Phosphorylation of Thr^{495} Regulates Ca^{2+}/Calmodulin-Dependent Endothelial Nitric Oxide Synthase Activity

Ingrid Fleming, Beate Fisslthaler, Stefanie Dimmeler, Bruce E. Kemp, Rudi Busse

Abstract—The activity of the endothelial nitric oxide synthase (eNOS) can be regulated independently of an increase in Ca^{2+} by the phosphorylation of Ser^{1177} but results only in a low nitric oxide (NO) output. In the present study, we assessed whether the agonist-induced (Ca^{2+}-dependent, high-output) activation of eNOS is associated with changes in the phosphorylation of Thr^{495} in the calmodulin (CaM)-binding domain. eNOS Thr^{495} was constitutively phosphorylated in porcine aortic endothelial cells and was rapidly dephosphorylated after bradykinin stimulation. In the same cells, bradykinin enhanced the phosphorylation of Ser^{1177}, which was maximal after 5 minutes, and abolished by the CaM-dependent kinase II (CaMKII) inhibitor KN-93. Bradykinin also enhanced the association of CaMKII with eNOS. Phosphorylation of Thr^{495} was attenuated by the protein kinase C (PKC) inhibitor Ro 31-8220 and after PKC downregulation using phorbol 12-myristate 13-acetate. The agonist-induced dephosphorylation of Thr^{495} was completely Ca^{2+}-dependent and inhibited by the PP1 inhibitor calyculin A. Little CaM was bound to eNOS immunoprecipitated from unstimulated cells, but the agonist-induced dephosphorylation of Thr^{495} enhanced the association of CaM. Mutation of Thr^{495} to alanine increased CaM binding to eNOS in the absence of cell stimulation, whereas the corresponding Asp^{495} mutant bound almost no CaM. Accordingly, NO production by the Ala^{495} mutant was more sensitive to Ca^{2+}/CaM than the aspartate mutant. These results suggest that the dual phosphorylation of Ser^{1177} and Thr^{495} determines the activity of eNOS in agonist-stimulated endothelial cells. Moreover, the dephosphorylation of Thr^{495} by PP1 precedes the phosphorylation of Ser^{1177} by CaMKII. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;88:e68-e75.)

Key Words: nitric oxide • phosphorylation • calmodulin-dependent kinase • protein kinase C • PP1

The constitutive endothelial nitric oxide (NO) synthase (eNOS) can be activated by receptor-dependent and -independent agonists as a consequence of an increase in the intracellular concentration of free Ca^{2+} ([Ca^{2+}]) and the association of a Ca^{2+}/calmodulin (CaM) complex with the enzyme (for review, see Fleming and Busse1). Indeed, both the agonist-induced NO formation and subsequent vasodilatation are abolished by the removal of Ca^{2+} from the extracellular fluid: as well as by CaM antagonists.4 eNOS was initially reported to be basally phosphorylated solely on serine residues5–9; however, eNOS can also be phosphorylated on threonine10 and tyrosine residues.10,11 To date, the functional consequences of eNOS Ser^{1177} phosphorylation have been studied in most detail; phosphorylation of this site on the reductase domain by Akt/protein kinase B (PKB), PKA, or the AMP-activated kinase (AMPK)6,12–16 enhances the specific activity of eNOS so that NO production is enhanced in the absence of a maintained increase in intracellular Ca^{2+}13,17 eNOS Ser^{1177} phosphorylation is however only associated with a modest increase in enzyme activity, ie, ~2 times the basal activity,13,17 and cannot account for the burst of NO production observed after the activation of endothelial cells by Ca^{2+}-elevating agonists. Because AMPK phosphorylates eNOS in vitro on Thr^{495} in the CaM-binding domain, leading to a reduction in eNOS activity,12 we determined whether the phosphorylation of eNOS Thr^{495} influences its association with CaM and thus the initial burst of enzyme activity in agonist-stimulated endothelial cells.

