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

ROLE OF PROTEIN KINASE A

Recently, we have shown that shear stress stimulates NO\textsuperscript{+} 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-Umar, V., and Jo, H. (2001) J. Appl. Physiol. 91, 1574–1581). Akt has been believed to regulate shear-dependent production of NO\textsuperscript{+} 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\textsuperscript{+} 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{+} production. In contrast, H89 did not inhibit phosphorylation of eNOS-S\textsuperscript{1179} induced by expressing a constitutively active Akt mutant (Akt\textsuperscript{MYV}) 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 (2–6). Although the exact mechanisms by which laminar shear stress prevents atherosclerosis are not known, NO\textsuperscript{+} 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\textsuperscript{+} from endothelial nitric-oxide synthase (eNOS)\textsuperscript{1} both in cultured cells and in intact vessels (7, 8), the molecular mechanisms by which shear stress regulates NO\textsuperscript{+} production have not been clearly elucidated. eNOS is known as a Ca\textsuperscript{2+}-calmodulin (CaM)-dependent form of NOS (9). Indeed, most humoral ligands, including bradykinin, acetylcholine, and ATP, stimulate NO\textsuperscript{+} production from eNOS 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\textsuperscript{+} from NO\textsuperscript{+} 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

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1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; Akt\textsuperscript{MYV}, 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-S1179 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 Ca2+-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 (17, 21–23). 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 been proposed that expression of a dominant negative Akt (Ad-AktAA) construct is sufficient to 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). BAEC were infected with adenoviral constructs expressing a constitutively active form of Akt (Ad-AktAA) or dominant negative forms of Akt mutants (Ad-AktAA and Ad-AktAAA). Because our initial results indicated that shear stress 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 were harvested from descending thoracic aortas that were maintained (37 °C, 5% CO2) in a growth medium (Dulbecco’s minimum Eagle medium (DMEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 2% fetal bovine serum (Atlanta Biologicals) 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 ~90% confluency. 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-AktAA, Ad-AktAA, and Ad-AktAAA) were used to overexpress Akt mutants. Ad-AktAA is a constitutively active Akt mutant generated by fusing a myristoylation signal to its amino terminus (29). Ad-AktAA and Ad-AktAAA were used to overexpress Akt mutants. AktAA is an Akt mutant generated by substituting Ala at two major regulatory phosphorylation sites (Thr308 and Ser473) (29). Ad-AktAAA is another Akt mutant in which the phosphate transfer residue in catalytic site (Lys179), in addition to Thr308 and Ser473, was replaced by Ala (22). Both AktAA and Ad-AktAAA 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 dish was exposed to non-pulsatile, laminar shear stress in a shear medium (pheno-red-free DMEM containing 0.5% fetal bovine serum and 25 mM HEPES, pH 7.4) by rotating a Teflon cone (0.5° cone angle) as described previously (20, 31).

**Preparation of Cell Lysates—**Following experimental treatments, BAEC were washed in ice-cold phosphate-buffered saline and lysed in 0.75 ml of lysis buffer A (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μM microcystin, and 1% Triton X-100). Cell lysates were clarified by spinning at 14,000 x g for 15 min at 4 °C. Protein content of each sample was measured by using a Bio-Rad DC assay (28).

**Immunoblotting—**Aliquots of cell lysates (20 μg of protein each) were resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore) (28). The membrane was incubated with a primary antibody overnight at 4 °C, and then with a secondary antibody conjugated with alkaline phosphatase (1 h at room temperature), which were detected by a chemiluminescence method (28). The bands were visualized using the National Institutes of Health IMAGE program. The following primary antibodies were used: polyclonal antibodies for phosphorylated forms of Akt-Thr308 (p-T-Akt), Akt-Ser473 (p-S-Akt), eNOS-S1179 (pS-eNOS), and ERK1/2 (pERK1/2) and total ERK1/2 from Cell Signaling Technology; a polyclonal antibody for total eNOS from Transduction Laboratories; and a monoclonal antibody against a hemagglutinin (HA) epitope from Roche Molecular Biochemicals.

**NO Assay—**A confluent BAEC monolayer grown in a 100-mm tissue culture dish was exposed to laminar shear stress by rotating Kreh-Ringer carbonate buffer (25 mM NaHCO3, pH 7.4, 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 11 mM glucose) with a cone in a 5% CO2 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-diaminonaphthalene 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 t-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-Thr308/Ser473—**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/cm2) 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 (Thr308 and Ser473) 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 Thr308 and Ser473 residues were essentially identical. In addition, the time course of Akt phosphorylation was very similar to that of eNOS phosphorylation.

