Reciprocal Phosphorylation and Regulation of Endothelial Nitric-oxide Synthase in Response to Bradykinin Stimulation*

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Endothelial nitric-oxide synthase (eNOS) is phosphorylated at Ser-1179 (bovine sequence) by Akt after growth factor or shear stress stimulation of endothelial cells, resulting in increased eNOS activity. Purified eNOS is also phosphorylated at Thr-497 by purified AMP-activated protein kinase, resulting in decreased eNOS activity. We investigated whether bradykinin (BK) stimulation of bovine aortic endothelial cells (BAECs) regulates eNOS through Akt activation and Ser-1179 or Thr-497 phosphorylation. Akt is transiently activated in BK-stimulated BAECs. Activation is blocked completely by wortmannin and LY294002, inhibitors of phosphatidylinositol 3-kinase, suggesting that Akt activation occurs downstream from phosphatidylinositol 3-kinase. BK stimulates a transient phosphorylation of eNOS at Ser-1179 that is correlated temporally with a transient dephosphorylation of eNOS at Thr-497. Phosphorylation at Thr-497 is blocked by wortmannin and LY294002. BK also stimulates a transient nitric oxide (NO) release from BAECs with a time-course similar to Ser-1179 phosphorylation and Thr-497 dephosphorylation. NO release is not altered by wortmannin. BK-stimulated dephosphorylation of Thr-497 and NO release are blocked by the calcineurin inhibitor, cyclosporin A. These data suggest that BK activation of eNOS in BAECs primarily involves dephosphorylation of the enzyme through calcineurin-mediated dephosphorylation at Thr-497.

Endothelial nitric-oxide synthase (eNOS) is an important regulator of cardiovascular homeostasis because it is the major source of nitric oxide (NO) production in vascular endothelial cells. eNOS plays a crucial role in the state of blood vessel vasodilation and hence blood pressure regulation (1). In addition, NO released from the endothelium modulates other processes including platelet aggregation (2), platelet and leukocyte adhesion to the endothelium (2, 3), endothelin-1 generation (4), vascular smooth muscle cell proliferation (5), and angiogenesis (6). Because of the important role of NO in each of these processes, abnormalities in vascular NO production are thought to contribute to the pathogenesis of certain vascular disorders such as those of atherosclerosis and hypertension (7).

eNOS is regulated by various cofactors and substrates, subcellular targeting, protein-protein interactions, and phosphorylation. Recently, specific sites for phosphorylation of eNOS and specific protein kinases that mediate the phosphorylation have been identified. Several laboratories have reported that eNOS is phosphorylated in endothelial cells at Ser-1179 (bovine sequence) by the Akt protein kinase, resulting in about a 2-fold increase in eNOS catalytic activity. Phosphorylation is accompanied also by a decrease in the dependence of the enzyme for Ca\(^{2+}\) calmodulin (CaM) (8–11). Akt is a well known downstream effector of signaling by growth factors that activate the phosphatidylinositol 3-kinase (PI3-kinase) pathway (12). Akt-mediated phosphorylation of eNOS in endothelial cells thus is stimulated by vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (8, 10) and also by fluid shear stress (9). Whether eNOS is phosphorylated by Akt at Ser-1179 in response to eNOS-activating agonists that signal through G-protein-coupled receptors such as those of bradykinin (BK), histamine, ATP, etc. is not known.

Purified eNOS is phosphorylated also by purified AMP-activated protein kinase. Phosphorylation in the presence of Ca\(^{2+}\)-CaM occurs primarily on Ser-1179, which similar to the phosphorylation catalyzed by Akt, results in increased eNOS activity and a decreased dependence on Ca\(^{2+}\)-CaM (13). However, when purified eNOS is phosphorylated by AMP-activated protein kinase in the absence of Ca\(^{2+}\)-CaM, Thr-497 is phosphorylated, resulting in decreased enzyme activity and increased dependence on Ca\(^{2+}\)-CaM. Whether Ser-1179 or Thr-497 phosphorylation of eNOS by AMP-activated protein kinase is regulated in endothelial cells in an agonist-dependent manner is not known. AMP-activated protein kinase is thought to be regulated by vigorous exercise, nutrient starvation, and ischemia/hypoxia when ATP levels drop and AMP accumulates (14).