Materials and Methods

The eNOS polyclonal antibody used for immunoprecipitation was generated from the keyhole limpet hemocyanin-conjugated peptide sequence VPWTFDPGDPDTPGP (position 1191 to 1205 in eNOS protein) by Eurogentec, and the phospho-specific antibodies recognizing Thr^{495} eNOS and Ser^{1177} eNOS were generated as described previously.12 Some experiments were performed using a Ser^{1177} specific antibody purchased from New England Biolabs. The monoclonal eNOS antibody for immunoblotting was purchased from Transduction Laboratories. Antibodies against Erk1/2 and Akt/PKB,
as well as the phosho-specific antibodies recognizing p-Erk1/2 (Thr202/Tyr204), p-Akt/PKB (Ser473), and p-Akt/PKB (Thr308), were from Cell Signaling. Bradykinin was purchased from Bachem Biochemica GmbH, and calmodulin, Ro 31-8220, PD 98059, U0126, and the anti-CaMKII antibody were from Calbiochem-Novabiochem. Protein A Sepharose was from Amersham Pharmacia, and protein G Sepharose was from Zymed. N\textsuperscript{-}nitro-I-arginine and all other substances were obtained from Sigma.

Organ Chamber Experiments
Porcine hearts were obtained from a local abattoir, and coronary epicardial artery segments were excised, cut into rings (4 mm), and preconditioned using (U64619, 1 to 3 \( \mu \)mol/L), as described.18 The bradykinin-induced, NO-mediated relaxant response was assessed in the absence and presence of PD 98059 and U0126.

Cell Culture
Human umbilical vein endothelial cells or porcine aortic endothelial cells, isolated as described,19 were seeded on fibronectin-coated dishes containing medium M-199 and 20% FCS supplemented with penicillin (50 \( \mu \)U/mL), streptomycin (50 \( \mu \)g/mL), L-glutamine (1 mmol/L), and L(-)-ascorbic acid (5 mg/mL). Because of the loss of several signaling pathways with time in culture, endothelial cells were used only after one passage.

COS-7 cells were cultured in DMEM:nutrient mixture-F12 (DMEM-F12, 1:1; GibCO BRL-Life Technology) with 10% FCS and supplemented with penicillin (50 \( \mu \)U/mL) and streptomycin (50 \( \mu \)g/mL).

eNOS Mutants and COS Cell Transfection
EhNOS mutants, based on the human eNOS wild-type sequence, were generated using a polymerase chain reaction–based mutagenesis kit (Stratagene). The mutation of Thr\textsuperscript{495} to alanine (Ala\textsuperscript{495}) or aspartate (Asp\textsuperscript{495}) was verified by sequencing. COS-7 cells were transfected with wild-type eNOS, eNOS\textsuperscript{Ala\textsuperscript{495}}, or eNOS\textsuperscript{Asp\textsuperscript{495}} mutants using either Superfect (Qiagen) or calcium phosphate coprecipitation, as described.20 Cells were cultured for an additional 48 hours before the isolation of eNOS protein.

Immunoblotting and Immunoprecipitation of eNOS
Cells were lysed in buffer containing Tris-HCl (pH 7.5; 50 mmol/L), NaCl (150 mmol/L), NaF (100 mmol/L), Na\textsubscript{2}PO\textsubscript{4} (15 mmol/L), Na\textsubscript{2}VO\textsubscript{4} (2 mmol/L), leupeptin (2 \( \mu \)g/mL), pepstatin A (2 \( \mu \)g/mL), trypsin inhibitor (10 \( \mu \)g/mL), phenylmethylsulfonyl fluoride (PMSF; 0.005%, wt/vol), CaMKII substrate peptide (100 \( \mu \)mol/L), and Triton X-100 (1% vol/vol), left on ice for 10 minutes, and centrifuged at 10,000 \( \times \)g for 10 minutes. In some experiments, eNOS was immunoprecipitated after preclarifying of the cell lysate with a mixture of protein A/G Sepharose. Proteins in the cell supernatant or immunoprecipitate were heated with SDS-PAGE sample buffer and separated by SDS-PAGE, as described.21 Proteins were detected using their respective antibodies as detailed in Results and were visualized by enhanced chemiluminescence using a commercial available kit (Amersham).

To reprobe Western blots with alternative primary antibodies, the nitrocellulose membranes were incubated at 50°C for 30 minutes in a buffer containing Tris-HCl (67.5 mmol/L, pH 6.8), \( \beta \)-mercaptoethanol (100 mmol/L), and SDS (2%). After extensive washing, the filters were incubated in blocking buffer containing BSA (3%) and subsequently with the primary antibody.

