Nitric oxide (NO) is a pleiotropic regulator of vascular function, and its overproduction by inducible nitric-oxide synthase (iNOS) in inflammatory conditions plays an important role in the pathogenesis of vascular diseases. iNOS activity is thought to be regulated primarily at the level of expression to generate "high output" NO compared with constitutive NO synthases. Here we show iNOS activity is acutely up-regulated by activation of the B1-kinin receptor (B1R) in human endothelial cells or transfected HEK293 cells to generate 2.5–5-fold higher NO than that stimulated by Arg alone. Increased iNOS activity was dependent on B1R activation of the MAPK ERK. In HEK293 cells transfected with human iNOS and B1R, ERK phosphorylated iNOS on Ser745 as determined by Western analysis using phospho-Ser antibody, in vitro kinase assays with activated ERK, and MALDI-TOF mass spectrometry. Mutation of Ser745 to Ala did not affect basal iNOS activity but eliminated iNOS phosphorylation and activation in response to B1R agonist. Mutation of Ser745 to Asp resulted in a basally hyperactive iNOS whose activity was not further increased by B1R agonist. ERK and phospho-ERK (after B1R activation) were co-localized with iNOS as determined by confocal fluorescence microscopy. Furthermore, ERK co-immunoprecipitated with iNOS. The discovery that iNOS can be phosphorylated by ERK and acutely activated by receptor-mediated signaling reveals a new level of regulation for this isoform. These findings provide a novel therapeutic target to explore in the treatment of vascular inflammatory diseases.

**Experimental Procedures**

**Materials**—Human iNOS cDNA in pcDNA3 was a gift from Dr. Timothy Billiar, University of Pittsburgh. Human B1R cDNA was a gift from Dr. Fredrik Leeb-Lundberg, University of Lund, Sweden. Des-Arg10-kallidin (DAK), phenol red-free DMEM/F-12, and protein A-agarose were from Sigma. PD98059, SB202190, and purified ERK1 were from EMD Biosciences. Effectene was from Qiagen. Arg-free and phenol red-free DMEM (custom-made), Lipofectamine 2000, Opti-MEM I, DMEM/F-12, and protein A-agarose were from Sigma. PD98059, SB202190, and purified ERK1 were from EMD Biosciences. Effectene was from Qiagen. Arg-free and phenol red-free DMEM (custom-made), Lipofectamine 2000, Opti-MEM I, and human IFN-γ were from Invitrogen. Anti-human iNOS antibodies were from Santa Cruz Biotechnology (polyclonal; SC-8310) or R&D Systems (monoclonal; MC-5225). IL-1β was served a protective function at low concentrations under normal conditions, but in inflammatory conditions and at high levels, NO may contribute to tissue damage, especially after reaction with superoxide to form peroxynitrite (5–7). Under normal conditions, the primary NO synthase (NOS) generating NO in the vasculature is endothelial NOS (eNOS). The activity of this constitutive isoform is closely regulated in a variety of ways, including changes in intracellular Ca2+ levels, Ser or Thr phosphorylation, S-nitrosylation, and by interaction with other proteins (4, 8–11). Under inflammatory conditions, endothelial cells can also express inducible NOS (iNOS) (12–14). In contrast to eNOS, iNOS is considered to be regulated primarily at the level of expression (15). Once expressed, iNOS is thought to continuously generate NO in the presence of sufficient cofactors and substrate until the protein is degraded (15, 16). These properties have led to the concept that iNOS generates high output NO in an unregulated fashion with primarily cytotoxic functions, for example in the host defense response (4). We showed that activation of the inducible kinin B1 receptor (B1R) with peptide agonists or angiotensin-converting enzyme inhibitors directly stimulates high output NO production in cytokine-treated human lung microvascular endothelial cells (HLMVEC) (13, 17). Surprisingly, the NO produced in response to B1R activation was generated primarily by iNOS (13), indicating the possibility of its acute, post-translational activation. We report here that B1R activation results in ERK1/2 activation and phosphorylation of Ser745 in iNOS, resulting in a profound activation and generation of “super-high output” NO. Receptor-dependent activation of iNOS via phosphorylation reveals a new layer of complexity in the regulation of this enzyme that can play an important role in inflammatory conditions.
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from Calbiochem. FBS was from Atlanta Biologicals. Phospho-Ser antibody was from BD Biosciences. Rabbit antibodies to human ERK1/2 and phospho-ERK1/2 were from New England Biolabs. Goat anti-mouse IgG conjugated with Alexa 488 and goat anti-rabbit IgG (H+L) conjugated with Alexa 543 were from Molecular Probes.