Because both the Ser-1179 and Thr-497 residues of bovine eNOS are phosphorylated with differential effects on enzyme activity, we wished to determine how these two phosphorylation events may work in concert to regulate eNOS activity in response to the traditional eNOS-activating agonist BK. Specifically, we investigated whether BK stimulation of bovine aortic endothelial cells (BAECs) activates Akt. Second, we investigated whether BK stimulation altered the phosphorylation of eNOS at Ser-1179 or Thr-497. Finally, we investigated whether BK-induced changes in phosphorylation altered the
the procedure described by Ishii et al. our study, passages 2–5 were used, whereas Bernier et al. (19) used passages 2–5. We have observed consistently that BAECs begin to lose their BK signaling capacity beyond passage 5.2Next, we determined whether BK-stimulated Akt activation results in phosphorylation of eNOS at Ser-1179 using an antibody that recognizes the Ser-1179-phosphorylated but not the nonphosphorylated form of eNOS (10, 13). BAECs were treated with BK (1 \mu M) for various times, and cell lysates were prepared. eNOS then was partially purified by affinity chromatography on 2',5'-ADP-Sepharose and immunoblotted (IB) with the anti-phospho-Ser-1179 eNOS antibody (A) and nonphosphospecific eNOS antibody (B). Similar results were obtained in three different experiments.

BK activation of Akt was also assessed by an assay of Akt activity. BAECs were treated with BK (1 \mu M) for various times, cells were lysed, and Akt was immunoprecipitated from lysates with anti-Akt antibody. Immunoprecipitates were then assayed for their ability to phosphorylate the well known Akt substrate, GSK-3\(\beta\). Phosphorylation of GSK-3\(\beta\) was measured utilizing a phosphospecific anti-GSK-3\(\beta\) (Ser-21/9) antibody. Similar results for each blot were obtained in three different experiments (Fig. 1B).

Fig. 2. Bradykinin-stimulated phosphorylation of eNOS at Ser-1179 in BAECs. BAECs were treated with BK (1 \mu M) for 0, 1, 5, 10, 15, or 30 min, and cell lysates were prepared. eNOS then was partially purified by affinity chromatography on 2',5'-ADP-Sepharose and immunoblotted (IB) with anti-phospho-Ser-1179 eNOS antibody (A) and nonphosphospecific eNOS antibody (B). Similar results were obtained in three different experiments.

recognizes both phosphorylated and nonphosphorylated Akt to confirm that there was no BK-dependent change in the total amount of Akt protein during the time-course of the experiments (Fig. 1B).

BK activation of Akt was also assessed by an assay of Akt activity. BAECs were treated with BK (1 \mu M) for various times, cells were lysed, and Akt was immunoprecipitated from lysates with anti-Akt antibody. Immunoprecipitates were then assayed for their ability to phosphorylate the well known Akt substrate, GSK-3\(\beta\), phosphorylation of GSK-3\(\beta\) was measured utilizing a phosphospecific anti-GSK-3\(\beta\) (Ser-21/9) antibody that recognizes the GSK-3\(\beta\) substrate only when it is phosphorylated on Ser-21 and/or Ser-9, previously shown to be sites of Akt-mediated phosphorylation (12). As shown in Fig. 1C, BK stimulated a transient increase in Akt activity and GSK-3\(\beta\) phosphorylation (32-kDa band) with maximal activity observed at 5 min. These results, using two different methods to show that BK activates the Akt protein kinase in cultured BAECs, are in contrast to those reported previously by Bernier et al. (19). In that study, BK (1 \mu M) stimulation of BAECs had no effect on Akt activation as revealed by immunoblotting with an anti-phospho-Akt antibody. In addition, pretreatment of BAECs with wortmannin had no effect on BK-induced complex formation between eNOS and Akt. The reason for this discrepancy may be because of differences in the passages of the BAECs used in their study versus those used in our study. In our study, passages 2–5 were used, whereas Bernier et al. (19) used passages 5–7. We have observed consistently that BAECs begin to lose their BK signaling capacity beyond passage 5.2

