

# Bradykinin-regulated Interactions of the Mitogen-activated Protein Kinase Pathway with the Endothelial Nitric-oxide Synthase\*

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Activation of the bradykinin B2 receptor in endothelial cells initiates a complex array of cellular responses mediated by diverse signaling pathways, including stimulation of the mitogen-activated protein (MAP) kinase cascade and activation of the endothelial isoform of nitric-oxide synthase (eNOS). Several protein kinases have been implicated in eNOS regulation, but the role of MAP kinases remains less well understood. We explored the interactions between eNOS and components of the MAP kinase pathway in bovine aortic endothelial cells (BAEC). Using co-immunoprecipitation experiments, we isolated eNOS in a complex with the MAP kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2) as well as the protein kinases Raf-1 and Akt. Within minutes of adding bradykinin to BAEC, the eNOS-Raf-1-ERK-Akt heteromeric complex dissociated, and it subsequently reassociated following more prolonged agonist stimulation. Bradykinin treatment of BAEC led to the activation of ERK, associated with an increase in phosphorylation of eNOS; phosphorylation of eNOS by ERK *in vitro* significantly reduced eNOS enzyme activity. Evidence for the direct phosphorylation of eNOS by MAP kinase in BAEC came from "back-phosphorylation" experiments using [ $\gamma$ - $^{32}$ P]ATP and ERK *in vitro* to phosphorylate eNOS isolated from cells previously treated with bradykinin or the MAP kinase inhibitor PD98059. The ERK-catalyzed *in vitro*  $^{32}$ P phosphorylation of eNOS isolated from BAEC treated with bradykinin was significantly attenuated compared with untreated cells, indicating that bradykinin treatment led to the phosphorylation of ERK-sensitive sites in cells. Conversely, eNOS isolated from endothelial cells pretreated with the MAP kinase inhibitor PD98059 showed increased ERK-promoted phosphorylation *in vitro*. Taken together, our results suggest that bradykinin-induced activation of ERK leads to eNOS phosphorylation and enzyme inhibition, a process influenced by the reversible associations of members of the MAP kinase pathway with eNOS.

The nonapeptide bradykinin is a key determinant of vascular

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function; bradykinin B2 receptors in endothelial cells modulate vasodilation, changes in vascular permeability, mitogenesis, and adhesion molecule expression, among other responses (1). The complex array of bradykinin's cellular responses is mediated by diverse signaling pathways, including stimulation of the MAP<sup>1</sup> kinase cascade and activation of the endothelial isoform of nitric oxide synthase (eNOS). Several essential transducers of the bradykinin response, the bradykinin B2 receptor (2), MAP kinase components (3), and eNOS, all can be localized in plasmalemmal caveolae in endothelial cells. Plasmalemmal caveolae serve as sites for the sequestration of diverse signaling proteins, including G protein-coupled receptors, growth factor receptors, G proteins, protein kinases, and nitric-oxide synthases (for a review, see Ref. 4). Caveolae represent spatially restricted membrane domains that may serve to coordinate signaling pathways and provide an additional level of discrimination and control in the cell's response to receptor-mediated activation of specific intracellular effectors. Caveolae-targeted proteins have been broadly implicated both in eNOS regulation and in signaling by components of the MAP kinase cascade (reviewed in Refs. 4 and 5). These facts suggested to us that there may be dynamic interactions of these signaling pathways that are modulated by the bradykinin B2 receptor.

The endothelial nitric-oxide synthase (eNOS) is a Ca<sup>2+</sup>-calmodulin-dependent enzyme that has been identified as an important determinant of vascular tone and platelet function. It has been established that bradykinin is a key endogenous activator of eNOS via B2 receptor-mediated increases in intracellular Ca<sup>2+</sup> consequent to the G protein-dependent activation of phospholipase C and the stimulation of downstream Ca<sup>2+</sup>-mobilizing pathways (6). It has also been clearly shown that bradykinin treatment of different cell types leads to activation of the MAP kinase cascade (7–9). Receptor-mediated stimulation of the MAP kinase pathway can be initiated by activation of the GTP-binding protein Ras; activated Ras then recruits the kinase Raf-1 to the plasma membrane, leading to Raf-1 phosphorylation and ultimately to the phosphorylation and activation of MAP kinases including ERK (10–12). An alternative pathway for MAP kinase activation by G protein-coupled receptors is independent of Ras and instead involves protein kinase C-dependent phosphorylation of Raf-1 (13). In addition to the existence of such stimulus-specific pathways for activation of MAP kinases, the signaling responses downstream from

<sup>1</sup> The abbreviations used are: MAP, mitogen-activated protein; NOS, nitric-oxide synthase; eNOS, endothelial isoform of NOS; ERK, extracellular signal-regulated protein kinase; BAEC, bovine aortic endothelial cell(s); TBS, Tris-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance.

MAP kinase can be importantly influenced by cell-specific protein interactions, adding to the complexity of this kinase cascade. In cultured vascular endothelial cells, bradykinin treatment has been found to activate the extracellular signal-regulated kinases ERK1 and ERK2 (14), also known as the p42/44 MAP kinase. ERK itself is phosphorylated following the upstream activation (by phosphorylation) of protein kinases Raf-1 and MAP kinase/ERK kinase (MEK).

The interrelationships of eNOS and MAP kinase pathways are less well understood. Although there are numerous putative MAP kinase phosphorylation consensus sequences in the eNOS molecule, it has not been previously established that eNOS itself is directly phosphorylated by MAP kinase in cells. Several other protein kinases have been more directly implicated in eNOS regulation, including kinase Akt (15–18), AMP-activated protein kinase (19–20), protein kinase C (21), and cyclic GMP-dependent protein kinase (19). Specific residues on eNOS have been identified that undergo phosphorylation by these kinases, but it appears that some residues may undergo phosphorylation by more than one kinase (*e.g.* Ser<sup>1177</sup> can be phosphorylated both by kinase Akt (15–18) and by the AMP-activated kinase (20)), yet other phosphorylated residues have no clear kinase consensus sequences. The proximity of eNOS and MAP kinases within plasmalemmal caveolae may facilitate their interactions and coordinate the regulation of these complex signaling pathways, both of which are known to be separately activated by bradykinin. In the present studies, we explore the role of bradykinin in modulating the interactions between eNOS and members of the MAP kinase pathway in cultured endothelial cells, and we also provide evidence indicating that ERK inhibits eNOS by phosphorylating the enzyme in endothelial cells.