Assay of eNOS Activity
EhNOS activity was monitored in intact cells by assessing the time-dependent and N\textsuperscript{-}nitro-I-arginine–sensitive accumulation of cGMP, as described22 and in cell lysates by monitoring the conversion of \( [\text{3H}] \)-arginine to \( [\text{3H}] \)-citrulline. After cell lysis, as described above, and centrifugation (100,000g), cell pellets were reuspended and incubated (double determination) in the absence and presence of N\textsuperscript{-}nitro-I-arginine (10 \( \mu \)mol/L) and calmodulin (up to 3 \( \mu \)mol/L) for 30 minutes with \( [\text{3H}] \)-arginine (0.5 \( \mu \)mol/L), NADPH (1 \( \mu \)mol/L), \( \delta \)-tetrahydrobiopterin (15 \( \mu \)mol/L), and flavine-adenine dinucleotide (1 \( \mu \)mol/L) in HEPES (50 mmol/L, pH 7.4) containing DTT (1 \( \mu \)mol/L), EGTA (0.2 \( \mu \)mol/L), EDTA (0.2 \( \mu \)mol/L), and CaCl\textsubscript{2} (174 to 402 \( \mu \)mol/L). The incubations (final volume 200 \( \mu \)L) were terminated by the addition of ice-cold HEPES buffer (100 mmol/L, pH 5.5) containing 10 mmol/L EGTA and 500 mg Dowex AG 50 W-X8 (counter-ion Na\textsuperscript{+}) cation exchange resin (Serva) and incubated for 5 minutes at 4°C. After centrifugation at 10,000g, \( [\text{3H}] \)-citrulline in the supernatant was quantified by liquid scintillation counting, and specific eNOS activity was calculated as the N\textsuperscript{-}nitro-I-arginine–sensitive formation of \( [\text{3H}] \)-citrulline per minute per milligram of protein.

CaMK-Dependent Kinase II (CaMKII) Assays
Protein kinase activity was determined in permeabilized endothelial cells using a synthetic peptide substrate derived from the autophosphorylated region of CaMKII (Calbiochem-Novabiochem) as described.21 To permeabilize cells and initiate the kinase assay, culture medium was replaced with assay buffer containing KCl (130 mmol/L), NaCl (10 mmol/L), glucose (5 mmol/L), MgCl\textsubscript{2} (10 mmol/L), EGTA (1 mmol/L), CaCl\textsubscript{2} (1.1 mmol/L), and HEPES-Tris (10 mmol/L, pH 7.4), supplemented with \( \beta \)-glycerophosphate (25 mmol/L), NaF (1 mmol/L), sodium vanadate (20 \( \mu \)mol/L), saponin (0.005%, wt/vol), CaMKII substrate peptide (100 \( \mu \)mol/L), and 100 \( \mu \)mol/L \( [\gamma\textsuperscript{32P}] \)ATP (22 000 cpm/mmol). After an initial incubation period (5 minutes), endothelial cells were stimulated with bradykinin as described in Results, and the reaction was terminated with 10% (vol/vol) TCA. Aliquots of the reaction mixture were then spotted onto phosphocellulose paper (Whatman P-81) and washed three times with 1% (vol/vol) phosphoric acid. Phosphopeptide bound to the paper strips was quantified by scintillation counting. Results are expressed as arbitrary units.

In some experiments, CaMKII activity was determined in eNOS immunoprecipitates isolated from bradykinin-stimulated endothelial cells. Incubations were performed in the absence and presence of CaMKII substrate peptide (100 \( \mu \)mol/L) at 30°C for 15 minutes in a buffer containing NaCl (140 mmol/L), glucose (5 mmol/L), MgCl\textsubscript{2} (10 mmol/L), EGTA (1 mmol/L), CaCl\textsubscript{2} (1.1 mmol/L), and HEPES-Tris (10 mmol/L, pH 7.4), supplemented with \( \beta \)-glycerophosphate (25 mmol/L), NaF (1 mmol/L), sodium vanadate (20 \( \mu \)mol/L), and 100 \( \mu \)mol/L \( [\gamma\textsuperscript{32P}] \)ATP (22 000 cpm/mmol). The reaction was stopped by transfer of the supernatant onto Whatman P-81 cellulose. Results are expressed as arbitrary units, which represent the difference in the signal obtained in the absence and presence of the specific substrate.