Next, we determined whether BK-stimulated Akt activation results in phosphorylation of eNOS at Ser-1179 using an antibody that recognizes the Ser-1179-phosphorylated but not the nonphosphorylated form of eNOS (10, 13). BAECs were treated with BK (1 \mu M) for various times, and cell lysates were prepared. eNOS then was partially purified by affinity chromatography on 2',5'-ADP-Sepharose and immunoblotted with the phosphospecific antibody. As shown in Fig. 2A, no phosphorylation of Ser-1179 was detected under basal conditions (time 0). BK, however, induced a rapid and transient phosphorylation of eNOS (130-kDa band) at Ser-1179 that was significant at 1 min and maximal at 5 min. Although phosphorylation of Ser-1179 was maximal at 5 min, a significant degree of phosphorylation was observed after only 1 min. However, as shown in Figs. 1 and 2, Akt is not activated significantly until 5 min after BK

Materials—Anti-Phospho-Ser-473 Akt antibody was purchased from New England Biolabs. Anti-Akt and anti-Phospho-Ser-21/9 glycoxygen synthase kinase-3\(\alpha/\beta\) (GSK-3\(\alpha/\beta\)) antibodies were prepared in a kit from New England Biolabs. Anti-eNOS antibody was purchased from Transduction Laboratories. Anti-Ser-1179 eNOS and anti-Thr-497 eNOS phosphospecific antibodies were described previously (13). BK, wortmannin, and cyclosporin A were purchased from Sigma. cGMP enzyme-immunoassay kits were purchased from Amersham Pharmacia Biotech. All other chemicals were purchased from Sigma. Rat fetal lung fibroblasts (RFL)-6 cells were obtained from the American Type Culture Collection. BAECs were passaged from primary cultures and used for experiments during passages 2–5.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were carried out as described previously (15, 16). Akt Activity Assay—Akt activity was measured using a nonradioactive immunoprecipitation–kinase assay kit (New England Biolabs). Endothelial NO Release—NO release from BAECs was measured by the procedure described by Ishii et al. (17).

RESULTS AND DISCUSSION

Previous reports have shown that eNOS is phosphorylated at Ser-1179 in cultured endothelial cells in response to the growth factors VEGF and insulin-like growth factor-1 and also in response to fluid shear stress. Phosphorylation seems to be catalyzed by the Akt protein kinase (8–11). Signal transduction pathways of growth factor receptors, however, can differ significantly from those of G-protein-coupled receptors such as the BK B2 receptor. Indeed, although it has been shown that BK activates Akt in HeLa cells (18), BK activation of Akt in endothelial cells has not been reported previously. We therefore determined whether BK activates Akt in endothelial cells using two different methods after BK (1 \mu M) stimulation of cultured BAECs (passages 2–5) for various times. In the first method, Akt activation was monitored by the state of Akt phosphorylation. BK-stimulated cells were lysed, and lysates were immunoblotted with a phosphospecific anti-Akt antibody that recognizes only the Ser-473-phosphorylated (and thus activated) form of Akt. As shown in Fig. 1A, BK stimulated a transient activation of Akt (60-kDa band) that was maximal at 5 min. Lysates were also immunoblotted with an Akt antibody that

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stimulation. Therefore, it seems that activation of only a minor subpopulation of total cellular Akt (seen at 1 min in longer time exposures of the blots shown in Fig. 1) is sufficient to produce near-maximal phosphorylation of eNOS at Ser-1179. Immunoblotting of lysates with a nonphosphospecific eNOS antibody confirmed that changes in phosphorylation were not caused by changes in the total amount of eNOS protein (Fig. 2B).