#### EXPERIMENTAL PROCEDURES

**Materials**—Fetal bovine serum, cell culture reagents, and media were purchased from Life Technologies, Inc. Anti-eNOS, anti-c-Raf-1 monoclonal, and anti-mouse IgG<sub>1</sub> antibodies were purchased from Pharmingen (Lexington, KY). Anti-MAP kinase (ERK1/2) polyclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-active MAP kinase polyclonal antibody was purchased from Promega (Madison, WI). Anti-phospho-Akt (Ser<sup>473</sup>) and anti-Akt polyclonal antibodies were purchased from New England BioLabs (Beverly, MA). Antibody 12CA5 directed against the HA epitope was from Roche Molecular Biochemicals. Recombinant MAP kinase (ERK2), PD98059, calphostin C, and wortmannin were purchased from Calbiochem. HOE 140 was purchased from Research Biochemicals International (Natick, MA). [ $\gamma$ -<sup>32</sup>P]ATP and [<sup>32</sup>P]orthophosphoric acid were purchased from ICN (Costa Mesa, CA). L-[<sup>3</sup>H]arginine was purchased from Amersham Pharmacia Biotech. Tris-buffered saline and phosphate-buffered saline were purchased from Boston BioProducts (Ashland, MA). Other reagents were from Sigma. The HA-tagged bovine eNOS cDNA has been previously described (22). This plasmid served as a template for the generation of the S1179A mutant by PCR-based site-directed mutagenesis using the manufacturer's protocols (Stratagene). The forward PCR primer for mutagenesis was 5'-CGTACCCAGGCCTTTCCCTG-3', and the reverse primer was 5'-CAGGGAAAAGCCTGGGTACG-3'. Following amplification, isolation, restriction digestion, and molecular cloning of the fragment containing the mutation, the nucleotide sequence of the amplified/mutated fragment was confirmed by dideoxynucleotide sequencing using standard techniques.

**Cell Culture and Transfection**—Bovine aortic endothelial cells (BAEC) were from Cell Systems (Kirkland, WA) and were maintained in culture as described (23). Cells were plated ( $8.5 \times 10^5$  cells/plate) onto gelatin-coated 100-mm plates and studied prior to cell confluence at passages 5–7. All drug treatments were carried out in culture medium; control cultures were incubated in parallel with vehicle-containing medium. Transfection of BAEC with plasmid DNA was performed using Lipofectin according to the manufacturer's protocols (Life Technologies). Transfected cells were studied 48 h following transfection.

**Preparation of Cellular Lysates, Immunoprecipitation, and Immunoblot Analyses**—Following various drug treatments as shown below, the BAEC were washed with phosphate-buffered saline, harvested in buffer

A (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml antipain, 2  $\mu$ g/ml soybean trypsin inhibitor, and 2  $\mu$ g/ml lima bean trypsin inhibitor), solubilized for 30 min at 4 °C, and centrifuged at  $14,000 \times g$ , 15 min. For immunoprecipitation using the eNOS monoclonal antibody, aliquots of cell homogenates were incubated with eNOS antibody at a final concentration of 4  $\mu$ g/ml. After 1 h at 4 °C, protein G-Sepharose beads (80  $\mu$ l of a 50% slurry) were added to the supernatant for a further 1-h incubation at 4 °C. Bound immune complexes were washed three times with buffer A and then eluted by boiling in electrophoresis sample buffer and resolved by SDS-PAGE on 9% gels, transferred onto nitrocellulose membranes, and then probed in immunoblots using eNOS, c-Raf-1, MAP kinase, or Akt antibodies using protocols provided by the suppliers. Immunoprecipitations using the 12CA5 antibody directed against the HA epitope tag were performed according to the manufacturer's instructions. For immunoblot analyses of cell lysates, 10–20 mg of cellular protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies as indicated. For the phospho-MAP kinase immunoblotting, the membranes were blocked for 4 h in 10 mM Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% skim milk. After the membranes had been washed with TBS-T, antibody against phosphorylated MAP kinase (0.2  $\mu$ g/ml) was added in TBS-T containing 0.1% skim milk for 2 h. After washing of the membranes with TBS-T, horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) was added at a 1:10,000 dilution in TBS-T and incubated for 1 h. After a final washing of the membranes with TBS-T, proteins were visualized with SuperSignal Western blot analysis system from Pierce. Immunoblot analyses using the phospho-Akt antibody were performed according to the protocol provided by the supplier.

**In Vitro eNOS Phosphorylation**—BAEC were harvested, lysed, and immunoprecipitated with eNOS antibodies, and the immune complexes were isolated using protein G-Sepharose and washed as described above. The eNOS immune complexes were incubated with recombinant ERK2 (0.1  $\mu$ g) in "phosphorylation buffer" (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 25  $\mu$ M ATP, and 2.5  $\mu$ Ci of  $\gamma$ -[<sup>32</sup>P]ATP at 30 °C for 30 min, in a final concentration of 40  $\mu$ l). The reaction was stopped by adding 15  $\mu$ l of 5 $\times$  concentrated electrophoresis sample buffer. Proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose membrane, and analyzed by autoradiography.

**Measurement of eNOS Enzymatic Activity**—NOS activity was assessed as described previously using anion exchange chromatography to measure the conversion of L-[<sup>3</sup>H]arginine into L-[<sup>3</sup>H]citrulline (24) with minor modifications. Briefly, the immunoprecipitated proteins were incubated at 30 °C for 30 min in 50 mM Tris-HCl, pH 7.4, 10  $\mu$ M tetrahydrobiopterin, 1 mM dithiothreitol, 1 mM CaCl<sub>2</sub>, 4  $\mu$ M flavin mononucleotide, 4  $\mu$ M flavin adenine dinucleotide, 0.5  $\mu$ M L-arginine, 1 mM  $\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form, and 0.2  $\mu$ Ci of L-[<sup>3</sup>H]arginine. The reaction was stopped by the addition of ice-cold sodium acetate (20 mM, pH 5.5) containing 1 mM L-citrulline, 2 mM EDTA, 2 mM EGTA, and the L-[<sup>3</sup>H]citrulline content was determined by anion exchange chromatography.

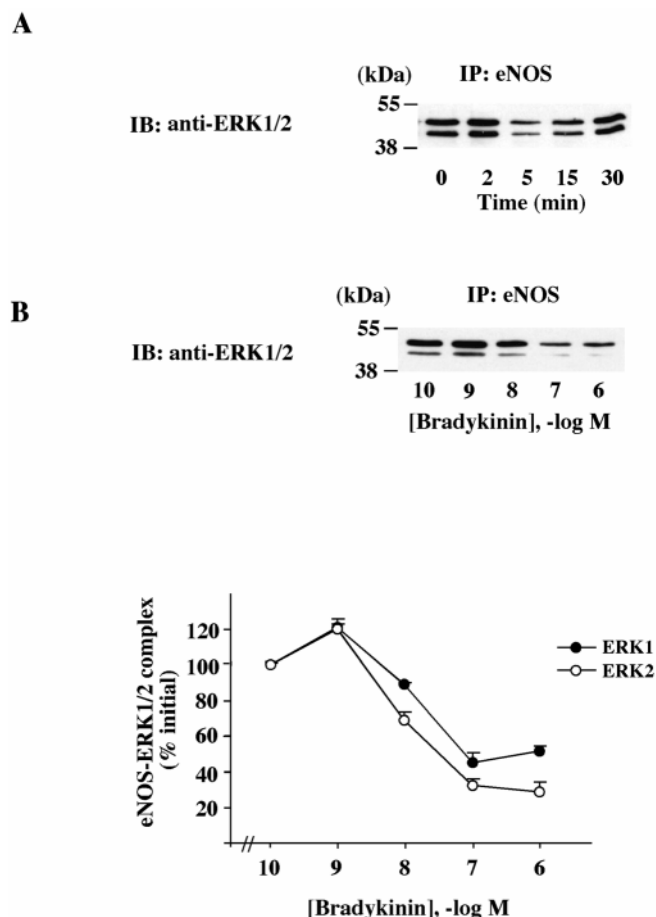
**Biosynthetic Labeling**—BAEC were incubated in phosphate-free Dulbecco's modified Eagle's medium containing 80  $\mu$ Ci/ml [<sup>32</sup>P]orthophosphoric acid (9000 Ci/mmol) for 4 h, exposed to drugs as indicated, and then harvested and immunoprecipitated with eNOS antibody as described above. The phosphorylated proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose membrane, and analyzed by autoradiography. The membrane was later probed by immunoblotting with the eNOS antibody to validate the identification of the eNOS band.

