Identification of Flow-dependent Endothelial Nitric-oxide Synthase Phosphorylation Sites by Mass Spectrometry and Regulation of Phosphorylation and Nitric Oxide Production by the Phosphatidylinositol 3-Kinase Inhibitor LY294002*

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Endothelial cells release nitric oxide (NO) acutely in response to increased laminar fluid shear stress, and the increase is correlated with enhanced phosphorylation of endothelial nitric-oxide synthase (eNOS). Phosphoamino acid analysis of eNOS from bovine aortic endothelial cells labeled with [32P]orthophosphate demonstrated that only phosphoserine was present in eNOS under both static and flow conditions. Fluid shear stress induced phosphate incorporation into two specific eNOS tryptic peptides as early as 30 s after initiation of flow. The flow-induced tryptic phosphopeptides were enriched, separated by capillary electrophoresis with intermittent voltage drops, also known as "peak parking," and analyzed by collision-induced dissociation in a tandem mass spectrometer. Two phosphopeptide sequences determined by tandem mass spectrometry, TQpSFSLQER and KLQTRPpSPGPPPAEQLLSQAR, were confirmed as the two flow-dependent phosphopeptides by co-migration with synthetic phosphopeptides. Because the sequence (RIR)TQpSFSLQER contains a consensus substrate site for protein kinase B (PKB or Akt), we demonstrated that LY294002, an inhibitor of the upstream activator of PKB, phosphatidylinositol 3-kinase (PKB or Akt), inhibits flow-induced eNOS phosphorylation by 97% and NO production by 68%. Finally, PKB phosphorylated eNOS in vitro at the same site phosphorylated in the cell and increased eNOS enzymatic activity by 15–20-fold.

Endothelial nitric-oxide synthase (eNOS1) or type III NOS is one of three isoenzymes that converts L-arginine to L-citrulline and nitric oxide (NO). Endothelial cells synthesize NO tonically and increase NO production in response to agonists and increased fluid shear stress (FSS). Endothelial NO contributes to blood vessel homeostasis by regulating vessel tone (1), cell growth (2), platelet aggregation (3), and leukocyte binding to endothelium (4). In vivo eNOS is both myristylated and palmitoylated. These modifications increase eNOS compartmentalization to plasmalemmal caveolae and facilitate release of NO from cells (5–7). In caveolae, which are small plasmalemmal invaginations that sequester signaling proteins (8), eNOS specifically interacts with the scaffolding protein caveolin-1 through a caveolin (9, 10) binding motif (11), located near the domain that binds Ca2+/calmodulin. Recent studies suggest that the activity of eNOS is regulated in a reciprocal manner through caveolin-1 inhibition and Ca2+/calmodulin stimulation (12–14).

Increased FSS stimulates an increase in free intracellular calcium [Ca2+]i, from intracellular stores (15, 16) leading to a Ca2+/calmodulin-dependent increase in eNOS activity. However, recent investigations show that increases in [Ca2+]i do not fully explain the rapid rise in NO production in response to FSS (17). Exposure of bovine aortic endothelial cells (BAEC) to 25 dynes/cm2 FSS for 30 s caused a 7-fold rise in NO production and a corresponding 2-fold increase in eNOS phosphorylation, whereas the calcium ionophore A23187 neither caused rapid NO production nor any net increase in eNOS phosphorylation (17). We therefore hypothesized that an increase in FSS could be transduced to increased NO production via activation of a protein kinase cascade resulting in phosphorylation and activation of eNOS.

