream regulator of Akt, which is then responsible for phosphorylating eNOS-S1179 directly. The other is that PKA, without involving Akt, is responsible for phosphorylation of eNOS-S1179 directly or indirectly. If the former were true, H89 would be expected to inhibit Akt phosphorylation. If the latter were true, then H89 would not prevent Akt phosphorylation. As shown in Fig. 6B, the PKA inhibitor did not inhibit phosphorylation of Akt at the two major regulatory sites (Thr308 and Ser473). This result favors the latter mechanism that PKA is responsible for phos-
cAMP as low as 250 μM. As shown in Fig. 8A, H89 significantly stimulated phosphorylation of eNOS-Ser1179, indicating that H89 inhibits shear-dependent phosphorylation of eNOS-Ser1179. H89 Does Not Inhibit Phosphorylation of eNOS-Ser1179 Induced by AktMyr. Therefore, this result strongly demonstrates the specificity of H89 as a PKA inhibitor without any significant effect on Akt kinase activity.

**PKA Mediates NO Production in Response to Shear Stress**—So far, our results showed that PKA mediates phosphorylation of eNOS-Ser1179 in response to shear stress. Does the PKA pathway play a functionally significant role in regulation of eNOS activity (NO production) in response to shear stress? To answer this question, BAEC were pretreated with 10 μM H89, 100 nM wortmannin, or vehicle control for 30 min. Cells were then exposed to shear stress for 30 min, and nitrite accumulating in the shear medium was determined. As shown in Fig. 10, treatment of BAEC with H89 prevented shear-dependent NO production to the same degree as wortmannin. This result provides a strong support for a critical role of PI3K and PKA-dependent mechanisms in NO production in response to shear stress.

**DISCUSSION**

The first significant finding of the present study is that overexpression of the dominant negative mutant of Akt (AktAA or AktAAA) did not inhibit phosphorylation of eNOS-Ser1179 if it was stimulated by shear stress (Fig. 5, A and C). In contrast, AktAA and AktAAA did inhibit eNOS-Ser1179 phosphorylation when it was stimulated by VEGF (Fig. 5, B and D). These results indicate that Akt is not the protein kinase phosphorylating eNOS-Ser1179 at least in response to shear stress. This result prompted us to search for the alternative mechanisms by which eNOS-Ser1179 is phosphorylated in response to shear stress. The second important finding is that we identified an
alternative protein kinase, PKA, that regulates phosphorylation of eNOS-Ser^{1179} and NO\textsuperscript{+} production.

Recent reports have clearly demonstrated the essential role of PI3K in regulation of eNOS in response to shear stress (13, 14). It was further proposed that Akt, which is activated by PI3K-dependent mechanisms, is directly responsible for phosphorylation of eNOS-Ser^{1179} and subsequent NO\textsuperscript{+} production (13, 14, 20, 31). However, this assumption has not been tested directly. Circumstantial evidence that has been used to support the role of Akt in phosphorylation of eNOS-Ser^{1179} and NO\textsuperscript{+} production in response to shear stress is following: 1) The PI3K inhibitors inhibit shear-dependent activation of Akt, phosphorylation of eNOS-Ser^{1179}, and production of NO\textsuperscript{+} (13, 14). 2) Overexpression of the constitutively active Akt^{145Y} mutant can increase phosphorylation of eNOS-Ser^{1179} and NO\textsuperscript{+} production (14, 17). 3) Overexpression of Akt^{AA} inhibits phosphorylation of eNOS on unknown Ser residues (20). These findings clearly demonstrate that the shear-dependent activation of eNOS is regulated by the PI3K-dependent mechanisms. Most of these findings have been reproduced, expanded, and confirmed in the
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The differential effects of the dominant negative Akt mutants on eNOS phosphorylation (Fig. 5) provide strong evidence supporting a new concept that phosphorylation of eNOS-S<sup>1179</sup> can be regulated by the Akt-independent as well as -dependent mechanisms depending upon each stimulus. In the case of VEGF, we confirm that phosphorylation of Akt is required for phosphorylation of eNOS-S<sup>1179</sup>. In the case of shear stress, however, Akt does not play an essential role in the phosphorylation of eNOS-S<sup>1179</sup>. Then, how does shear stress regulate phosphorylation of eNOS-S<sup>1179</sup> in an Akt-independent manner?