**"Back-phosphorylation" of eNOS**—BAEC were exposed to drugs as indicated and then harvested and solubilized with detergent as described above; the soluble preparation was then immunoprecipitated with eNOS antibody and protein G-Sepharose, and the complexes were washed three times. eNOS phosphorylation was performed using recombinant ERK and [ $\gamma$ -<sup>32</sup>P]ATP as described above. The reaction was stopped by adding 15  $\mu$ l of 5 $\times$  concentrated electrophoresis sample buffer. Proteins were resolved by SDS-PAGE, electroblotted onto nitrocellulose membrane, and analyzed by autoradiography. The membrane was later probed by immunoblotting with the eNOS antibody to validate the identification of the eNOS band.

#### RESULTS

We began our exploration of the possible associations between eNOS and signaling proteins of the MAP kinase pathway by performing a series of co-immunoprecipitation experiments in endothelial cells treated with bradykinin. In resting





**FIG. 2. Effects of bradykinin on interactions of eNOS with ERK.** Shown are the results of co-immunoprecipitation experiments performed in BAEC treated with bradykinin as indicated; immunoprecipitates are isolated using the eNOS antibody, and the immune complexes are resolved by SDS-PAGE followed by immunoblotting with the ERK antibody as described in detail under "Experimental Procedures." **A**, time course of the effects of bradykinin on eNOS-ERK co-immunoprecipitation from BAEC. Cells were incubated with bradykinin ( $1 \mu\text{M}$ ) for the indicated times; eNOS was immunoprecipitated (IP), and immunoblot (IB) analysis was performed with ERK and eNOS antibodies as shown. The experiment shown is representative of four independent experiments. **B**, bradykinin dose response for eNOS-ERK dissociation. BAEC were incubated with indicated concentrations of bradykinin for 5 min, and eNOS was immunoprecipitated, resolved on SDS-PAGE, and subjected to immunoblot analysis using the ERK antibody. The experiment shown is representative of four independent experiments; the lower panel shows the results of densitometric analyses pooled from four experiments, plotting the percentage of the initial co-immunoprecipitation between eNOS and ERK remaining at the indicated doses of bradykinin. Each data point represents the mean  $\pm$  S.E. derived from four independent experiments.

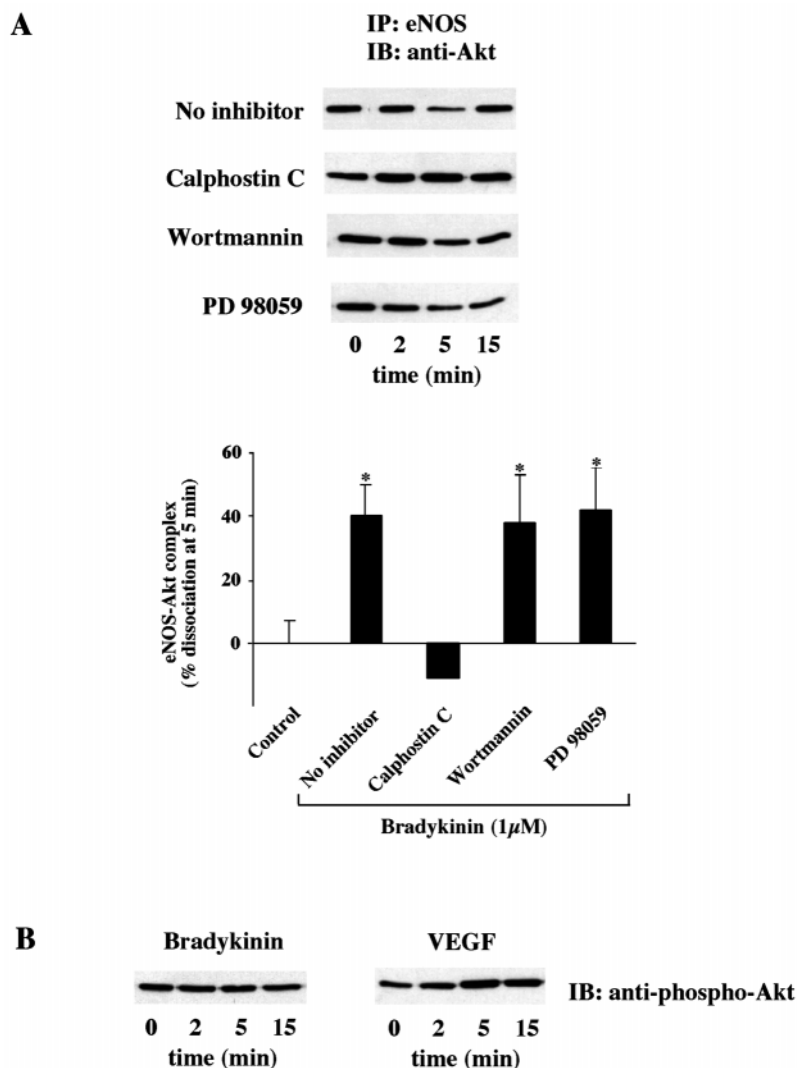
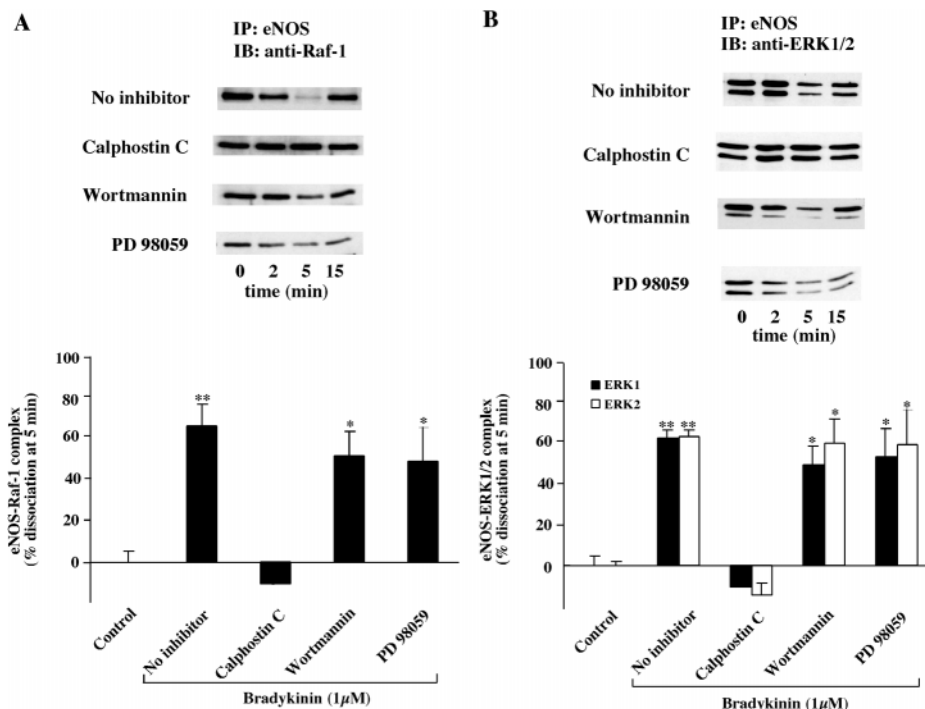
plex reforms with more prolonged agonist treatment. Pretreatment of BAEC with the protein kinase C inhibitor calphostin C blocks the bradykinin-induced dissociation of eNOS and Akt, whereas the kinase inhibitors wortmannin and PD98059 are without effect (Fig. 4A). We next investigated whether kinase Akt itself is activated by bradykinin in BAEC. Akt activity is controlled by phosphorylation of specific threonine and serine residues (27). Using a specific antibody recognizing active (phosphorylated) Akt, we found absolutely no increase in Akt phosphorylation at any time following bradykinin treatment of BAEC (Fig. 4B). In contrast to the lack of effect of bradykinin on Akt activation, we found (Fig. 4B) that vascular endothelial growth factor, which has been previously shown to activate eNOS and to increase Akt phosphorylation (15, 17), leads to a robust increase in Akt phosphorylation.