Statistics
Data are expressed as mean±SEM, and statistical evaluation was performed using Student’s \( t \) test for unpaired data, one-way ANOVA, followed by a Bonferroni \( t \) test, or ANOVA for repeated measures where appropriate. Values of \( P<0.05 \) were considered statistically significant.

Results
Time Course of the Bradykinin-Induced Changes in eNOS Phosphorylation
Using phospho-selective antibodies, we found that eNOS in unstimulated porcine aortic endothelial cells was strongly phosphorylated on Thr\textsuperscript{495} but only weakly, if at all, on Ser\textsuperscript{1177} (Figure 1). Both of the currently available antibodies that specifically recognize eNOS Ser\textsuperscript{1177} occasionally gave a double band. However, as the upper band recognized by the phospho-antibody is (1) larger than eNOS, (2) evident in some unstimulated cells (see Figure 1), (3) can be observed in cells expressing unphosphorylatable Ser\textsuperscript{1177} mutants (data not
Effect of Kinase Inhibitors on the Phosphorylation and Activity of eNOS

The bradykinin-induced serine phosphorylation of eNOS was not associated with the activation of Akt/PKB. In fact, although this kinase is basally active in unstimulated endothelial cells, Akt/PKB phosphorylation (Ser1177) was decreased after stimulation with bradykinin (Figure 2A). No bradykinin-induced increase in the phosphorylation of Akt/PKB could be detected using a second phospho-specific antibody that recognizes Akt/PKB Thr495 (data not shown). The selective phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, which abolishes the shear stress–induced phosphorylation of Akt/PKB as well as the phosphorylation and activation of eNOS,13 affected neither the bradykinin-induced phosphorylation of eNOS Ser1177 (Figure 2A) nor the intracellular accumulation of cGMP (Figure 2B).

Effect of Ca²⁺ Removal on the Bradykinin-Induced Alterations in eNOS Phosphorylation

As the agonist-induced activation of eNOS in endothelial cells is exquisitely Ca²⁺-dependent, we determined whether the phosphorylation/dephosphorylation of eNOS was equally affected by the removal of extracellular Ca²⁺.

In a nominally Ca²⁺-free solution ([Ca²⁺]₀ ≈ 10 nmol/L), bradykinin induced a significantly smaller increase in eNOS Ser1177 phosphorylation than that observed in Ca²⁺-containing medium (Figure 3). This finding suggests that although the influx of extracellular Ca²⁺ is required for the maximal phosphorylation of Ser1177, a small increase in [Ca²⁺], attributable to the release of intracellularly stored Ca²⁺, may be sufficient to elicit eNOS phosphorylation. KN-93 (30 μmol/L), a selective CaMKII inhibitor, abolished the agonist-induced phosphorylation of Ser1177, Akt/PKB, and Erk1/2 in bradykinin-stimulated porcine aortic endothelial cells (Figure 2B).
phosphorylation of Ser\textsuperscript{1177} and attenuated the bradykinin-induced accumulation of cGMP. The intracellular concentration of cGMP in porcine endothelial cells was 0.26±0.02 versus 0.25±0.02 pmol/well under basal conditions and 5.56±0.37 versus 1.18±0.13 pmol/well after stimulation with bradykinin (100 nmol/L, 3 minutes) in the absence and presence of KN-93, respectively (P<0.001, n=8). To show that bradykinin activates CaMKII in endothelial cells, we monitored the phosphorylation of a synthetic CaMKII substrate in permeabilized endothelial cells. Bradykinin enhanced the activity of CaMKII by \textasciitilde2.5-fold within 1 minute of cell stimulation (Figure 4A). Immunoprecipitation of eNOS from unstimulated porcine endothelial cell lysates resulted in the coprecipitation of CaMKII (Figure 4B). The CaMKII/eNOS association transiently increased after bradykinin stimulation before decreasing after \textasciitilde5 minutes. In some experiments, CaMKII activity was assessed in eNOS immunoprecipitates and the changes in kinase activity measured displayed a similar time course to that of the CaMKII/eNOS association (Figure 4B).