The objectives of the present work were to elucidate the eNOS sites phosphorylated in response to increased FSS and to identify potential protein kinase mediators of the mechan-
transduction of FSS to NO production. The amino acid residues phosphorylated in small proteins may be determined by fractionation of tryptic peptides by high pressure liquid chromatography (HPLC) and mass spectrometry (18). However, tryptic fragmentation of large proteins such as eNOS creates numerous peptides whose rapid elution from HPLC exceeds the scan rate of the triple quadrupole mass spectrometer. This prevents acquisition of sequences, by collision-induced dissociation (CID), of a substantial fraction of the peptides. Additionally, phosphopeptides from proteins isolated from a cell will nearly always exist as minor ions due to sub-stoichiometric phosphorylation compared with nonphosphorylated peptides and will not be scanned as major parent ions. In order to identify phosphorylation sites in eNOS, tryptic phosphopeptides were first enriched by immobilized metal affinity chromatography (IMAC). The peptides were then separated by peak parking solid phase extraction capillary electrophoresis (SPE-CE), which extends the peak analysis time to provide for both increased scan times of parent ions and increased MS/MS analyses of each selected peptide according to optimized CID parameters. The identity of two specific eNOS residues phosphorylated in response to flow was determined, and evidence was obtained linking FSS to eNOS phosphorylation and NO production via activation of phosphatidylinositol 3-kinase phosphotyrosine in the cell and that the phosphorylation increases eNOS enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Materials—** The monoclonal antibody (H32) to eNOS was purchased from Biomol (Plymouth Meeting, NJ). Anti-phosphoserine 473-PKB rabbit polyclonal antibody was purchased from New England Biolabs (Beverly, MA), and anti-PKB (pleckstrin homology domain) sheep polyclonal antibody and activated recombinant PKB were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Sequencing grade modified trypsin and compound U0126 were obtained from Promega (Madison, WI). Sequencing grade trypsin and compound U0126 were obtained from Promega (Madison, WI). Phosphoserine, phosphothreonine, phosphoarginine, ninyhydrin, polyvinylpyrrolidone (PVP40, Mf = 40,000), silver nitrate ultra, sodium thiosulfate ultra, sodium carbonate, and 8-(chlorophenylthio)-guanosine 3':5'-cyclic monophosphate (cGMP) were purchased from Calbiochem. The synthetic peptides RIRTQpSFSLQER and KLQTRPpSPGPPPAEQLLSQAR were purchased from SynPep Corp. (Dublin, CA). Formaldehyde and ammonium bicarbonate were purchased from J. T. Baker Inc. High purity acetone for HPLC was obtained from Burdick and Jackson (Muskegon, MI). L-2[14C]arginine monohydrochloride (278 mCi/mmol) were purchased from Amersham Pharmacia Biotech. [32P]Orthophosphoric acid (185 MBq) was purchased from NEN Life Science Products. Phosphoserine, phosphothreonine, ninyhydrin, polyvinylpyrrolidone (PVP40, Mf = 40,000), silver nitrate ultra, sodium thiosulfate ultra, sodium carbonate, and 8-(chlorophenylthio)-guanosine 3':5'-cyclic monophosphate (cGMP) were purchased from Calbiochem. Phosphopeptides were obtained from Life Technologies, Inc. Polyvinylidene difluoride (PVDF) Immobilon P was purchased from Millipore (Bedford, MA). DETA NONOate was purchased from Alexis Biochemicals (San Diego, CA). Detergent and ammonium bicarbonate were purchased from J. T. Baker Inc. High purity acetone for HPLC was obtained from Burdick and Jackson (Muskegon, MI).

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**BAEC Culture and Exposure to Fluid Stress Stress—** BAEC cultures were established and maintained in culture in medium 199 (Life Technologies, Inc.) supplemented with fetal calf serum as described (17). Cells from passage 3–5 were seeded in 100-mm tissue culture dishes and used at 60–80% confluence for labeling and shear stress experiments. Cells were exposed to laminar flow in a cone and plate viscometer as described (19). The cells were rapidly washed with ice-cold phosphate-buffered saline and lysed in 0.1 M phosphate-buffered saline containing 1% Triton X-100, 50 mM β-glycerophosphate, 200 μM NaN3, 10 μM leupeptin, 10 μM soybean trypsin inhibitor, 2 mM benzamidine, 1 mM Na3VO4, and 2 mM EDTA. Lysates were centrifuged for 5 min at 14,000 rpm at 4°C. Equivalent amounts of protein as determined by Bradford assay were used for all immunoprecipitations. The supernatants were precleared as described (20) and incubated for 4 h at a final NaCl concentration of 400 mM. The immuno-precipitates were washed with 0.1 M acetic acid, and the eluates from the immune pellets were stored at −20°C.

**Cell Labeling, Lysis, and Immunoprecipitation of eNOS—** eNOS was immunoprecipitated from six [32P]orthophosphate-labeled 100-mm culture dishes and 12 non-labeled culture dishes of BAEC exposed to 15 dynes/cm² FSS for 1 min. The pooled immunoprecipitates were separated on a 0.75-mm thick, 9% SDS-polyacrylamide gel. The eNOS was located by silver staining (24), excised from the gel, and the incorporation of ³²P was determined by Cerenkov counting. The gel slices were washed in 1% ammonium bicarbonate, pH 8.3, and 1% acetic acid. The buffer was exchanged to 1% acetic acid and 25 mM dithiothreitol and 10 mM EDTA, containing 50% ethylene glycol. The tryptic phosphopeptides were recovered from the gel slices, as determined by Cerenkov counting.