We next explored whether bradykinin treatment of BAEC would lead to activation of ERK, a response previously observed following bradykinin B2 receptor activation in other cell types (7–9). We treated endothelial cells with bradykinin ( $1 \mu\text{M}$ , 5 min) and quantitated ERK activation by performing immunoblots of cell lysates probed with an antibody specific for activated (phosphorylated) ERK (Fig. 5A). As shown in Fig. 5A, bradykinin leads to ERK activation, a response blocked by the kinase inhibitor PD98059 (a MEK inhibitor) but not by the protein kinase C inhibitor calphostin. We also found that recombinant ERK could phosphorylate eNOS *in vitro*; immunoprecipitated eNOS was incubated with purified recombinant ERK in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , and the phosphorylation of eNOS was analyzed by SDS-PAGE followed by autoradiography (Fig. 5B). To assess the influence of phosphorylation on eNOS activity, we performed NOS enzyme assays following incubation of immunoprecipitated eNOS with ERK (or vehicle) plus (unlabeled) ATP. As shown in Fig. 5C, incubation of eNOS with MAP kinase leads to a significant decrease in NOS activity, measured as the formation of  $[\text{L}\text{-}^3\text{H}]\text{citrulline}$  from  $[\text{L}\text{-}^3\text{H}]\text{arginine}$  ( $43 \pm 2\%$  decrease in enzyme activity; mean  $\pm$  S.E.,  $p < 0.05$ ,  $n = 3$ ).

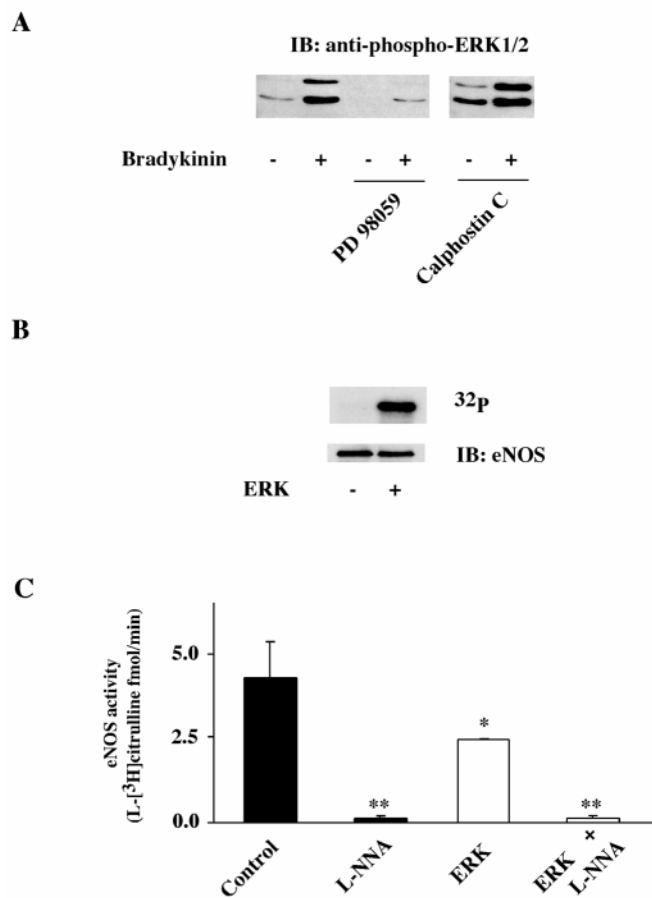
Treatment of  $[\text{P}^{32}]\text{orthophosphate}$  biosynthetically labeled BAEC with bradykinin leads to an increase in eNOS phosphorylation, as we have previously shown (23); this effect of bradykinin was blocked by PD98059 (Fig. 6A). We developed additional evidence for the phosphorylation of eNOS by MAP kinase in BAEC by performing "back-phosphorylation" experiments, in which we used  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and ERK *in vitro* to phosphorylate eNOS that had been isolated from cells that were pretreated with bradykinin or the MAP kinase inhibitor PD98059. As shown in Fig. 6B, the ERK-catalyzed *in vitro*  $^{32}\text{P}$  phosphorylation of eNOS isolated from BAEC that had been pretreated with bradykinin ( $1 \mu\text{M}$ , 5 min) was significantly attenuated relative to untreated cells ( $30 \pm 5\%$  decrease in  $^{32}\text{P}$  labeling of eNOS;  $p < 0.05$ ,  $n = 3$ ), indicating that the bradykinin treatment of endothelial cells leads to the phosphorylation of ERK-sensitive sites in eNOS. Conversely, eNOS isolated from endothelial cells pretreated with the MAP kinase inhibitor PD98059 showed a robust increase in ERK-promoted  $^{32}\text{P}$  phosphorylation *in vitro* (radiolabeling of eNOS following PD98059 treatment was  $162 \pm 6\%$  relative to control cells;  $p < 0.05$ ,  $n = 3$ ). We next performed experiments in BAEC transfected with the mutant eNOS-S1179A, in which serine 1179 (the putative site for phosphorylation by Akt and other kinases) had been changed to alanine (S1179A). We constructed this S1179A mutant eNOS in a plasmid in which eNOS is fused at its C terminus to the HA epitope tag; we have previously validated this construct in detail (22, 28). The use of an epitope tag permits the selective immunoprecipitation of the transfected eNOS, allowing the features of the transfected mutant to be distinguished from the endogenous enzyme expressed in endothelial cells. The transfected eNOS-S1179A mutant still undergoes phosphorylation in  $^{32}\text{P}_i$  biosynthetically labeled BAEC (data not shown), and we therefore performed back-phosphorylation experiments to determine whether the remaining phosphorylation sites were ERK-sensitive. These back-phosphorylation experiments in PD98059-treated BAEC, shown in Fig. 6C, reveal that the S1179-HA-eNOS mutant undergoes ERK-sensitive phosphorylation, just like the wild-type endogenous enzyme.