Cell Culture and Transfection—HEK293 cells were maintained in DMEM with 10% heat-inactivated FBS, 100 μg/ml penicillin, and 100 units/ml streptomycin. HLMVEC (Cambrex) were cultured in dishes coated with 0.1% gelatin in basal medium with growth factors and antibiotics from EGM-2 BulletKits (Cambrex) and 10% FBS. HLMVEC were treated with 20 ng/ml IL-1β and 200 units/ml IFN-γ for 16–24 h to induce B1R and iNOS expression (13, 17).

HEK293 cells were co-transfected with iNOS and B1R cDNA plasmids with Effectene transfection reagent per the manufacturer’s instructions. After 24 h, cells were washed and maintained in DMEM/F-12 with 0.5% FBS overnight and then changed to the medium indicated for experiments. HEK293 cells stably expressing B1Rs were established by transfecting with human B1R cDNA and selecting with 500 μg/ml G418 (Invitrogen) and responsiveness to B1R agonist (increased intracellular Ca2+).

HLMVEC were transfected with either B1R or iNOS cDNA plasmids by electroporation with a Nucleofector (Amxa Biosystems) using the manufacturer’s kit and protocol optimized for HLMVEC. Experiments were carried out 48 h after transfection.

Generation of Constructs—Overlapping PCR was used to mutate Ser745 to either Ala or Asp using human iNOS cDNA as template. The PCR fragment was re-introduced into iNOS/pcDNA3.1 between the HindIII and NotI restriction sites. iNOS was tagged at the N terminus by inserting iNOS cDNA in-frame into the 3’ end of a PDsRed-Monomer vector (Clontech). For GST fusion constructs, the iNOS sequence encoding amino acids 721–761 was amplified by PCR using (Clontech). For GST fusion constructs, the iNOS sequence encoding amino acids 721–761 was amplified by PCR using (Clontech). Insertion of the PCR fragment was confirmed by sequencing.

Western Blotting—Cells were washed, collected, and lysed for 30 min on ice in 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) protease inhibitor mixture (Sigma) and centrifuged at 16,000 × g for 15 min at 4 °C. Supernatant proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected with appropriate primary antibodies followed by horseradish peroxidase-labeled secondary antibody and enhanced chemiluminescence.

Detection of ERK and iNOS Phosphorylation—HEK293 cells co-transfected with human iNOS and B1R were serum-starved in DMEM/F-12 for 3 h and then treated with 100 nM DAKD as indicated. Cells were washed twice with ice-cold PBS containing 100 mM sodium vanadate, collected by centrifugation, and lysed as above with buffer containing 20 mM p-nitrophenyl phosphate, 25 mM NaF, 20 mM β-glycerol phosphate, and 1 mM sodium vanadate to inhibit phosphatases. Samples were centrifuged (16,000 × g, 4 °C, 15 min), and the supernatants were used. For detection of ERK1/2, aliquots were added to an equal volume of 2× SDS-PAGE sample buffer, boiled for 5 min, and subjected to Western blotting with antibody to total or phospho-ERK1/2. For detection of phospho-iNOS, supernatants were pre-cleared by rotating for 30 min with 25 μl of protein A-agarose bound to normal rabbit IgG followed by centrifugation. Supernatants were incubated with 3 μg of polyclonal anti-human iNOS antibody overnight and then with 25 μl of protein A-agarose for 3 h. The immunoprecipitates were washed with 500 μl of lysis buffer five times and once with 50 mM Tris, pH 7.5, 150 mM NaCl. Proteins were eluted as above and analyzed by Western blotting with primary antibodies to either phospho-Ser or iNOS (monoclonal).