**PI3K Regulates Phosphorylation of eNOS-S1179 and Akt-Thr308/Ser473 in Response to Shear Stress—**To examine whether the shear-dependent phosphorylation of eNOS and Akt are regulated by PI3K-dependent mechanisms, BAEC were pretreated with 30 min with the PI3K 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-Thr308 and Ser473 (Fig. 2B). In contrast, wortmannin did not block shear-dependent phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (Fig. 2C). It is noteworthy that wortmannin caused a decrease even in the basal 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-Thr308/Ser473 by a PI3K-dependent mechanism, confirming previous reports (14, 31).
Expression of a Constitutively Active Akt Mutant (Akt\textsuperscript{Myr})
Induces Phosphorylation of eNOS-S\textsuperscript{1179}—To address the issue whether Akt regulates phosphorylation of eNOS-S\textsuperscript{1179} in response to shear stress, we used three different recombinant adenoviral constructs to transiently express a constitutively active Akt mutant (Akt\textsuperscript{Myr} tagged with HA) or two dominant negative constructs (Akt\textsuperscript{AAA} and Akt\textsuperscript{AA} tagged with HA) in BAEC. First, BAEC were infected with Ad-Akt Myr (10 or 50 m.o.i.) or Ad-H\textsubscript{9252}-gal (50 m.o.i.) as a control. The dose-dependent expression of Akt\textsuperscript{Myr} was confirmed by Western blot as described in the legend of Fig. 1. As controls for equal loading, the same membranes were re-probed with antibodies that detect the total amount of each protein. The phosphorylated bands were quantified as in Fig. 1, and the graphs show mean ± S.E. (n = 3).

FIG. 2. A PI3K inhibitor blocks phosphorylation of eNOS and Akt, but not ERK, in response to shear stress. BAEC were pretreated with vehicle (Me\textsubscript{2}SO) or a PI3K inhibitor (100 nM wortmannin) for 30 min before exposure to shear stress (15 dyn/cm\textsuperscript{2}) for the time periods indicated. Phosphorylation of eNOS, Akt, and ERK was determined by Western blot as described in the legend of Fig. 1. As controls for equal loading, the same membranes were re-probed with antibodies that detect the total amount of each protein. The phosphorylated bands were quantified as in Fig. 1, and the graphs show means ± S.E. (n = 3).

PKA-dependent Phosphorylation of eNOS-S\textsuperscript{1179} by Shear Stress

FIG. 1. Shear stress stimulates phosphorylation of eNOS and Akt. Confluent monolayers of BAEC were exposed to laminar shear stress (15 dyn/cm\textsuperscript{2}) with a cone-and-plate viscometer for the time periods indicated. Cell lysates (20 μg/lane) were analyzed by Western blot with antibodies specific for phosphorylated forms of eNOS-S\textsuperscript{1179} (pS-eNOS), Akt-Thr\textsuperscript{308} (pT-Akt), and Akt-Ser\textsuperscript{473} (pS-Akt). The membranes were re-probed with antibodies detecting total eNOS and Akt to monitor equal loading of samples. Densitometry was performed to quantify phosphorylated bands, and the graphs show mean ± S.E. (n = 3).
FIG. 3. 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 AktAA was tagged with an HA epitope, an HA antibody was used to show expression of the transfected protein.

FIG. 4. Expression of a dominant-negative Akt mutant inhibits shear stress-dependent NO production. BAEC were infected with 50 m.o.i. each of Ad-AktAA and Ad-β-gal. One day after the infection, cells were exposed to stationary control or laminar shear stress (15 dyn/cm²) for 1 h in Krebs-Ringer buffer as shear medium. During shear or static exposure, medium (1 ml) was collected and replenished with fresh buffer at indicated time points to determine the accumulation of nitrite in the medium by a fluorescent 2,3-diaminonaphthalene assay. The line graphs show means ± S.E. (n = 3). The above results showing that shear stress stimulates phosphorylation of eNOS-S1179 may 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 is responsible for phosphorylation of eNOS-S1179 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-

demonstrate that the dominant negative Akt mutants prevent eNOS-S1179 phosphorylation if it is stimulated by VEGF but not by shear stress. It is interesting to find that AktAA blocks shear-dependent NO production without inhibiting eNOS-S1179 phosphorylation. Taken together, these results show that shear stress stimulates phosphorylation of eNOS-S1179 in an Akt-independent manner.

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-
phorylation of eNOS-S1179 without involving an Akt pathway.

Interestingly, H89 augmented the phosphorylation of Akt both in basal and sheared cells (Fig. 6B). This result showing that, even when Akt was intensely stimulated by H89, eNOS phosphorylation was not increased (rather, it was completely inhibited) provides further evidence against the role of Akt as the protein kinase directly phosphorylating eNOS-S1179.