The next series of experiments investigated the effects of various protein kinase inhibitors on eNOS enzyme activity. We speculated that agonist-induced phosphorylation might lead to a sustained alteration in eNOS enzyme activity that could be measured in assays with the isolated enzyme *in vitro*. BAEC

**FIG. 3. Effects of protein kinase inhibitors on bradykinin-induced dissociation of eNOS and protein kinases Raf-1 or ERK.** BAEC were incubated with protein kinase inhibitors as indicated for 30 min at 37 °C: protein kinase C inhibitor calphostin C (200 nM), phosphatidylinositol 3-kinase inhibitor wortmannin (500 nM), or the MAP kinase inhibitor PD98059 (10  $\mu$ M). Bradykinin (1  $\mu$ M) was then added and the cells harvested at the indicated times following the addition of the agonist. eNOS was immunoprecipitated, and the immune complexes were resolved by SDS-PAGE and analyzed in immunoblots probed with antibodies against Raf-1 (A) or ERK (B). The data shown are representative data of three independent experiments. The lower panels show the results of densitometric analyses from pooled data, plotting the percentage of the eNOS-kinase complex remaining at 5 min following the addition of bradykinin relative to the amount present at  $t = 0$ . Results are shown for blots probed with antibodies against Raf-1 (A) or ERK (B); each data point represents the mean  $\pm$  S.E. derived from three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus value at  $t = 0$  (ANOVA).



**FIG. 4. Effects of protein kinase inhibitors on bradykinin-induced dissociation of eNOS and protein kinase Akt.** A, BAEC were incubated with protein kinase inhibitors as indicated for 30 min at 37 °C: calphostin C (200 nM), wortmannin (500 nM), or PD98059 (10  $\mu$ M). Bradykinin (1  $\mu$ M) was then added, and the cells were harvested at the indicated times following the addition of the agonist. eNOS was immunoprecipitated, and the immune complexes were resolved by SDS-PAGE and analyzed in immunoblots probed with antibodies against Akt. The data shown are representative data of three independent experiments. The middle panel shows the results of densitometric analyses from pooled data, plotting the percentage of the eNOS-Akt complex remaining at 5 min following the addition of bradykinin relative to the amount present at  $t = 0$ ; each data point represents the mean  $\pm$  S.E. derived from three independent experiments. \*,  $p < 0.05$  versus value at  $t = 0$  (ANOVA). B shows an immunoblot probed for activated kinase Akt in cell lysates prepared from BAEC incubated with either bradykinin (1  $\mu$ M) or vascular endothelial cell growth factor (VEGF, 40 ng/ml) for the indicated times. Cell lysates (20  $\mu$ g) were analyzed in immunoblots probed with an antibody directed against activated (phosphorylated) kinase Akt. The experiment shown is representative of three independent experiments, which yielded equivalent results.



**FIG. 5. Activation of ERK by bradykinin and inhibition of eNOS by ERK.** *A* shows the results of immunoblots prepared from BAEC treated with bradykinin and various protein kinase inhibitors and probed with an antibody against activated (phosphorylated) ERK. BAEC were pretreated with PD98059 (10  $\mu$ M) or calphostin C (200 nM) for 30 min and then stimulated without (–) or with (+) bradykinin (1  $\mu$ M, 5 min). Cell lysates (10  $\mu$ g) were resolved on SDS-PAGE, and immunoblots were probed using an anti-phospho-ERK antibody. Representative data from four independent experiments are shown. *B* shows the results of *in vitro* phosphorylation of eNOS by ERK. eNOS was immunoprecipitated from BAEC with the eNOS antibody and incubated in the absence (–) or in the presence (+) of recombinant ERK for 30 min at 30 °C, as described under “Experimental Procedures.” Following SDS-PAGE and transfer to a nitrocellulose membrane, the membrane was analyzed by autoradiography and then probed in an immunoblot with eNOS antibody, as shown. The experiment shown is representative of three independent experiments. *C* shows the results of an eNOS activity assay in which eNOS was immunoprecipitated and incubated with or without recombinant ERK for 30 min at 30 °C, following which eNOS enzymatic activity was measured as described under “Experimental Procedures.” Each data point represents the mean  $\pm$  S.E. of L-[<sup>3</sup>H]citrulline production, derived from three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus control (ANOVA test).

were pretreated with different protein kinase inhibitor for 30 min, treated for varying (brief) times with 1  $\mu$ M bradykinin, harvested, lysed, and immunoprecipitated with the eNOS antibody as described above. NOS enzyme activity was then measured for the immunoprecipitated eNOS (using the [<sup>3</sup>H]arginine-[<sup>3</sup>H]citrulline assay) following various cell treatments. As shown in Fig. 7, bradykinin treatment of BAEC yielded eNOS that showed a transiently increased eNOS activity; a 2-fold increase in enzyme activity was seen at 2 min of bradykinin stimulation, and activity returned to base line by 5 min. The bradykinin-induced increase in eNOS enzyme activity was completely abolished by pretreatment with calphostin C; by contrast, wortmannin did not block the bradykinin-induced tran-