In Vitro Kinase Assay—The generation and isolation of GST fusion proteins and in vitro kinase assay were done as described (18) with the following modifications. Induction with isopropyl 1-thio-β-D-galactopyranoside was done at 30 °C for 3 h, and 0.5 μg of purified GST fusion protein was incubated with 50 ng of active or inactive ERK1 in kinase assay buffer containing 10 μCi of [γ-32P]ATP for 30 min at 30 °C. Samples were subjected to SDS-PAGE, stained with GelCode Blue (Pierce), and dried, and bands were detected by autoradiography.

Measurement of NO Production—For nitrite measurements, aliquots of medium (500 μl) were removed, and nitrate was reduced to nitrite using a Cd-Cu reducer (Nitralyzer II kit, World Precision Instruments) following the manufacturer’s instructions. Nitrite was measured in 50-μl aliquots using the Griess reagent (nitrite detection kit; Promega).

NO production was measured directly in real time with a porphyrinic microsensor as described (17, 19, 20). The porphyrinic sensor was positioned with a micromanipulator close to the cell culture surface (20 ± 1 μm). In some studies, cells were preincubated in L-Arg-free and phenol red-free DMEM for 2–3 h. After establishing a base line, 1 mM L-Arg (± 100 nM DAKD was added, and the response (current versus time) was recorded continuously.

Immunostaining and Confocal Microscopy—HEK293 cells stably expressing WT human B1R were transfected with DsRed-MFP-iNOS and ERK1 cDNA. After 24 h, cells were serum-starved with 0.5% FBS in DMEM/F-12 medium overnight and then treated with 100 nM DAKD for the indicated times. HLMVEC seeded on coverslips were cytokine-treated for 16 h as above, then serum-starved for 3 h in EBM-2 with 0.5% FBS, and treated with 100 nM DAKD for the indicated times. Cells were washed twice with PBS and fixed on ice for 30 min with 4% paraformaldehyde in PBS. HEK293 cells were stained with rabbit anti-phospho-ERK1/2 or anti-ERK1/2 antibody (1:1000 dilution) followed by goat anti-rabbit IgG second antibody conjugated with Alexa 488 (1:750 dilution). HLMVEC were stained with mouse monoclonal anti-human iNOS antibody (1:200 dilution) and rabbit anti-ERK1/2 antibody (1:200 dilution) followed by goat anti-mouse IgG (H+L) conjugated with Alexa 488 (1:750 dilution) for iNOS and goat anti-rabbit IgG (H+L) conjugated with Alexa 543 (1:750 dilution) for ERK1/2. Coverslips were mounted on slides with mount medium (Vectashield) and scanned using an Olympus LMS510 Meta confocal microscope.

MALDI-TOF Analysis—HEK293 cells stably expressing B1Rs were transfected with WT, S745A, or S745D iNOS and then incubated with or without 100 nM DAKD for 5 min. Cells were lysed in buffer containing phosphatase inhibitors and immuno-
precipitated with anti-iNOS antibody as above. An aliquot of immunoprecipitate from cells transfected with WT iNOS but not treated with DAKD was incubated with purified active ERK1 as described above. Samples were separated by SDS-PAGE on 7% gels and stained with GelCode Blue, and the iNOS band at 130 kDa was excised and digested with trypsin as described (21). Tryptic peptides were subjected to MALDI-TOF mass spectrometry using a cyano-4-hydroxycinnamic acid matrix and analyzed by a Voyager DE-PRO mass spectrometer (Applied Biosystems) equipped with a nitrogen laser as described (21).

RESULTS

HLMVEC were treated with IL-1β and IFN-γ for 16 h to up-regulate expression of iNOS and B₁R (13, 17). After incubation in Arg-free medium for 3 h, addition of 1 mM Arg resulted in prolonged high output NO (Fig. 1A), reaching a maximum of 295 ± 22 nM NO (n = 3). In HLMVEC not preincubated in Arg-free medium, addition of 100 nM B₁R agonist DAKD, caused a greater increase in NO production that peaked between 40 and 60 min (Fig. 1B), reaching a maximum of 832 ± 37 nM NO (n = 3). Total NO output (measured as area under the curve) was much greater for cells treated with DAKD than cells treated with Arg (Fig. 1C). The specific iNOS inhibitor, 4 µM 1400W, blocked the response to either DAKD or Arg but only the response to DAKD was inhibited by B₁R antagonist des-Arg¹⁰-Leu⁸-kallidin (Fig. 1, A–C).