**Off-line IMAC and HPLC for Enrichment of eNOS Phosphopeptides—** An ion metal affinity column (IMAC) was constructed and operated as described previously (22) with the following exceptions. To each end of a 10-cm-long piece of Teflon tubing (1/16 inches outer diameter × 0.0001 inches inner diameter) a piece of polyimide-coated fused silica capillary (Pluronic Technologies, Tucson, AZ) was inserted and held in place by a union (Valco, Houston, TX). Prior to fixing the second of the two polyimide capillaries in place the open Teflon end was placed in a slurry of POROS-MC (PerSeptive) inside a vessel pressurized by helium, and the IMAC column was packed to a length of 5 cm under 500 pounds/square inch pressure. The second piece of fused silica capillary was then fixed in place with a second union. For operation one of the two polyimide capillaries was placed in a helium pressure vessel and the other served as an outlet. The IMAC column was prepared for use by washing (5 min/wash at 5 pounds/square inch) sequentially with water, 0.1 M EDTA, water, 0.1 M acetic acid, 0.1 M FeCl₃, and 0.1 M acetic acid. The sample, reconstituted in 0.1 M acetic acid, was loaded in entirety and followed by washing with 0.1 M acetic acid, water, 1.0 M NH₄H₂PO₄ for elution of bound phosphopeptides, water, and 0.1 M acetic acid. The eluted phosphopeptides were oxidized with 0.4% heptfluorobutyric acid (solvent A) and 0.005% heptfluorobutyric acid (solvent A) and injected onto the HPLC column. HPLC was carried out on a Michrome Bioresources instrument (Auburn, CA) equipped with a 0.5-mm C18 column. A linear gradient from 0 to 60% acetonitrile, which contained 0.4% acetic acid and 0.005% heptfluorobutyric acid wash, was used to elute peptides from the column. One-minute fractions were collected and ³²P content was determined by Cerenkov counting.
Solid Phase Extraction and Capillary Electrophoresis (SPE-CE)—Fractions from the HPLC separation intended for SPE-CE were concentrated slightly to remove excess acetonitrile, as described (26). The sample was pressure-injected on a C18 cartridge (1 mm × 250 μm) placed at the head of the CE capillary, a series of washing steps followed, and the prepared sample for CE which began when a small plug of organic solvent was pressure-injected onto the SPE cartridge (27, 28).

Mass Spectrometry—Peptides were sequenced on a Finnigan (San Jose, CA) TSQ 7000 triple quadrupole mass spectrometer equipped with a home-built electrospray ionization device described previously (26). The mass spectrometer was computer-controlled using a program written in instrument control language that automatically provided data-dependent ion selection and varied capillary electrophoretic voltage. Ion selection and CE voltage were dependent on ion intensity such that an ion reached a given intensity the mass spectrometer simultaneously performed ion selection and varied capillary electrophoretic voltage. Ion selection and CE voltage were dependent on ion intensity such that an ion reached a given intensity the mass spectrometer simultaneously switched from full scan mode to MS/MS mode and the CE voltage decreased from −20 to −5 V. When the ion signal decreased below a preset threshold, the mass spectrometer returned to initial scanning/electrophoretic conditions.

Measurement of Nitric Oxide—NO release by BAEC was measured as its nitrogen oxide (NOx) metabolites, using a chemiluminescence detector as described in detail (17).