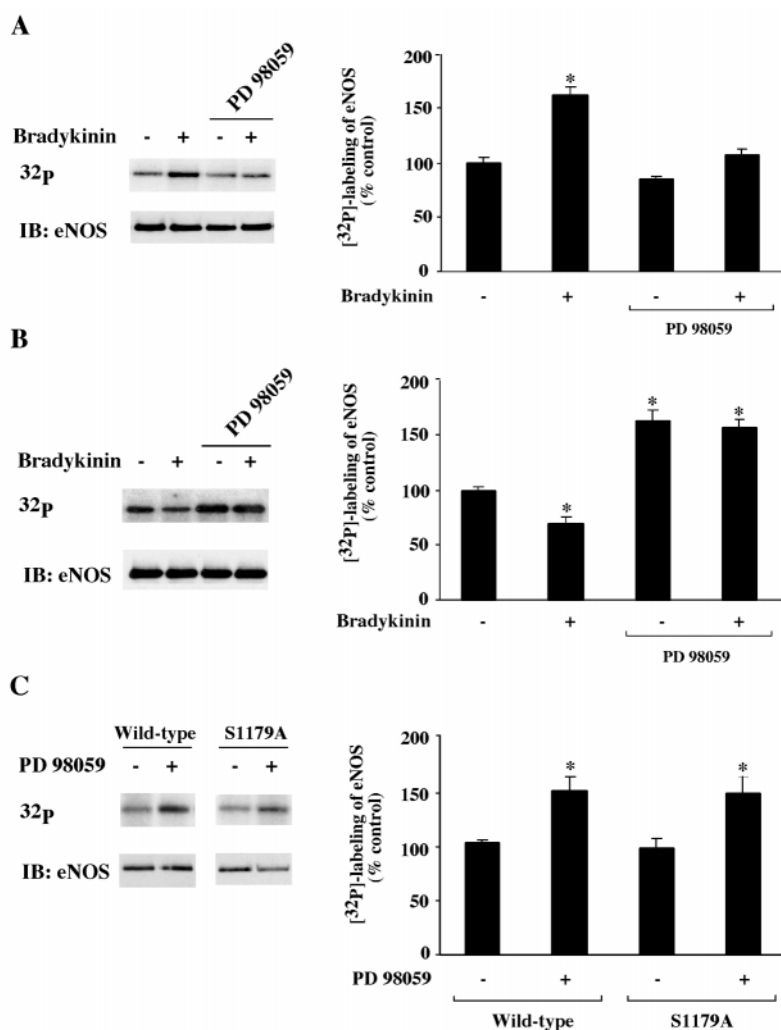
sient enzyme activation. Importantly, pretreatment of BAEC with the MAP kinase inhibitor PD98059 (10  $\mu$ M) induced a marked and sustained increase in eNOS activity (2.2-fold increase;  $p < 0.01$ ,  $n = 3$ ) even in the absence of bradykinin (Fig. 7).

#### DISCUSSION

These studies have provided multiple lines of evidence establishing the existence of regulatory interactions between components of the MAP kinase pathway and eNOS. We have shown that antibodies against eNOS co-immunoprecipitate the MAP kinases ERK1/2 as well as the MAP kinase kinase kinase Raf-1 (Figs. 1 and 2). Control experiments showed that nonimmune serum yields no co-immunoprecipitation, establishing that the immunoprecipitation of these kinases by the eNOS antibody is specific (Fig. 1). Further confidence in the specificity and relevance of these observations comes from experiments that explore the effects of bradykinin on these eNOS-kinase interactions. As shown in Figs. 1 and 2, the addition of bradykinin led to the dissociation of the eNOS-kinase heteromeric complex, which subsequently reassociates. The dose response for the effect of bradykinin on dissociation of the eNOS-kinase complex showed an  $EC_{50}$  of  $\sim 30$  nM, similar to that observed for many physiological responses to bradykinin in cultured cells (29). The antagonist compound HOE140 completely blocks the effect of bradykinin on dissociation of the eNOS-kinase complex, indicating that the response is mediated by B2 bradykinin receptors.

We characterized the bradykinin-regulated interactions of eNOS with several kinases both within the MAP kinase pathway (Raf-1, ERK1, ERK2), as well as with the phosphatidylinositol 3-kinase-activated protein kinase Akt (Figs. 1, 2, and 4). We have previously used cellular imaging approaches to establish that bradykinin promotes the translocation of eNOS from plasmalemmal caveolae to intracellular membranes (30). It is plausible that the approaches used in the current study reveal a biochemical correlate (or consequence) of bradykinin-induced eNOS subcellular translocation, in which bradykinin-induced changes in eNOS lead to its dissociation from heteromeric complexes involving several caveolae-targeted proteins including members of the MAP kinase pathway (Figs. 1 and 2) and protein kinase Akt (Fig. 4). The targeting of these different signaling proteins to caveolae may serve to facilitate their regulatory interactions both with caveolin (4, 5) and with one another and thereby coordinate the multiple cellular responses that are elicited following activation of the B2 bradykinin receptor. The time course of bradykinin-induced eNOS phosphorylation (minutes) is slower (23) than the time course of eNOS activation following B2 receptor stimulation (within several seconds). This temporal sequence of bradykinin-mediated eNOS activation followed by enzyme phosphorylation is consistent with a model wherein ERK phosphorylation serves to attenuate eNOS activation. It appears less likely that eNOS phosphorylation by ERK is necessary for its translocation; we have previously shown that an eNOS mutant expressed only in the cytosol (myristoylation-deficient eNOS) still undergoes phosphorylation (22), and the present studies indicate that an inhibitor of the MAP kinase pathway does not block dissociation of the eNOS-kinase heteromeric complex (Fig. 3). Indeed, since eNOS is reversibly associated with protein kinases that are implicated in opposing regulatory effects on the enzyme, leading to both eNOS activation (Akt) and inhibition (ERK), the proximity of these kinases to eNOS and their reversible associations must be accompanied by a higher level of control.

Different protein kinase inhibitors have distinct effects on bradykinin-induced dissociation of eNOS-kinase complexes in agonist-treated endothelial cells (Figs. 3 and 4). Neither the phosphatidylinositol 3-kinase inhibitor wortmannin nor the