Further proof that B₁Rs can activate iNOS was obtained in transfected HEK293 cells. DAKD (100 nM) generated only low levels of NO in mock-transfected cells or cells transfected with either iNOS alone or B₁R alone (Fig. 1D). However, in cells co-transfected with B₁R and iNOS, DAKD generated a profound output of NO that was blocked by the iNOS inhibitor
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1400W (Fig. 1D). To confirm these results, nitrite accumulation was measured in HEK293 cells transfected with B1R and iNOS. Cells incubated with fresh medium alone (containing 0.7 mM Arg) generated 1.2 ± 0.1 μM (n = 4) nitrite in 20 min, reflecting basal iNOS activity. Addition of 100 nM DAKD with fresh medium increased nitrite accumulation to 6.2 ± 0.3 μM, which was inhibited 77% by a B1 antagonist, 1 μM des-Arg¹⁰-Leu⁸-kallidin (1.4 ± 0.1 μM nitrite; n = 4). Finally, in B1R- and iNOS-transfected cells, 1 mM Arg stimulated a slow increase in NO concentration as measured with the NO electrode, but addition of 100 nM DAKD 5 min later caused a further 3-fold increase in the slope of the rate of NO production (Fig. 1E). Taken together, these data show that B1R stimulation results in acute activation of iNOS, a novel mode of regulation for this enzyme.

It has been reported that B1Rs can activate eNOS transfected into adventitial fibroblasts of cerebral arteries (22), and we reported that B1R-dependent NO production was because of eNOS activation in bovine pulmonary artery endothelial cells (13). However, in HLMVEC, B1R-dependent NO production is blocked by the highly specific iNOS inhibitor 1400W (Fig. 1) and not by the relatively eNOS/nNOS-specific inhibitor L-N⁶-nitroarginine (L-NNA) (13). To further rule out the involvement of eNOS activation in the HLMVEC response, we transfected HLMVEC (not cytokine-treated) with the B1R alone and stimulated with 100 nM DAKD. As shown in Fig. 2A, B1R-transfected cells generated relatively low levels of NO that were only slightly higher than that in control HLMVEC or vector-transfected cells. NO production was not because of eNOS activation as it was not inhibited by 4 μM L-NNA (Fig. 2A). These results are consistent with our previous report of a low level of prolonged B1R-dependent NO output in control HLMVEC (17), which constitutively express low levels of iNOS (not shown). To confirm that L-NNA does inhibit eNOS in HLMVEC, cells were stimulated with 10 μM A23187, the calcium ionophore, to acutely activate eNOS, which resulted in a sharp pulse of NO output reaching a maximum of 218 ± 13 nM within 3 s and returning to base line within 20 s (not shown). L-NNA (4 μM) pretreatment substantially reduced the response to A23187 (70 ± 7 nM NO), whereas 50 μM PD98059 had no effect (222 ± 13 nM NO). To show that B1Rs were efficiently transfected, HLMVEC were cotransfected with B1Rs + iNOS and then stimulated with either 1 mM Arg or 100 nM DAKD. Under these conditions, DAKD stimulated high output NO (Fig. 2B) similar to that seen in cytokine-treated HLMVEC (Fig. 1).

We explored the involvement of ERK as the B1R is known to activate this MAPK (23). In HEK293 cells transfected with B1R and iNOS, 100 nM DAKD stimulated a large increase in ERK1/2 phosphorylation within 5 min and was elevated at 20 min (Fig. 3A). In transfected HEK293 cells or cytokine-treated HLMVEC preincubated with the MEK inhibitor PD98059, NO production in response to 100 nM DAKD was substantially reduced (Fig. 3B), indicating ERK activation is required to stimulate iNOS activity. To determine whether iNOS is phosphorylated in response to B1R activation, we used phospho-Ser-specific antibody to probe immunoprecipitated iNOS. As shown in Fig. 3C, there was substantial serine phosphorylation of iNOS within 5 min of DAKD stimulation, which remained elevated at 20 min. A phosphorylated dimer form of iNOS was also seen under these denaturing conditions (Fig. 3C), which may represent the nondissociable dimer form of iNOS recently reported in HEK293 cells expressing iNOS (24). The iNOS phosphorylation was ERK-dependent as PD98059 inhibited it (Fig. 3D).