Construction of the eNOS Expression Plasmid—pCeNOS, the plasmid in which expression of His6 eNOS in Escherichia coli, was constructed using two sequential two-piece ligations. An NdeI-SfiI fragment including the internal 533 nucleotides of bovine eNOS cDNA (29) was created using the following sense and antisense primers: 5′-TGA- TTACCATATGGC[CATCAC]AACTTGAAGATGGTGGGCAGAG and 5′-GGCCGAGCCGTGCTTGGCCCGCACAG. This fragment was purified and used as a template to create a HindIII-SfiI fragment using additional polymerase chain reaction, using this sense primer 5′-TATCCCAAGCTTGGTGATACCATATGGCCCA and the former antisense primer. This fragment (a HindIII-SfiI fragment with an internal NdeI site) was purified and cut with HindIII and SfiI. pBlue-script eNOS (29) was similarly cut and dephosphorylated. Ligation products were transformed into DH5α cells, and colonies were screened for positive recombinants via restriction digestion. A positive clone (pBlue-script NOD) was maxi-prepped and then cut with NdeI and XhoI. pcW ori+ (30) was cut similarly, and the two pieces ligated, transformed, and screened as before. A positive clone (pCeNOS) allowed isopropyl-1-thio-β-D-galactopyranoside-induced eNOS expression at 23 °C (31) as judged by immunoblot analysis. The clone was sequenced through the region that was amplified by polymerase chain reaction, and this sequence was found to be perfectly consistent with the published bovine eNOS sequence (29) in the region.

Expression and Purification of Recombinant eNOS—eNOS was expressed and purified from E. coli as described (32, 33) with minor modifications. pCeNOS was transformed into BL21(DE3)pLysS cells. A colony was grown to log phase and inoculated into 2-liter flasks containing 500 ml of Terrific broth and 100 μg/ml ampicillin. At an A600 of 0.8, 250 μg of isopropyl-1-thio-β-D-galactopyranoside, 500 μM aminolevulinic acid, and 3 μM riboflavin were added. The culture was shaken at 23 °C for 24 h at 200 rpm, and bacteria were pelleted and lysed in 50 mM Tris, pH 8.0, a protease mixture (1 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin, pepstatin A, chymostatin, benzamidine, antipain, 2.5 mM ml lysozyme, 30 units/ml DNase I, and 0.15 mg/ml RNase A) for 15 min at 37 °C. Then 5 μl flavin adenine dinucleotide, 5 μM flavin mononucleotide, 20 μM (6-R,5,6,7,8-tetrahydro-1,3-biotin), and 40 μM (S)-β-mercaptoethanol were added, and the lysate was centrifuged at 50,000 × g for 20 min at 4 °C. eNOS in the supernatant was purified using 2′,5′-ADP-Sepharose chromatography as described (32). The protein was further purified by Ni2+ chelate chromatography as described (33). eNOS was 95% pure by silver stain after SDS-PAGE and was stored in aliquots in 10% glycerol at −80 °C. Identification and Regulation of eNOS Phosphorylation Sites

In Vitro Phosphorylation of eNOS and Arginine-Citrulline Conversion Assay—Kinase reactions using PKB to phosphorylate eNOS were performed according to the manufacturer's instructions. For determination of stoichiometry 150 ng of PKB was used to phosphorylate 10 μg of eNOS with [γ-32P]ATP (90 μCi) at a specific activity of 2800 cpm/nmol. At the indicated time points, aliquots of the reaction were boiled in SDS sample buffer, and eNOS was separated by SDS-PAGE and then localized by silver staining. The eNOS bands were excised, and incorporation was determined by Cerenkov counting. For activation of eNOS by PKB, 300 ng of PKB (specific activity 560 nmol/min mg using eNOS as a substrate) was incubated with 2.5 μg of recombinant eNOS in a 20-min protein kinase reaction at 30 °C. Reactions without PKB were run in parallel. After phosphorylation, 1-μg aliquots of eNOS from the kinase reactions with and without PKB were diluted into 100-μl eNOS assays containing 20 μM L-[1-14C]Arginine (1375 cpm/nmol), 3 μM CaCl2, 4 μM FAD, 4 μM FMN, 4 μM BH4, 1 mM NADPH, 50 mM Tris, pH 7.5. 3 μM calmodulin, and reactions were carried out exactly as described (34) at 30 °C. Labeled arginine was separated from labeled citrulline by thin layer chromatography (34) and [14C]citrulline counts/min formed were determined by scraping and counting the silica gel containing [14C]citrulline in scintillant after localization by autoradiography.

RESULTS

Time Course and Phosphoamino Acid Analysis of eNOS Phosphorylation in Response to FSS—Previously we and others (17, 35) have shown that eNOS is phosphorylated in BAEC in static conditions and that the phosphorylation of eNOS is enhanced in response to flow (17). BAEC were labeled with [32P]orthophosphate in phosphate-free DMEM containing 200 μM Na2VO4. Cells were maintained in static condition or subjected to laminar FSS (19). The cells were lysed; eNOS was immunoprecipitated with a monoclonal antibody, and the immunoprecipitate was fractionated on an SDS-polyacrylamide gel. The proteins in the gel were transferred to nitrocellulose and detected by autoradiography (Fig. 1A). eNOS was basally phosphorylated in cells maintained in static culture (Fig. 1A, lane 1), and exposure to laminar FSS caused a rapid, nearly 2-fold increase (17) in eNOS phosphorylation (see Fig. 1A and legend).