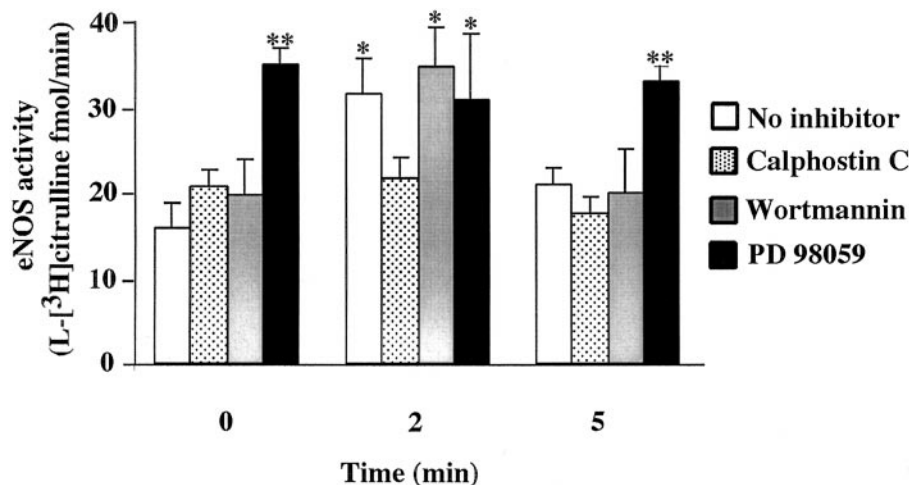


**FIG. 6. Phosphorylation of eNOS in endothelial cells.** The experiments shown in *A* involve <sup>32</sup>P<sub>i</sub> biosynthetic labeling of BAEC, which are treated as indicated and then immunoprecipitated with eNOS antibodies followed by SDS-PAGE and autoradiography. The *left-hand panel* shows a representative experiment in which <sup>32</sup>P<sub>i</sub> biosynthetic labeled BAEC were treated with or without PD98059 (10 μM) for 30 min and then incubated with (+) or without (-) bradykinin (1 μM) for 5 min and then harvested, immunoprecipitated with eNOS antibodies, analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by autoradiography and in immunoblots probed with the eNOS antibody. The *right panel* shows a histogram of phosphorylation data derived from three independent experiments; each data point represents the mean ± S.E. \*, *p* < 0.05 versus control (ANOVA). *B* shows a series of back-phosphorylation experiments, as described in detail under "Experimental Procedures." The *left panel* shows a representative experiment in which BAEC were pretreated with PD98059 (10 μM) for 30 min and then stimulated with (+) or without (-) bradykinin (1 μM) for 5 min. eNOS was immunoprecipitated and subjected to phosphorylation *in vitro* using [γ-<sup>32</sup>P]ATP plus recombinant ERK as described under "Experimental Procedures" and then analyzed by SDS-PAGE followed by autoradiography and subsequent immunoblotting with the eNOS antibody, as shown. Pooled data from three independent experiments are shown in the *histogram* in the *middle panel*; each data point represents the mean ± S.E. \*, *p* < 0.05 versus control (ANOVA). *C* shows the results of back-phosphorylation experiments in BAEC transfected with wild-type or S1179A mutant eNOS cDNAs. BAEC were transfected either with a cDNA construct encoding the HA epitope-tagged S1179A eNOS mutant (*S1179A*) or with the cDNA encoding HA-tagged wild-type eNOS (*Wild-type*) and analyzed in back-phosphorylation experiments in cells treated with PD98059, exactly as above, except that immunoprecipitation was performed with the antibody directed against the HA epitope to selectively immunoprecipitate the transfected construct. Pooled data from three independent experiments are shown in the *histogram panel*; each data point represents mean ± S.E. \*, *p* < 0.05 versus control (ANOVA).

MEK inhibitor PD98059 have substantive effects on bradykinin-induced eNOS dissociation in endothelial cells incubated with these inhibitors prior to treatment with bradykinin. Although PD98059 has no effect on bradykinin-induced eNOS-kinase dissociation, we reproducibly observed that PD98059 markedly delayed the reassociation of the complex, and we found that less of the eNOS-kinase complex was present to begin in cells treated with this kinase inhibitor (Fig. 3). Perhaps this reflects some involvement of the MAP kinase pathway in reassembly of the eNOS-kinase heteromeric complex and/or a role for this pathway in the retargeting of eNOS to plasmalemmal caveolae. The fact that neither wortmannin nor PD98059 attenuate the bradykinin-induced dissociation of eNOS from ERK or Raf-1 (Fig. 3) or from kinase Akt (Fig. 4)

indicates that neither the MAP kinase pathway nor the kinase Akt pathway are directly involved in disassembly of eNOS-kinase complexes, despite their being involved in eNOS inhibition or activation, respectively. By contrast, the protein kinase C inhibitor calphostin completely blocks the bradykinin-induced dissociation of eNOS-protein kinase heteromeric complexes. The inhibitory effect of calphostin suggests a role for protein kinase C somewhere in the pathway leading to the dissociation of eNOS from its associated protein kinases. Activation of protein kinase C may lead to the activation of the MAP kinase pathway (13), but in the present studies, using inhibitor treatments of intact cells, the locus of the calphostin effect cannot be definitively established. Indeed, although we have used these various protein kinase inhibitors at concentra-

**FIG. 7. Effects of protein kinase inhibitors on bradykinin-stimulated eNOS activity.** Shown are the results of *in vitro* NOS activity assays using eNOS immunoprecipitated from BAEC that had been treated for 30 min with various kinase inhibitors and then stimulated with bradykinin (1  $\mu$ M) for varying lengths of time, as indicated. Cells were incubated with vehicle (no inhibitor), calphostin C (200 nM), wortmannin (500 nM), or PD98059 (10  $\mu$ M) and then treated with bradykinin (1  $\mu$ M) for the indicated times, harvested, and immunoprecipitated. eNOS activity (assayed as L-[<sup>3</sup>H] citrulline production) was measured directly on the immunoprecipitates as described under "Experimental Procedures." Each data point represents the mean  $\pm$  S.E. from three independent experiments, each performed in triplicate. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus no inhibitor at time 0 (ANOVA).



tions unlikely to yield significant nonspecific effects (8, 25, 26), the pleiotropic effects often seen with these compounds make it difficult to draw definitive conclusions about the direct involvement of any particular kinase pathway on the basis of kinase inhibitor data alone. We pursued several additional lines of investigation to establish more direct evidence for the roles of specific protein kinases in regulation of eNOS in endothelial cells.

The involvement of protein kinase Akt in eNOS phosphorylation has been effectively established in several independent studies (15–18), although the evidence for involvement of kinase Akt in activation of eNOS by cell surface receptors in endothelial cells is less compelling. We have shown in these studies (Fig. 4) that bradykinin does not activate kinase Akt, whereas vascular endothelial cell growth factor does activate this kinase. Thus, bradykinin, a key endogenous activator of eNOS, does not appear to modulate the enzyme via activation of Akt. Moreover, other kinases, including the AMP-activated protein kinase, protein kinase A, and a cyclic GMP-activated kinase have each been implicated in eNOS activation in prior reports (19, 20), although the relevance of these kinases to regulation of eNOS in intact endothelial cells remains less well understood. Indeed, the patterns of eNOS phosphorylation in endothelial cells may be distinctly different from features of eNOS phosphorylation studied in heterologous cell systems or *in vitro* (31).

Activation of bradykinin B2 receptors in endothelial cells leads to the activation of ERK, as shown in the current studies (Fig. 5) and in prior reports in other endothelial cell systems (14). Since bradykinin is such an important endogenous activator of eNOS (6) and in the context of our data showing the dynamic regulation of eNOS-ERK interactions by bradykinin, we sought additional evidence for the direct involvement of ERK in eNOS regulation. There are at least 14 plausible ERK consensus sequences in eNOS, and previous reports identifying phosphorylated residues in eNOS do not permit the definitive exclusion or assignment of any of these putative ERK-phosphorylated sites. However, simply showing that eNOS can be phosphorylated by ERK *in vitro* (Fig. 5B) or even documenting that bradykinin-stimulated eNOS phosphorylation is blocked by PD98059 (Fig. 6A) does not establish definitively that eNOS is directly modulated by ERK in BAEC. Indeed, the large number of potential eNOS phosphorylation sites and the diversity of kinases implicated in eNOS regulation confound attempts to directly study eNOS phosphorylation in intact cells. We therefore performed a series of back-phosphorylation experiments.