Although eNOS activity is regulated by phosphorylation (25), none of the identified phosphorylation sites are conserved in iNOS. Analysis of the human iNOS sequence with Scansite (26) and NetPhos (27) revealed one highly significant potential MAPK phosphorylation site at Ser²⁴⁵, which is conserved in all known iNOS sequences (Fig. 4A). This Ser is also conserved in nNOS and eNOS (Fig. 4A) but has not been reported to be phosphorylated. We mutated Ser²⁴⁵ to Ala (S745A) and found that phosphorylation was greatly diminished in response to B1R

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stimulation (Fig. 3 A). To prove that ERK can directly phosphorylate Ser745, WT and S745A GST-iNOS fusion proteins containing residues 721–761 were generated. When incubated with purified active (phospho)-ERK1 in the presence of \(^{32}\)P-ATP, a phosphorylated band was seen with either WT GST-iNOS-(721–761) or ATF-2 (positive control) but not with S745A GST-iNOS-(721–761) (Fig. 5 A). Inactive (de-phospho) ERK1 did not yield any radioactive bands (not shown). Further evidence was obtained from MALDI-TOF mass spectrometry of tryptic peptides from immunoprecipitated iNOS. A tryptic peptide with the sequence QNLQSPTSSR (where the underlined S indicates Ser at position 745) was at \(m/z\) 1117.560 in all samples.  

**FIGURE 3.** B1R stimulation activates ERK-dependent phosphorylation of iNOS. A, HEK293 cells transfected with B1R and iNOS were preincubated for 30 min with or without p38 inhibitor SB202190 or MEK inhibitor PD98059 and then stimulated with 100 nM DAKD for the indicated time. Cells were lysed, and Western analysis was carried out using antibodies to phospho-ERK or total ERK. B, cytokine-stimulated HLMVEC or HEK293 cells transfected with B1R, and iNOS were preincubated for 30 min without or with MEK inhibitor PD98059, and 100 nM DAKD was added, and NO production was recorded continuously with a porphyrinic electrode for 20 min. C and D, HEK293 cells transfected with B1R and either WT iNOS (without or with 30 min of preincubation with PD98059) or S745A mutant iNOS were stimulated with 100 nM DAKD for the indicated time. Cells were lysed and immunoprecipitated (IP) with polyclonal antibody to iNOS followed by Western blotting (WB) with phospho-Ser–specific antibody (top panel). Blots were stripped and re-probed with monoclonal antibody to iNOS (lower panel). (The lower apparent level of iNOS phosphorylation in Fig. 3C compared with Fig. 3D is possibly due to use of a different lot of phospho-Ser antibody from the same manufacturer.) B shows mean values ± S.E. for \(n = 3\). All other panels are representative of three separate experiments.  

**FIGURE 4.** Ser745 is conserved in iNOS from all species and is present on an exposed loop in the reductase domain. A, comparison of NOS sequences around Ser745 (arrow). B, model of the structure of the human iNOS reductase domain was generated using the EsyPred3D homology modeling program (50) based on the structure of the rat nNOS reductase domain (42, 43). The Ser745 side chain is colored purple and marked with an arrow. The cofactor binding domains and the connecting domain (CD) as identified in rat nNOS are indicated by arrows.)
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A

GST-iNOS

WT

S745A

GST-ATF-2

GST-iNOS

GST

GST-ATF-2

B

FIGURE 5. Activated ERK phosphorylates Ser745 in iNOS. A, WT and S745A GST-iNOS-(721–761) or GST-ATF-2 fusion proteins were incubated with [γ-32P]ATP and active ERK for 30 min followed by SDS-PAGE. The gel was stained for protein (bottom panel) and then analyzed for radioactivity by autoradiography (top panel). B, HEK293 cells stably expressing B1Rs and transfected with iNOS were untreated (WT control) or treated with 100 nM DAKD for 5 min. iNOS immunoprecipitated from untreated cells was also incubated with active ERK in vitro. Immunoprecipitated iNOS was resolved by SDS-PAGE, and tryptic peptides were analyzed by MALDI-TOF mass spectrometry. Peaks corresponding to iNOS residues 741–750 (m/z = 1117.560, nonphosphorylated, 1197.526 phosphorylated) and residues 222–229 (m/z = 1121.487) are indicated by arrows. The peak at m/z = 1198.6 was not identified. Results shown are representative of three separate experiments.