Various investigators have labeled endothelial cells under static conditions with [32P]orthophosphate in the presence of phenylarsine oxide (36) or pervanadate (37) and have shown that eNOS contains predominantly phosphoserine and a small amount of phosphotyrosine. BAEC were labeled with [32P]orthophosphate in the presence of Na2VO4 (see "Experimental Procedures") and maintained under static condition or exposed to FSS at 15 dynes/cm2 for 1 min. Fig. 1B shows that


Identification and Regulation of eNOS Phosphorylation Sites

**Fig. 2. Two-dimensional tryptic phosphopeptide maps of eNOS isolated from BAEC under static and flow conditions.** BAEC were labeled with [32P]orthophosphate, maintained in static culture, or exposed to FSS at 15 dynes/cm² for the indicated times. The eNOS was immunoprecipitated, size fractionated by SDS-PAGE, and transferred to nitrocellulose as in Fig. 1A. The eNOS protein was excised from the blots and digested with trypsin; typically ~85% of the eNOS cpm were recovered after trypsinization. Tryptic digests were spotted onto thin layer plates and separated by HVE in the first dimension and by TLC in the second dimension, and autoradiography was performed as shown: A, static condition; B, FSS 1 min; C, FSS 2.5 min; and D, FSS 10 min. These data are representative of two similar experiments (see Table 1).

eNOS contains only phosphoserine under both static and flow conditions (n = 3). No other phosphoamino acid was detected in eNOS.

Two-dimensional Tryptic Phosphopeptide Maps of eNOS under Static and Flow Conditions—The eNOS transferred to nitrocellulose (shown in Fig. 1A) was subjected to tryptic cleavage and the tryptic peptides were spotted onto a thin layer plate. The peptides were fractionated in the first dimension by HVE and in the second dimension by TLC. Autoradiograms of the thin layer plates are shown in Fig. 2. Under static conditions eNOS contained 8–10 phosphopeptides (Fig. 2A), two of which, F1 and F2, increased with time when BAEC are exposed to 15 dynes/cm² FSS (Fig. 2, B–D).

The relative volumes of each of the basal and flow-stimulated phosphopeptides were quantified by PhosphorImager analysis in two independent experiments examining phosphate incorporation as a function of shear stress duration (Table 1). Whereas the incorporation of phosphate into eNOS during a 10-min time course was approximately 2-fold, the specific incorporation into the flow-dependent phosphorylation sites F1 and F2 was more pronounced. Flow-dependent phosphorylation of peptide F1 was stimulated by severalfold to 30-fold, and the stimulation of phosphorylation into peptide F2 ranged from 3- to 6-fold (Table 1). The substantial experimental variation is due to the fact that the peptide mapping procedure is semi-quantitative. Additionally, the fold stimulation of phosphorylation depends upon the basal level of phosphate incorporation, which is significantly modulated by the state of BAEC growth. Nonetheless, substantial increases in phosphorylation occurred in both peptides F1 and F2 as early as 30 s and more than 90% of the incorporation of phosphate into the peptides occurred by 2.5 min. These data (Fig. 2 and Table 1) demonstrate that flow-induced eNOS phosphorylation is very rapid and that flow enhances the phosphorylation of two specific eNOS tryptic peptides.

Preparation of eNOS for Mass Spectrometry and Rationale for Enrichment of eNOS Phosphopeptides Prior to Mass Spectrometric Analysis—To obtain enough eNOS for mass spectrometric analysis of the phosphopeptides, eNOS was immunoprecipitated from 18 100-mm tissue culture dishes of BAEC. Six of the dishes were labeled with [32P]orthophosphate; 12 dishes were unlabeled, and all dishes were subjected to 15 dynes/cm² for 1 min. Pooled lysates were immunoprecipitated; eNOS was fractionated by SDS-polyacrylamide gel electrophoresis, and the gel was silver-stained (Fig. 3, inset). Comparison of intensities of silver-stained eNOS to molecular weight markers of known amount on the gel provided an estimation of 10–20 pmol of eNOS recovered from 18 culture dishes (data not shown). The silver-stained eNOS was excised from the gel and digested in situ with trypsin. Typically 95% of the phosphopeptides were recovered. The phosphopeptides were bound to and eluted from an immobilized metal (Fe³⁺) affinity column (IMAC) with cpm recoveries of ~70%. The peptides were further fractionated by reversed phase-HPLC, and the radioactivity in the individual fractions was determined by Cerenkov counting (Fig. 3, bar graph).