Next, we tested whether ERK pathway regulates the shear stress-dependent phosphorylation of eNOS-S1179. BAEC were pretreated with 0–40 μM of PD98059, a selective inhibitor of MEK1/2 (the upstream protein kinase regulating ERK1/2), and then sheared at 15 dyn/cm² for 20 min. Phosphorylations of eNOS-S1179 and Akt-Ser473 stimulated by shear stress were not affected by this inhibitor (Fig. 7, A and B), whereas ERK phosphorylation was inhibited in a concentration-dependent manner (Fig. 7C). These results demonstrate that ERK pathway does not play a significant role in shear-dependent phosphorylation of Akt-Ser1176 and eNOS-S1179.

Treatment of BAEC with cAMP Induces Phosphorylation of eNOS-S1179—To further examine the role of PKA in phosphorylation of eNOS-S1179, we stimulated the protein kinase by incubating BAEC with a direct activator, a cell-permeable cAMP analog (8-Br-cAMP). As shown in Fig. 8A, treatment of cells with 8-Br-cAMP alone maximally stimulated phosphorylation of eNOS-S1179 within 2–5 min, which was sustained for as long as 60 min. In contrast, during the same early incubation period (up to 5 min) Akt phosphorylation was not at all stimulated by 8-Br-cAMP (Fig. 8A). Only after 15-min incubation with 8-Br-cAMP, a modest increase in Akt phosphorylation was observed (Fig. 8A). These results demonstrate that eNOS-S1179 can be rapidly phosphorylated in the absence of Akt phosphorylation in response to cAMP. These also suggest that eNOS-S1179 phosphorylation can be stimulated by an Akt-independent, but by a PKA-dependent, mechanism. In additional dose curve studies, it was also found that a concentration of 8-Br-cAMP as low as 250 μM significantly stimulated phosphorylation of eNOS-S1179 (data not shown).

Next, to determine the specificity of PKA pathway, cells were pretreated with or without H89 (5–20 μM) for 30 min before incubating with 8-Br-cAMP. H89, as low as 5 μM, significantly inhibited phosphorylation of eNOS-S1179 induced by cAMP (Fig. 8B). On the other hand, the PKA inhibitor did not inhibit Akt phosphorylation induced by 8-Br-cAMP (Fig. 8B). In fact, as shown above in Fig. 6B, H89 augmented phosphorylation of Akt induced by cAMP. These results demonstrate that cAMP stimulated eNOS-S1179 phosphorylation by the PKA-dependent mechanisms in an Akt-independent manner.
alternative protein kinase, PKA, that regulates phosphorylation of eNOS-S\textsuperscript{1179} and NO\textsuperscript{\cdot} 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-S\textsuperscript{1179} and subsequent NO\textsuperscript{\cdot} 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-S\textsuperscript{1179} and NO\textsuperscript{\cdot} production in response to shear stress is following: 1) The PI3K inhibitors inhibit shear-dependent activation of Akt, phosphorylation of eNOS-S\textsuperscript{1179}, and production of NO\textsuperscript{\cdot} (13, 14). 2) Overexpression of the constitutively active Akt\textsuperscript{\textsuperscript{557}} mutant can increase phosphorylation of eNOS-S\textsuperscript{1179} and NO\textsuperscript{\cdot} production (14, 17). 3) Overexpression of Akt\textsuperscript{\textsuperscript{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

Fig. 7. ERK pathway does not regulate shear stress-dependent phosphorylation of eNOS-S\textsuperscript{1179}. Confluent BAEC were pretreated for 30 min with increasing concentrations of PD98059, a MEK1/2 inhibitor, and then exposed to shear stress (15 dyn/cm\textsuperscript{2}) for 20 min. Phosphorylation of eNOS, Akt, and ERK was determined by Western blot as described in Fig. 2. As controls for equal loading, the same membranes were re-probed with antibodies that detect the total amount of each protein. The phosphorylated bands were quantified as in Fig. 1, and the bar graphs show means ± S.E. (n = 3).

Fig. 8. A cell-permeable cAMP analog alone induces eNOS phosphorylation in a PKA-dependent manner. In A, BAEC were stimulated with 1 mM 8-Br-cAMP for indicated time. In B, BAEC were pretreated with the PKA inhibitor, H89 (0–20 μM) for 30 min, and then treated with 1 mM 8-Br-cAMP for another 30 min. Cell lysates (20 μg/lane) were analyzed by Western blot with antibodies specific for the phosphorylated form of eNOS-S\textsuperscript{1179} (pS-eNOS) and pS-Akt and pT-Akt. The membranes were re-probed with antibodies detecting total eNOS and Akt to monitor equal loading of samples. In A, the phosphorylated bands (pS-eNOS and pS-Akt) were quantified as in Fig. 1, and the bar graphs show means ± S.E. (n = 3).
FIG. 9. PKA inhibitor does not block phosphorylation of eNOS-Ser1179 induced by AktMyr. BAEC were infected with 50 m.o.i. of Ad-AktMyr, and 2 days after the infection, cells were treated with 0–20 μM H89 for 60 min, and cell lysates were analyzed by Western blot with an antibody specific for the phosphorylated form of eNOS-S1179 (pS-eNOS). The membranes were re-probed with antibodies detecting total eNOS and Akt to monitor equal loading of samples and expression of AktMyr, respectively. H89 has virtually no effect on eNOS-S1179 phosphorylation induced by AktMyr overexpression.