samples from cells transfected with WT iNOS (Fig. 5B), but not in those transfected with either S745A or S745D mutants, which yielded instead peaks at 1101.564 or 1145.569, respectively, because of altered masses of the mutated residues (not shown). A peak corresponding to the phosphorylated form of the tryptic peptide (80 Da larger at m/z 1197.526) was detected in WT iNOS treated in vitro with activated ERK or from cells stimulated with 100 nM DAKD but not in WT iNOS from untreated cells (Fig. 5B) or cells transfected with the S745A or S745D mutants and treated with DAKD (not shown).

To determine whether phosphorylation at Ser745 is required for B1R-mediated activation of iNOS, HEK293 cells were co-transfected with B1Rs and either WT iNOS, the S745A mutant (to block phosphorylation), or S745D mutant (to mimic phosphorylation). The basal activities of WT and S745A iNOS were equal in response to 1 mM Arg, but 100 nM DAKD + 1 mM Arg with 100 nM DAKD for 10 min, cells stained brightly with anti-phospho-ERK1/2 antibody in both the cytosol and nucleus, and a high degree of co-localization of iNOS and phospho-ERK1 was apparent in the cytosol (Fig. 7A). Evidence for interaction of iNOS and ERK was obtained in co-immunoprecipitation studies. Antibodies to iNOS co-immunoprecipitated ERK1 only in cells co-expressing iNOS and ERK1, both in the absence and presence of 100 nM DAKD (Fig. 7B).

To explore this interaction in HLMVEC, cytokine-treated cells were serum-starved for 3 h and were then treated with 100 nM DAKD. As shown in Fig. 8A, there was robust ERK activation within 1–5 min in response to B1R agonist. Immunostaining of the cells with antibodies to iNOS and ERK showed co-localization that intensified after 1 and 5 min of treatment with 100 nM DAKD (Fig. 8B). In addition, antibodies to iNOS co-immunoprecipitated ERK both in the absence
and presence of 100 nM DAKD (Fig. 8C) as in the transfected HEK293 cells.

**DISCUSSION**

We found that B1R-dependent stimulation of iNOS activity is mediated by ERK activation in cytokine-treated HLMVEC or transfected HEK293 cells. In transfected HEK293 cells, B1R-dependent ERK activation resulted in phosphorylation of Ser745 in iNOS, leading to a prolonged high output of NO. This effect was mimicked by transfection of cells with the phospho-mimicking mutant iNOS-S745D and blocked by transfection of the non-phosphorylatable mutant iNOS-S745A. Although this phosphorylation event has not been directly demonstrated in cytokine-treated HLMVEC, the similar dependence on ERK activation and the co-localization and co-immunoprecipitation of iNOS and ERK in this cell type indicate a similar mechanism is involved.

This discovery reveals a novel regulatory mechanism for iNOS, previously thought to be restricted to nNOS and eNOS (15, 16). iNOS is known to be regulated at the level of expression through transcriptional, post-transcriptional, and translational mechanisms (15), but recent studies suggest that post-translational regulation is also important. For example, iNOS dimerization and activity are inhibited by S-nitrosylation (28) but enhanced by interaction with hsp90 (29). iNOS is also downregulated by sequestration in aggresomes (30), rapid cellular turnover (31), and enhanced degradation by interaction with caveolin (32). Co-transfection of iNOS and activated Src in HEK293 cells caused phosphorylation of Tyr151, increased iNOS expression, and a 28% decrease in activity by redistribution to detergent-insoluble domains, but insulin-mediated activation of Src did not have this effect (33). These mechanisms of post-translational regulation, affecting basal iNOS activity or protein levels, differ fundamentally from our finding of an acute, B1R-dependent activation of iNOS via phosphorylation. Although numerous phosphorylation sites have been reported to regulate eNOS activity (4, 8, 9, 11), these mechanisms do not apply to iNOS. For example, Akt phosphorylation sites in eNOS...
are not conserved in iNOS, and co-transfection of constitutively active Akt with iNOS in COS-7 cells did not enhance iNOS activity (34). In addition, cGMP- and cAMP-dependent protein kinases can activate eNOS but do not phosphorylate or activate iNOS in vitro (35). Although iNOS in primary mouse macrophages was found to be phosphorylated on serine or threonine residues (36), the specific residues were not determined, and effects on activity were not measured. iNOS in Raw264.7 mouse macrophages contained phosphorytrosine under basal conditions, and pervanadate treatment enhanced tyrosine phosphorylation and iNOS activity (37), but the kinases/phosphatases involved and phosphorylation sites were not identified.