In initial experiments, when individual fractions of eNOS tryptic phosphopeptides isolated by reversed phase-HPLC were analyzed off-line by MS/MS, no eNOS phosphopeptides could be identified by CID, even though tryptic peptides representing 40% coverage of the amino acid sequence of eNOS were identified (data not shown), and more importantly, the majority of the incorporated phosphate was recovered in the fractions analyzed. Typically the MS/MS instrument is programmed to isolate peptides for CID in order of decreasing abundance, thus limiting the numbers of peptides that are sequenced from each chromatographic peak in the case where several peptides co-elute. Since each analytical cycle (consisting of peptide mass analysis, peptide ion selection, CID, and acquisition of MS/MS spectra derived from selected peptide ions) requires on the order of 3 s, only the most abundant peptide ions from a typical chromatographic peak can be analyzed. Because the phosphorylation sites in phosphoproteins may be modified only to low stoichiometry, many phosphopeptides from proteins isolated from cells will not be sequenced without technical innovations.

The failure to identify eNOS phosphopeptides of low abundance was overcome by intermittently extending the time available for peptide analysis by reducing the sample flow rate to the mass spectrometer (26), a procedure known as “peak parking” (38). Briefly, each HPLC fraction in Fig. 3, bar graph, containing significant cpm (labeled 1–7 above the peaks) was submitted to solid phase extraction-capillary electrophoresis (SPE-CE) for peptide concentration and separation. A program was written so that as each peak entered the mass spectrometer by SPE-CE the voltage dropped, providing for acquisition by CID of the seven most abundant peptides in each fraction. This procedure is described in more detail elsewhere (26).

Mass Spectra and Sequences of the Flow-dependent Phosphorylation Sites of eNOS—HPLC fractions containing radioactive counts above background were analyzed by automated SPE-CE-MS/MS. The CID spectra generated from these analyses were initially screened by SEQUEST for the presence of eNOS-derived phosphopeptides using an 80-atomic mass unit tag for serine, threonine, or tyrosine. Fractions 18 and 25 were found to each contain a single, mono-phosphorylated peptide in addition to several non-phosphorylated peptides. These spectra were then analyzed manually (39) to confirm the SEQUEST results. The CID spectrum for the phosphopeptide, [M + 2H]⁺ 558 m/z, from fraction 18 is shown in Fig. 4 and corresponds to bovine eNOS residues Thr-1177 through Arg-1185. Location of phosphate to Ser-1179 is unambiguously derived by observation of y series fragment ions, y1–y7, and the b2 ion (40). Both the y7 ion, 946 m/z, resulting from fragmentation be-

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2. B. Gallis and M. Corson, unpublished observations.
Identification and Regulation of eNOS Phosphorylation Sites

Table I
Time course of phosphorylation of two eNOS peptides in response to 15 dynes/cm² FSS

| Time  | Peptide F1 | Stimulation | Relative volume | Peptide F2 | Stimulation |
|-------|------------|-------------|----------------|------------|-------------|
|       |            | fold        |                |            | fold        |
| 0     |            | 1.0         | 576,344        |            | 335,189     |
| 0.5   |            | 2.7         | 1,573,726      |            | 885,617     |
| 1.0   |            | 2.7         | 3,009,441      |            | 1,067,813   |
| 2.5   |            | 5.2         | 3,224,277      |            | 1,154,124   |
| 10.0  |            | 5.6         | 92,203         |            | 138,863     |
|       |            | 22.3        | 2,057,316      |            | 460,429     |
| 1.0   |            | 29.4        | 2,717,982      |            | 701,923     |
| 2.5   |            | 33.4        | 3,078,855      |            | 916,095     |
| 10.0  |            | 6.6         | 0 92,203 1.0  |            | 138,863     |

* The 30-s time point was lost in Experiment 1.
* The 10.0-min time point was lost in Experiment 2.