The removal of extracellular Ca\textsuperscript{2+} completely abolished the agonist-induced dephosphorylation of eNOS Thr\textsuperscript{495}, whereas the CaMKII inhibitor was without effect (Figure 5).

Role of PKC in the Regulation of eNOS Activity and Phosphorylation

Bradykinin-induced changes in eNOS phosphorylation were assessed in the absence and presence of the PKC inhibitor Ro 31-8220. The time-dependent phosphorylation of eNOS Ser\textsuperscript{1177} was unaffected by Ro 31-8220 (data not shown), whereas the basal phosphorylation of Thr\textsuperscript{495} was attenuated by the inhibitor, as was the rephosphorylation of this residue in bradykinin-stimulated endothelial cells (Figure 6A). These effects were associated with an increase in the accumulation of cGMP (Figure 6B). Acute incubation of endothelial cells with phorbol 12-myristate 13-acetate (PMA) for up to 30 minutes, which activates PKC, enhanced the phosphorylation of Thr\textsuperscript{495} (data not shown). Depleting PKC by incubating endothelial cells with PMA for 24 hours, on the other hand, significantly attenuated the phosphorylation of Thr\textsuperscript{495} in unstimulated cells and abolished its bradykinin-induced dephosphorylation and rephosphorylation (Figure 6C). In parallel experiments, both basal cGMP levels and the...
bradykinin-induced increase in cGMP were found to be significantly greater in PMA-treated than in solvent-treated cells (Figure 6D). Prolonged treatment with PMA did not affect the bradykinin-induced phosphorylation of Ser1177 (data not shown).

Effect of Thr495 Phosphorylation on the Association of CaM With eNOS

In coimmunoprecipitation experiments, we observed that only a small amount of CaM is associated with the eNOS isolated from unstimulated endothelial cells (Figure 7A). The recovery of CaM in eNOS precipitates was markedly increased when the endothelial cells were briefly stimulated (1 minute) with either bradykinin (data not shown) or histamine (Figure 7A), the time point at which the dephosphorylation of Thr495 was maximal. Ten minutes after cell stimulation, the phosphorylation of Thr 495 had returned to control levels and almost no CaM was associated with eNOS. KN-93 did not affect the agonist-induced increase in the binding of CaM to eNOS (data not shown).

Effect of Thr495 Phosphorylation on the Phosphorylation and Activation of eNOS

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Okadaic acid, which selectively inhibits PP2A, enhanced the phosphorylation of both Akt/PKB (data not shown) and eNOS Ser1177 (Figure 8). This effect was associated with a 2-fold increase in N^ω-nitro-L-arginine–sensitive cGMP accumulation, as previously reported.23 Calyculin A, which inhibi-
its PP1 and PP2A, markedly enhanced the basal phosphorylation of eNOS Thr$^{495}$ and prevented the bradykinin-induced dephosphorylation of this residue (Figure 8). Calyculin A also enhanced the phosphorylation of both Akt/PKB and eNOS Ser$^{1177}$. However, as calyculin A markedly attenuates the bradykinin-induced Ca$^{2+}$ response, it was not possible to assess the effect of this inhibitor on the bradykinin-induced production of NO using intact cells. Cyclosporin A, which inhibits the Ca$^{2+}$-dependent phosphatase calcineurin, did not affect the phosphorylation of either Thr$^{495}$ or Ser$^{1177}$ (Figure 8) or the agonist-induced accumulation of cGMP (data not shown).

**Effect of Thr$^{495}$ Phosphorylation on eNOS Activity**

The effects of phosphatase inhibitors on the phosphorylation of Thr$^{495}$ and the conversion of [H]$\text{L}$-arginine to [H]$\text{L}$-citrulline were assessed using unstimulated cultured porcine aortic endothelial cells. Incubation of endothelial cell lysates for 60 minutes at 37°C in assay buffer resulted in the dephosphorylation of eNOS Thr$^{495}$ (Figure 9A). Under these conditions, eNOS activity could be stimulated by the addition of CaM and was abolished by the CaM antagonist calmidazolium. The inclusion of calyculin A (100 nmol/L) in the assay samples greatly reduced the dephosphorylation of Thr$^{495}$ and abolished the sensitivity of eNOS to the addition of CaM (Figure 9A). Neither okadaic acid nor cyclosporin A prevented the activation of eNOS by CaM (data not shown).