FIG. 10. Shear stress-dependent NO production is regulated by PI3K- and PKA-dependent mechanisms. BAEC were pretreated with vehicle (Me2SO) or a PI3K inhibitor (100 nM wortmannin) or a PKA inhibitor (10 μM H89) for 30 min before exposure to stationary control or laminar shear stress (15 dyn/cm2) for 30 min. The accumulation of nitrite was determined as described in Fig. 4. The bar graphs show means ± S.E. (n = 3).

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-S1179 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-S1179. In the case of shear stress, however, Akt does not play an essential role in the phosphorylation of eNOS-S1179. Then, how does shear stress regulate phosphorylation of eNOS-S1179 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 p70S6 kinase (36, 37). Moreover, it is becoming increasingly clear that eNOS-S1179 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-S1179 (Fig. 7). This is consistent with our previous finding that inhibition of ERK1/2 pathway by treating BAEC with pertussis toxin (inhibitor of Gαi/o-protein family) had no effect on shear-dependent NO production (31). In comparison, the ERK1/2 pathway has been shown to stimulate eNOS phosphorylation at sites other than the Ser1179 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-S1179.

Next, we found that PKA plays a critical role in shear-dependent phosphorylation of eNOS-S1179 in BAEC. Treatment of BAEC with H89 blocked shear-dependent phosphorylation of eNOS-S1179 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-S1179 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-S1179 (1) 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-S1179 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-S1179 phosphorylation induced by the constitutively active Akt. However, this possibility is highly unlikely, because H89 did not have any effect on phosphorylation of eNOS-S1179 if it was induced by AktMyr (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...
PKA-dependent Phosphorylation of eNOS-Ser\(^{1179}\) by Shear Stress

FIG. 11. Proposed roles of PKA and Akt in eNOS regulation by shear stress. A, shear stress is proposed to activate PI3K and PDK1/2 in sequence, which in turn stimulate both Akt and PKA. PKA is proposed to regulate phosphorylation of eNOS-S\(^{1179}\) directly or indirectly, whereas Akt phosphorylates eNOS at sites (\(\gamma\)) other than S\(^{1179}\) residue. A coordinated regulation of eNOS by both the PKA and Akt-dependent mechanisms is proposed to result in production of NO. The link between PDK1/2 and PKA has not been established as described in the text. B, in contrast, VEGF stimulates eNOS-S\(^{1179}\) phosphorylation by PI3K- and Akt-dependent mechanisms. Specific inhibitors (wortmannin, Akt\(^{AA}\), H89) and stimulators (cAMP and Akt\(^{Myr}\)) are indicated.

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-S\(^{1179}\).

Collectively, our results indicate that PKA plays a critical role in phosphorylation of eNOS-S\(^{1179}\). In support of this finding, eNOS-S\(^{1179}\) 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-S\(^{1179}\) 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-S\(^{1179}\) 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-S\(^{1179}\) directly or indirectly. However, it is not clear at this time whether phosphorylation of eNOS-S\(^{1179}\) 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-S\(^{1179}\) (26, 35). For example, bradykinin has been shown to stimulate phosphorylation of eNOS-S\(^{1179}\) 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-S\(^{1179}\) in response to bradykinin (26, 34).

Interestingly, we found that expression of Akt\(^{AA}\) inhibited shear-dependent stimulation of NO\(^*\) production without affecting eNOS-S\(^{1179}\) phosphorylation (Figs. 4 and 5). These findings demonstrate that, despite the lack of its effect on eNOS-S\(^{1179}\) 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-S\(^{1179}\)? 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 Ser\(^{1179}\), eNOS has been shown to contain several other phosphorylation sites, including Ser\(^{116}\), Thr\(^{497}\), and Ser\(^{635}\) (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. PKA 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-S\(^{1179}\) directly or indirectly. In addition, Akt is proposed to regulate NO\(^*\) production by unknown mechanisms, including phosphorylation of eNOS at sites other than the S\(^{1179}\) residue. As comparison, VEGF-dependent stimulation of eNOS-S\(^{1179}\) 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-S\(^{1179}\) 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-S\(^{1179}\) 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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