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Fig. 3. Preparation of eNOS and resultant tryptic phosphopeptides for mass spectrometry. *Inset,* the eNOS was immunoprecipitated from six 100-mm tissue culture dishes labeled with [32P]orthophosphate and 12 unlabeled dishes. Fractionation of the immunoprecipitate by SDS-PAGE and silver staining revealed a single major eNOS band (arrow) and the anti-eNOS heavy chain (IgG, arrow). The eNOS bands were excised, pooled, and contained 19,000 cpm by Cerenkov counting. The bar graph shows the tryptic phosphopeptides which were enriched by IMAC and fractionated by HPLC. The numbers 1–7 at the tops of the bars represent the seven fractions individually fractionated by SPE-CE prior to MS/MS analysis. These data are representative of three similar experiments.

Fig. 4. Tandem mass spectra of eNOS phosphopeptide F1. Flow-dependent phosphopeptide F1 was sequenced by interpretation of fragment ions produced during CID in a triple quadrupole mass spectrometer. Samples were introduced by SPE-CE electrospray ionization. Annotated ions are shown as nominal m/z values and result from fragmentation of an amide bond with charge retention on the carboxyl (y series ion) or amino terminus (b series ion).

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The 10.0-min time point was lost in Experiment 2.
observe a decrease in flow-dependent phosphorylation at this site even though MAP kinase was inhibited by 95% in an immune complex kinase assay (data not shown). Other inhibitors and activators of protein kinases were used to modulate phosphorylation levels of eNOS tryptic phosphopeptides. Since laminar FSS causes a rapid increase in BAEC [Ca^{2+}], cells were incubated prior to flow with the group-specific inhibitor of the Ca^{2+}/calmodulin kinases (45) KN-62 (10 µM, n = 4), but no inhibition of phosphorylation of any phosphopeptide was observed (data not shown). Because NO decreases levels of cyclic GMP and may activate the cyclic GMP-dependent protein kinase, we used the NO donor 2,2'-hydroxynitrosobenzidine/bis-ethanamine-NONOate (100 µM, n = 3) and stable cyclic GMP analogue 8-(chlorophenylthio)-guanosine 3’5’-cyclic monophosphate (500 µM, n = 3) to determine whether the level of phosphorylation of any eNOS tryptic peptide could be enhanced by these agents. No increases in the level of phosphorylation of any tryptic peptide were observed (data not shown). However, three novel eNOS phosphopeptides, not observed under static or flow conditions, appeared in the presence of these compounds (data not shown). These findings suggest that under the conditions used for these studies eNOS is neither a sub-
FIG. 8. PKB phosphorylates eNOS stoichiometrically in vitro at Ser-1179 and increases its arginine-citrulline conversion activity. A, recombinant, activated PKB was used to phosphorylate 1 μM eNOS. The stoichiometry was calculated and displayed (closed circles) as mol of phosphate per mol of eNOS (mol P/mol eNOS). B, the eNOS phosphorylated in A was isolated by SDS-PAGE, digested with trypsin in the gel slice, and the eluted phosphopeptides were separated by two-dimensional peptide mapping. An autoradiogram is shown. The separated phosphopeptides were scraped and sequenced; F1 was the major phosphopeptide of eNOS. The stoichiometry was calculated and displayed (Fig. 8A). The eNOS phosphorylation via a pathway involving PI3-kinase and PKB by 15–20-fold (Fig. 8C). Stoichiometric phosphorylation of eNOS by PKB activated eNOS by 15–20-fold (Fig. 8C).

**DISCUSSION**

Through the use of innovative modifications of mass spectrometry, we have identified two flow-dependent phosphorylation sites in eNOS. We have confirmed the identities of the tryptic phosphopeptides by co-migration of in vivo labeled tryptic phosphopeptides of eNOS with synthetic peptides based on the sequences discovered by mass spectrometry. One tryptic phosphopeptide, designated F1, TQpSFSLQER, cleaved from the sequence, RIQTQpSFSLQER, is consistent with the PKB substrate consensus phosphorylation sequence RXRXXXX (where X is F, I, or V) (46). Because PI3-kinase is an upstream activator of PKB (46), we used the PI3-kinase inhibitor, LY294002 (48), to partially inhibit flow-dependent PKB activity. When BAEC were pretreated with LY294002 prior to flow, the inhibitor decreased flow-dependent phosphorylation of PKB by 80%, eNOS phosphorylation by 97%, and NO production by 68%. These data suggest that flow-dependent regulation of NO production in BAEC occurs in part by regulation of the level of phosphorylation of Ser-1179 in eNOS. Indeed, in vitro phosphorylation of eNOS by PKB occurred at Ser-1179 and activated the enzyme by 15–20-fold.