It is important to emphasize that Akt is not the only downstream target of PI3K in endothelial cells. It has been demonstrated that PI3K activates phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates and activates not only Akt, but also many other target kinases, including PKA, PKG, PKC, serum- and glucocorticoid-inducible kinase, and p<sup>70</sup>S6 kinase (36, 37). Moreover, it is becoming increasingly clear that eNOS-S<sup>1179</sup> can be phosphorylated by other protein kinases, including PKA, PKG, and AMP kinase in addition to Akt depending on each stimulus (24, 26, 27). Therefore, we began to screen the role of other protein kinases in shear-dependent phosphorylation and activation of eNOS.

In this study, we examined two protein kinases, PKA and ERK, because they have been implicated in regulation of phosphorylation and activity of eNOS (27, 34). First, we showed that the ERK1/2 pathway (by using the MEK1/2 inhibitor) does not play a significant role in shear-dependent phosphorylation of eNOS-S<sup>1179</sup> (Fig. 7). This is consistent with our previous finding that inhibition of ERK1/2 pathway by treating BAEC with pertussis toxin (inhibitor of G<sub>a<sub>q</sub></sub> protein family) had no effect on shear-dependent NO<sup>+</sup> production (31). In comparison, the ERK1/2 pathway has been shown to stimulate eNOS phosphorylation at sites other than the Ser<sup>1179</sup> residue in response to bradykinin, and that inhibition of ERK1/2 activity stimulated eNOS activity (34). Taken together, these results suggest that the ERK1/2 pathway is not involved in phosphorylation of eNOS-S<sup>1179</sup>.

Next, we found that PKA plays a critical role in shear-dependent phosphorylation of eNOS-S<sup>1179</sup> in BAEC. Treatment of BAEC with H89 blocked shear-dependent phosphorylation of eNOS-S<sup>1179</sup> without inhibiting Akt phosphorylation (Fig. 6, A and B), suggesting a role for PKA. It was further demonstrated that stimulation of PKA by using a cell-permeable cAMP analog, 8-Br-cAMP, alone maximally stimulated phosphorylation of eNOS-S<sup>1179</sup> within a few minutes (2–5 min) (Fig. 8A). It should be noted that Akt phosphorylation was not at all stimulated by 8-Br-cAMP during the same time period (Fig. 8A). Only after 15 min or longer incubation, 8-Br-cAMP modestly increased phosphorylation of Akt (Fig. 8A). These results clearly illustrate two points that phosphorylation of eNOS-S<sup>1179</sup> does not have to require Akt and 2) can be regulated by a PKA-dependent manner in response to shear stress.

In our study we tested whether the effects of H89 on phosphorylation of eNOS-S<sup>1179</sup> were due to its direct inhibition of Akt activity. If this possibility were true, treatment of cells with the inhibitor would have prevented the eNOS-S<sup>1179</sup> phosphorylation induced by the constitutively active Akt. However, this possibility is highly unlikely, because H89 did not have any effect on phosphorylation of eNOS-S<sup>1179</sup> if it was induced by Akt<sup>Myr</sup> (Fig. 9). Interestingly, we observed that H89 treatment alone strongly increased phosphorylation of Akt (Figs. 6B and 8B). At this time the mechanism underlying the stimulatory effect of H89 on Akt phosphorylation is not known. One of our speculations is that basal PKA activity may be involved in controlling dephosphorylation of Akt and that H89 may inhibit...
the dephosphorylation pathway. Consistent with this speculation, PKA has been shown to regulate protein phosphatases (27, 38). Nevertheless, what is clear at this point is that, even when Akt phosphorylation was maximally stimulated by H89, eNOS phosphorylation was completely inhibited. This argues against the role of Akt as the protein kinase directly phosphorylating eNOS-S1179.