The effect of BK on phosphorylation of Thr-497 was also investigated using a second antibody that recognizes the Thr-497-phosphorylated, but not the nonphosphorylated, form of eNOS (13). Experiments were performed on BAECs as described above for Ser-1179. Fig. 3A shows that, unlike the case of Ser-1179, Thr-497 was phosphorylated significantly under basal conditions (time 0), and after BK stimulation, eNOS (130-kDa band) was dephosphorylated almost completely within 5 min, after which it became rephosphorylated. Differences observed were not caused by differences in the total amount of eNOS protein, because when lysates were probed with a nonphosphospecific anti-eNOS antibody, equal amounts of eNOS protein were detected for all time points (Fig. 3B).

To determine how changes in phosphorylation of Ser-1179 and Thr-497 affect endothelial NO release, we evaluated potential inhibitors of BK-induced changes in phosphorylation. Akt activation generally occurs downstream from activation of PI3-kinase (12). Akt, however, can be activated through pathways that do not involve activation of PI3-kinase (20). To determine whether BK-stimulated Akt activation and eNOS Ser-1179 phosphorylation in BAECs occurs downstream from PI3-kinase activation, we utilized the specific PI3-kinase inhibitor, wortmannin (21). BAECs were treated with BK (1 μM) for various times after either no pretreatment or pretreatment with wortmannin (100 nM) for 30 min. Akt activities of cell lysates then were assayed using the GSK-3α/β substrate as described earlier. Wortmannin significantly blocked BK stimulation of Akt activity (Fig. 4A) in contrast to BK stimulation alone (Fig. 5B). LY294002 had a similar inhibitory effect on phosphorylation (data not shown). These data support the view that BK stimulates Akt-mediated phosphorylation of eNOS at Ser-1179 in cultured BAECs and that if other protein kinases are involved, they are involved only to a very minor extent.

Previously, it has been shown that wortmannin inhibits growth factor-stimulated NO release from cultured endothelial cells (8, 23). Inhibition is partial (about 50%) and has been attributed to a blockade of Akt-mediated phosphorylation of eNOS at Ser-1179 in cultured BAECs and that if other protein kinases are involved, they are involved only to a very minor extent.
BAECs were preincubated with or without wortmannin (100 nM) for 30 min prior to treatment with BK (1 μM) for 0, 1, 5, 10, 15, or 30 min. Cell culture medium then was transferred to confluent RFL-6 cell cultures for 3 min and removed. RFL-6 cells were lysed, and cGMP was measured in the cell lysates using an enzyme immunoassay. Data represent the mean ± S.E. from three experiments.

As shown in Fig. 6, maximal BK activation of eNOS is at least 6-fold, whereas maximal VEGF activation of eNOS in cultured endothelial cells consistently has been reported to be only about 2-fold (8, 24).

We also examined the effects of VEGF stimulation on phosphorylation of eNOS at Ser-1179 and Thr-497. BAECs were treated with VEGF (20 ng/ml) for various times, and cells were lysed. eNOS phosphorylation then was examined with the two different phosphospecific anti-eNOS antibodies. As shown in Fig. 7A, VEGF stimulated a transient phosphorylation of eNOS at Ser-1179, which was consistent with the results of previous reports (8, 10). However, in contrast to BK, VEGF did not stimulate dephosphorylation of eNOS at Thr-497 (Fig. 7B). Furthermore, wortmannin had no effect on the BK-stimulated dephosphorylation of Thr-497 (data not shown), suggesting that BK-stimulated NO release may in fact be regulated through the effects of dephosphorylation at the Thr-497 site. Taken together, these results may help to explain the fact that BK activates eNOS to a much greater extent than does VEGF. The high level of activation induced by BK may require a deinhibitory component consisting of the dephosphorylation of eNOS at the Thr-497 inhibitory phosphorylation site. The lower level of activation of eNOS by VEGF (as compared with BK) may be caused by the absence of this dephosphorylation component in the VEGF signal transduction pathway. Support for this hypothesis is provided also by the observation that the time-course of BK-stimulated Thr-497 phosphorylation (Fig. 3) is correlated more closely with the time-course of BK-stimulated NO release (Fig. 6) than is the time-course of BK-stimulated Ser-1179 phosphorylation (Fig. 2).