Flow-dependent phosphorylation of eNOS occurs near the amino terminus, at Ser-116, and near the carboxyl terminus, at Ser-1179. The amino acid residues surrounding Ser-1179 suggest that eNOS may be phosphorylated by PKB as well as possibly other protein kinases (49). Unlike Ser-1179, the sequence surrounding Ser-116 contains a proline residue at the n + 1 position, suggesting that a proline-directed protein kinase may phosphorylate this site. Thus FSS appears to activate at least two different protein kinases that phosphorylate eNOS. The significance of these modifications for regulation of eNOS function will be better understood when a crystal structure of the enzyme or site-directed eNOS mutants become available. Recently the crystal structure of an eNOS fragment (amino acid residues 39–482) revealed that pairs of cysteine residues (96 and 101) at the dimer interface tetrahedrally coordinate a zinc ion that stabilizes the tetrahydrobiopterin-binding site that interacts with Ser-104 and Val-106 (50). We speculate that incorporation of phosphate at Ser-116 may alter the structure of the tetrahydrobiopterin-binding site to enhance eNOS catalytic activity.

The two phosphorylation sites identified in this study and a third site described elsewhere (26) are the first in vivo phosphorylation sites identified for any isoenzyme of nitric-oxide synthase. The other two isoenzymes of nitric-oxide synthase, inducible nitric-oxide synthase (iNOS) and neuronal nitric-oxide synthase (nNOS), share 50–60% amino acid identity (51–53) with eNOS. The mechanism of regulation of nNOS remains unclear, but this enzyme contains the conserved PKB consensus sequence (51) we have identified as being phosphorylated in eNOS. nNOS lacks the F2 site and iNOS contains neither the F1 nor the F2 sites we have identified as being phosphorylated in eNOS in response to flow. Indeed, levels of NO produced by iNOS are believed to be regulated not by phosphorylation of iNOS but by the levels of mRNA for this protein (53). In contrast to the sequence divergence between eNOS and the other isoforms of NOS, the primary amino acid sequences of mammalian eNOS are more than 90% conserved (52) and contain the phosphorylation sites we have described.
Previous investigators have shown that eNOS contains predominantly phosphorysine with phosphotyrosine as a minor modification (36, 37). In these studies, either phenylarsine oxide or pervanadate was used. Both of these protein tyrosine phosphatase inhibitors greatly increase the overall tyrosine phosphate content of many cellular proteins (54, 55). We have found only phosphorysine in eNOS under both static and flow conditions in BAEC. Tandem MS/MS analysis of eNOS tryptic peptides derived from cells under static or flow conditions yielded CID spectra that covered 40% of the amino acid sequence of eNOS (data not shown). These spectra were screened and matched against the known sequence of eNOS using the SEQUEST (26) program, for addition of phosphate to serine, threonine, and tyrosine. No phosphothreonine or phosphotyrosine residues were found. Whereas such modifications may exist and may not be detected by the mass spectrometer because of low stoichiometry, the SEQUEST search did confirm the phosphoamino acid analysis in this study by identifying three phosphorysine-containing peptides (26).

Repeated attempts to identify tryptic phosphopeptides of eNOS by MS/MS analysis after conventional means of enrichment (IMAC) and separation (HPLC) were not successful, despite a 40% coverage of the eNOS primary amino acid sequence. In this study, the data-dependent modulation of the ratio at which peptide ions entered the MS/MS, produced by a peptide peak-generated drop in the electric field of the CE, expanded the analytical window for each peptide to be analyzed. This peak parking procedure (26, 38) should be applicable to identification of in vivo phosphorylation sites in other large phosphoproteins or in cases in which the phosphoprotein cannot be purified to homogeneity.

It has recently been shown (57) that the AMP-activated kinase phosphorylates Ser-1179 on eNOS in vitro and increases eNOS activity. While this manuscript was under review, two groups (58, 59) showed that Ser-1179 is phosphorylated in response to VEGF and shear stress, that PI3-kinase inhibitors decrease agonist-induced phosphorylation of eNOS and NO production, and that PKB phosphorylates and activates eNOS in vitro.

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