iNOS is considered to produce “high output” NO compared with eNOS and nNOS, so further activation can lead to super-high output NO in cells expressing high levels of enzyme. iNOS is highly expressed in rodent macrophages in response to LPS or cytokines, but it is much lower in human macrophages (15, 38). Many human cell types express iNOS in varying amounts in response to inflammatory mediators (15) but usually only at low levels in endothelial and vascular smooth muscle cells because of epigenetic silencing (12). Like iNOS, B1Rs are not constitutively expressed but are also induced under inflammatory conditions (23). Receptor-dependent activation of iNOS provides a mechanism for generating high output NO in inflammation, even in cells that express only modest levels of enzyme.

All NOS isoforms contain an N-terminal oxygenase domain that binds heme, tetrahydrobiopterin, and Arg and a C-terminal reductase domain that binds cofactors NADPH, FAD, and FMN and supplies electrons to the oxygenase domain (39, 40). Ser745 resides in the reductase domain (Fig. 4), but the mechanism by which phosphorylation increases iNOS activity is unknown. The oxygenase domain crystal structures have been determined for iNOS, eNOS, and nNOS, but the reductase domain structure is known only for rat nNOS (40–43). Based on known mechanisms of NOS regulation (40, 41), phosphorylation of Ser745 could increase activity in several ways as follows: by enhancing iNOS dimerization, by causing a conformational change that promotes electron transfer, or by generating a binding site for a protein that alters iNOS activity or localization. Regulation of dimerization seems unlikely as the oxygenase domain is the primary determinant of iNOS dimerization (44). In addition, Ser1005 (the nNOS equivalent of Ser745) is on the outside of the molecule opposite the nNOS dimer interaction interface (42).

A molecular model of the human iNOS reductase domain (based on rat nNOS (42, 43)) reveals Ser745 to be in the FAD binding domain in the middle of an exposed loop that connects two anti-parallel β-strands, adjacent to the NADPH binding domain (Fig. 4B). In this model, Ser745 would not directly interact with NADPH or FAD, but it is near Arg750/1010 (iNOS/nNOS numbering) and forms two H-bonds with residues in the loop containing Arg1017/1284. Arg1010 and Arg1284 are conserved residues critical for NADPH binding in nNOS (43, 45). Phosphorylation of Ser745 might alter its interaction with the loop containing Arg1017, which in turn could cause a conformational change, altering the alignment of NADPH and FAD or the orientation of the C-terminal inhibitory tail to enhance electron transfer and iNOS activity. Whether Ser745 is in a location that could influence the orientation of and electron transfer between the reductase and oxygenase domains will require determination of the crystal structure of a NOS holoenzyme, which has remained elusive (41).
The vascular endothelium comprises an important semipermeable barrier that dynamically regulates the passage of fluid and macromolecules and also participates in the regulation of vascular tone, host-defense reactions, and angiogenesis (46). NO generated by endothelial cells plays important roles in these processes via its ability to activate guanylate cyclase and increase cGMP or nitrosylate cysteine residues on critical signaling molecules (3, 4, 47). Although the cardiovascular effects of NO effects are generally beneficial, it becomes more complicated during inflammatory conditions where oxidants such as superoxide can combine with NO to form the damaging oxidant, peroxynitrite (5, 6).

In summary, the ability to generate super-high output NO by receptor-dependent ERK phosphorylation and activation of iNOS provides a new therapeutic target to explore in the treatment of vascular inflammatory diseases. The fact that angiotensin-converting enzyme inhibitors, drugs widely used for the treatment of hypertension, congestive heart failure, and many other cardiovascular disorders, can also activate this pathway as direct agonists of B,Rs (13, 48, 49) enhances the potential significance of these findings.

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