To examine further the role of Thr-497 dephosphorylation in the BK-stimulated eNOS activation process, we sought to identify the protein phosphatase responsible for catalyzing the dephosphorylation reaction. Four major serine/threonine-specific protein phosphatases are found in mammalian cells termed protein phosphatase-1, -2A, -2C, and -2B/CaM-dependent phosphatase known as protein phosphatase-2B or calcineurin (25). Protein phosphatase-1 and -2C are inhibited potently by okadaic acid, whereas protein phosphatase-2A and calcineurin are not (26). BAECs were preincubated with okadaic acid (100 nM) for 1 h prior to stimulation with BK (1 μM) for various times, and cell lysates were prepared. Samples then were processed as described earlier and immunoblotted with the phosphospecific anti-eNOS antibody that recognizes the Thr-497-phosphorylated, but not the nonphosphorylated, form of eNOS. Okadaic acid pretreatment had no effect on BK-stimulated dephosphorylation of Thr-497 (data not shown), suggesting that neither protein phosphatase-1 nor protein phosphatase-2A is responsible for catalyzing the dephosphorylation reaction. Next, we utilized cyclosporin A, an immunosuppressive drug that is a specific inhibitor of calcineurin (27), to determine whether calcineurin may be the responsible phos-
phatase. BAECs were pretreated with cyclosporin A (100 nM) for 30 min prior to treatment with BK (1 μM) for various times. Immunoblotting experiments then were carried out utilizing the phosphospecific antibody. Cyclosporin A pretreatment completely blocked BK-stimulated dephosphorylation of eNOS at Thr-497 (Fig. 8A), implicating calcineurin as the phosphatase responsible for mediating the dephosphorylation event. Equal loading of eNOS protein on the gel was confirmed by immunoblotting with a nonphosphospecific anti-eNOS antibody (Fig. 8B).

To determine whether calcineurin-mediated dephosphorylation of eNOS at Thr-497 has a role in agonist stimulation of eNOS activity, we examined the effect of cyclosporin A on BK-stimulated NO release from BAECs. Cells were either pretreated or not pretreated with cyclosporin A (100 nM) for 30 min and then exposed to BK (1 μM) for various times. BK-stimulated NO release then was quantitated by reporter cell assay of cGMP production. As shown in Fig. 9, cyclosporin A almost completely blocked BK stimulation of NO release, suggesting that calcineurin-mediated dephosphorylation of eNOS at Thr-497 may in fact play an important role in activation of eNOS after BK stimulation of BAECs.

Previously, Dawson et al. (28) have shown that neuronal NOS (nNOS) is a calcineurin substrate and that dephosphorylation of nNOS by calcineurin (at an as yet unknown residue) increases NOS catalytic activity in primary neuronal cultures. Thus, evidence exists for regulation of an NOS enzyme by calcineurin-mediated dephosphorylation. However, the Thr-497 residue found in bovine eNOS (and conserved in human and mouse eNOS) is not found in either human, rat, or mouse nNOS (29). The mechanism of calcineurin regulation of eNOS by calcineurin has not been reported. Furthermore, prior to the work presented here, endothelial cell and agonist-dependent regulation of eNOS by calcineurin have not been reported. The potential role of calcineurin in eNOS regulation also may help to explain the underlying mechanism involved in the development of arterial hypertension in organ transplant recipients who are administered cyclosporin A (30). This negative side effect seems to be caused by reduced NO release from the endothelium (31, 32). The molecular mechanism by which cyclosporin A affects NO release is not known but likely involves the drug acting as a selective inhibitor of the Ca2+-CaM-dependent serine/threonine-specific protein phosphatase calcineurin (27). Thus, the results of this study demonstrating that calcineurin-mediated dephosphorylation of Thr-497 is associated with attenuated NO release in cultured endothelial cells suggests an in vivo mechanism of cyclosporin A in promoting endothelial dysfunction.

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