The design of these back-phosphorylation experiments is based upon the premise that if phosphorylation is stimulated

by a given agonist in the intact (unlabeled) cell, this should yield an increase in the phosphorylation state of the protein of interest. When this unlabeled phosphoprotein is then isolated from the treated cell and subsequently radiolabeled in an *in vitro* phosphorylation reaction using a candidate kinase plus [ $\gamma$ -<sup>32</sup>P]ATP, the protein should undergo less incorporation of radioactivity if the kinase used *in vitro* modifies the same sites as those that had undergone phosphorylation in the intact cell. Conversely, pretreatment of intact unlabeled cells with the appropriate kinase inhibitor should lead to the candidate phosphoprotein's having a relatively lower stoichiometry of endogenous phosphorylation and therefore to the protein's being more robustly radiolabeled when subsequently incubated *in vitro* with the [ $\gamma$ -<sup>32</sup>P]ATP plus the appropriate kinase. Our back-phosphorylation experiments show that bradykinin pretreatment of endothelial cells reduces subsequent phosphorylation of eNOS by ERK, and, conversely, treatment of endothelial cells with the MEK inhibitor PD98059 increases the subsequent phosphorylation by ERK (Fig. 6). Taken together, these data provide strong evidence for a direct role of ERK in eNOS phosphorylation in endothelial cells. Importantly, the S1179A mutant showed an identical pattern of back-phosphorylation as the wild-type enzyme, thereby providing evidence that the inactivation of the putative Akt phosphorylation state does not block eNOS phosphorylation by ERK.

The residue in eNOS that has most commonly been found to be a substrate for *in vitro* phosphorylation is Ser<sup>1179</sup> (which corresponds to Ser<sup>1177</sup> in human eNOS); in the present studies, we have characterized the eNOS phosphorylation site mutant S1179A in intact endothelial cells. In back-phosphorylation experiments (Fig. 6C), we found that the S1179A eNOS mutant still shows evidence of ERK-sensitive phosphorylation in intact endothelial cells, and <sup>32</sup>P<sub>i</sub> biosynthetic labeling experiments in BAEC showed that this mutant still undergoes phosphorylation. Clearly, phosphorylation of eNOS at Ser<sup>1179</sup> is only part of the story, and it seems highly likely that several additional residues in eNOS undergo phosphorylation in endothelial cells, suggesting that diverse kinase pathways might importantly influence the enzyme. The present studies have provided evidence indicating that ERK may play a key role in eNOS regulation.

Although our data strongly suggest that ERK-catalyzed phosphorylation of eNOS can lead to enzyme inhibition, these studies have not explicated the precise mechanisms whereby this inhibition is achieved. We showed that *in vitro* phosphorylation of eNOS by ERK is associated with a reduction in enzyme activity (Fig. 4); this finding is consistent with our

observation in intact cells that the MEK inhibitor PD98059 increases eNOS enzyme activity (Fig. 7). However, defining the mechanisms whereby ERK inhibits eNOS must await the identification of the site(s) of phosphorylation by this kinase.

These studies have identified a new level of complexity in the regulation of eNOS by phosphorylation and have provided new evidence for the inhibition of eNOS activity by MAP kinase phosphorylation. The multiplicity of candidate kinases and potential sites for eNOS phosphorylation indicates that interactions between kinase (and phosphatase) pathways may be important determinants of nitric oxide signaling in the vascular wall.

## REFERENCES

- Kaplan, A. P., Joseph, K., Shibayama, Y., Nakazawa, Y., Ghebrehwet, B., Reddigari, S., and Silverberg, M. (1998) *Clin. Rev. Allergy Immunol.* **16**, 403–429
- De Weerd, W. F. C., and Leeb-Lundberg, L. M. F. (1997) *J. Biol. Chem.* **272**, 17858–17866
- Liu, P., Ying, Y.-S., and Anderson, R. G. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 13666–13670
- Shaul, P. W., and Anderson, R. G. W. (1998) *Am. J. Physiol.* **275**, L843–L851
- Couet, J., Li, S., Okamoto, T., Scherer, P. E., and Lisanti, M. P. (1997) *Trends Cardiovasc. Med.* **7**, 103–110
- Mombouli, J.-V., and Vanhoutte, P. M. (1999) *J. Mol. Cell Cardiol.* **31**, 61–74
- Jaffa, A. A., Miller, B. S., Rosenzweig, S. A., Naidu, P. S., Velarde, V., and Mayfield, R. K. (1997) *Am. J. Physiol.* **273**, F916–F924
- Graness, A., Adomeit, A., Heinze, R., Wetzker, R., and Leibmann, C. (1998) *J. Biol. Chem.* **273**, 32016–32022
- Naraba, H., Ueno, A., Kosugi, Y., Yoshimura, M., Murakami, M., Kudo, I., and Oh-ishi, S. (1998) *FEBS Lett.* **435**, 96–100
- Seger, R., and Krebs, E. G. (1995) *FASEB J* **9**, 726–735
- Blenis, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5889–5895
- Davis, R. J. (1993) *J. Biol. Chem.* **268**, 14553–14556
- Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993) *Nature* **364**, 249–252
- Fleming, I., Fisslthaler, B., and Busse, R. (1995) *Circ. Res.* **76**, 522–529
- Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *Nature* **399**, 597–601
- Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) *Nature* **399**, 601–605
- Michell, B. J., Griffiths, J. E., Mitchelhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., Ortiz de Montellano, P. R., Kemp, B. E., and Pearson, R. B. (1999) *Curr. Biol.* **9**, 845–848
- Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G., Berk, B. C., Aebersold, R., and Corson, M. A. (1999) *J. Biol. Chem.* **274**, 30101–30108
- Butt, E., Bernhardt, M., Smolenski, A., Kotsonis, P., Frohlich, L. G., Sickmann, A., Meyer, H. E., Lohmann, S. M., and Schmidt, H. H. H. W. (2000) *J. Biol. Chem.* **275**, 5179–5187
- Chen, Z.-P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., de Montellano, P. R. O., and Kemp, B. E. (1999) *FEBS Lett.* **443**, 285–289
- Hirata, K.-I., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., and Yokoyama, M. (1995) *Hypertension* **25**, 180–185
- Robinson, L., Busconi, L., and Michel, T. (1995) *J. Biol. Chem.* **270**, 995–998
- Michel, T., Li, G. K., and Busconi, L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6252–6256
- Balligand, J. L., Kobzik, L., Han, X., Kaye, D. M., Belhassen, L., O'Hara, D. S., Kelly, R. A., Smith, T. W., and Michel, T. (1995) *J. Biol. Chem.* **270**, 14582–14586
- Sakamoto, N., Uemura, H., Hara, Y., Saito, T., Masuda, Y., and Nakaya, H. (1998) *Br. J. Pharmacol.* **125**, 283–292
- Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689
- Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15**, 6541–6551
- Lee, C. M., Robinson, L. J., and Michel, T. (1995) *J. Biol. Chem.* **270**, 27403–27406
- Morgan-Boyd, R., Stewart, J. M., Vavrek, R. J., and Hassid, A. (1987) *Am. J. Physiol.* **253**, C588–C598
- Prabhakar, P., Thatte, H. S., Goetz, R. M., Cho, M. R., Golan, D. E., and Michel, T. (1998) *J. Biol. Chem.* **273**, 27383–27388
- Robinson, L. J., Ghanouni, P., and Michel, T. (1996) *Methods Enzymol.* **268**, 436–448