Collectively, our results indicate that PKA plays a critical role in phosphorylation of eNOS-S1179. In support of this finding, eNOS-S1179 has shown to be phosphorylated either directly by PKA catalytic subunits in vitro or by treatment with isobutylmethylxanthine (increases cAMP level in cells) in BAEC (25, 27). However, the significance of PKA-dependent phosphorylation of eNOS-S1179 in response to physiological stimuli has not been determined until the current study. Our study establishes for the first time that PKA pathway plays a critical role in eNOS-S1179 phosphorylation under a physiologically relevant condition, shear stress. Furthermore, we established the functional significance of PKA pathway in regulation of eNOS activity (NO production) under shear stress condition. We showed that treatment of H89 prevented shear-dependent NO production in BAEC (Fig. 10). In support of our finding, the cAMP and PKA pathway has been shown to induce NO production in isolated arteries (39).

One potential mechanism by which shear stress stimulates NO production is through activation of PKA, which in turn regulates phosphorylation of eNOS-S1179 directly or indirectly. However, it is not clear at this time whether phosphorylation of eNOS-S1179 is directly responsible for NO production in response to shear stress. This caution is especially important in light of the previous reports showing that NO production from eNOS can be regulated independently of the phosphorylation status of eNOS-S1179 (26, 35). For example, bradykinin has been shown to stimulate phosphorylation of eNOS-S1179 as well as NO production (26, 34). However, treatment of the cells with a PI3K inhibitor (wortmannin) did not inhibit NO production, whereas it blocked the phosphorylation of eNOS-S1179 in response to bradykinin (26, 34).

Interestingly, we found that expression of AktAA inhibited shear-dependent stimulation of NO production without affecting eNOS-S1179 phosphorylation (Figs. 4 and 5). These findings demonstrate that, despite the lack of its effect on eNOS-S1179 phosphorylation, Akt still is a critical mediator of shear-dependent NO production. Then, how does Akt regulate eNOS activation in response to shear stress without regulating phosphorylation of eNOS-S1179? One potential mechanism is that there are unidentified amino acid residues in eNOS that can be phosphorylated by Akt. Alternatively, Akt may activate other protein kinases and phosphatases, which then regulate phosphorylation of eNOS on other sites. In support of these ideas, in addition to Ser1179, eNOS has been shown to contain several other phosphorylation sites, including Ser116, Thr497, and Ser335 (14, 17, 19, 26, 27, 40) and some unknown sites as well (20, 34). Another possibility is that Akt may regulate eNOS activity by regulating other regulatory molecules such as caveolin, CaM, and heat shock protein 90 (10, 41). These speculative ideas await further studies.

Based on our results as well as previous findings reported by other investigators, we now propose a following scenario by which shear stress regulates eNOS phosphorylation and NO production as depicted in Fig. 11A. Shear stress stimulates activation of PI3K, which in turn activates PDK1/2. PDK1/2 is then proposed to stimulate both Akt and PKA. PI3K has been shown to phosphorylate and activate PKA (36), although it has not been studied whether shear stress activates PKA by the same mechanisms. Establishing this pathway will require further studies. PKA is then proposed to stimulate phosphorylation of eNOS-S1179 directly or indirectly. In addition, Akt is proposed to regulate NO production by unknown mechanisms, including phosphorylation of eNOS at sites other than the S1179 residue. As comparison, VEGF-dependent stimulation of eNOS-S1179 phosphorylation and subsequent NO production by the PI3K/Akt-dependent pathway are shown in Fig. 11B.

In conclusion, the current study demonstrates that both PKA and Akt play critical roles in regulating phosphorylation of eNOS-S1179 and the enzyme activity (NO production). However, the mechanisms by which these two protein kinase pathways regulate eNOS phosphorylation and enzyme activity seem to be quite different, depending on the given stimulus. In the case of shear stress, phosphorylation of eNOS-S1179 is regulated by a PKA-dependent, but Akt-independent, mechanism, whereas the NO production is regulated by the mechanisms dependent on both PKA and Akt. The activity of eNOS in cells may be controlled through a coordinated regulation and interaction between the two protein kinase pathways, Akt and PKA.

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