The sensitivity of the eNOS Ala$^{495}$ and Asp$^{495}$ mutants to activation by CaM was also determined. The Ala$^{495}$ mutant was concentration-dependently activated by Ca$^{2+}$ and CaM, but the sensitivity of the Asp$^{495}$ mutant to these cofactors was significantly reduced, and activation of the enzyme was only apparent in the presence of 300 nmol/L Ca$^{2+}$ and 100 nmol/L CaM (Figure 9B). Calyculin A did not affect the activity of either eNOS mutant (data not shown), and differences in the enzymatic activity of the mutants were not due to differences in their expression, as determined by Western blotting (Figure 9B). Moreover, in the presence of supraphysiological concentrations of Ca$^{2+}$ and CaM (each 1 μmol/L), there was no difference in the maximal activity of the two enzymes.

**Discussion**

The results of the present investigation show that rapid changes in the phosphorylation of eNOS on two separate residues (Thr$^{495}$ and Ser$^{1177}$) precede its activation in response to the stimulation of endothelial cells with Ca$^{2+}$-elevating agonists, such as bradykinin, histamine, and ionomycin. Although Ser$^{1177}$ phosphorylation increases the specific activity of eNOS at any given Ca$^{2+}$ concentration, the association of CaM with eNOS in agonist-stimulated endothelial cells, and thus the initiation of NO production, is regulated by the Ca$^{2+}$-dependent dephosphorylation of eNOS Thr$^{495}$.

eNOS is basally phosphorylated on threonine and serine residues, and although Ser$^{1177}$ can be phosphorylated by several protein kinases in actively proliferating and stimulated cells, this residue is generally only weakly phosphorylated under resting conditions. Different stimuli appear to elicit distinct kinetics of Ser$^{1177}$ phosphorylation, for example, the phosphorylation of this residue in shear stress–stimulated cells is maintained, whereas Ser$^{1177}$ phosphorylation in bradykinin-stimulated cells is transient. The activation of eNOS in endothelial cells stimulated by fluid shear stress or with bradykinin also differs with respect to their sensitivity to the removal of extracellular Ca$^{2+}$, the former being relatively insensitive to changes in [Ca$^{2+}$]$_{i}$ and the latter highly Ca$^{2+}$-sensitive. These apparent differences can be attributed to the kinetics and the Ca$^{2+}$ sensitivity of the signal transduction cascade activated by a given stimuli. Although the activation of PI3K and Akt/PKB can account entirely for the phosphorylation of eNOS Ser$^{1177}$ observed in response to fluid shear stress and vascular endothelial growth factor, we found no evidence to suggest that endothelial cell stimulation with bradykinin activated Akt/PKB or that preventing the PI3K-mediated activation of Akt/PKB had any consequence on eNOS phosphorylation and NO generation. Although bradykinin has been reported to activate Akt/PKB in bovine aortic endothelial cells, this response was slower than the agonist-induced phosphorylation of eNOS Ser$^{1177}$, and bradykinin-...
induced NO production was unaffected by PI3K inhibitors. Therefore, signaling through the PI3K/Akt/PKB pathway does not appear to be relevant for the regulation of eNOS by agonists of G protein–coupled receptors such as bradykinin or histamine.

Experiments in which eNOS phosphorylation was monitored in the absence and presence of extracellular Ca$$^{2+}$$ suggested that the kinase which phosphorylates Ser$$^{1177}$$ in bradykinin- and histamine-stimulated endothelial cells is activated after an increase in intracellular Ca$$^{2+}$$. Moreover, the CaMKII inhibitor KN-93 abolished the bradykinin-induced phosphorylation of Ser$$^{1177}$$ and attenuated the bradykinin-induced accumulation of cGMP, indicating that CaMKII phosphorylates eNOS Ser$$^{1177}$$ in bradykinin-stimulated endothelial cells. Indeed, CaMKII could be coprecipitated with eNOS, and the CaMKII activity of eNOS precipitates was transiently increased after bradykinin stimulation. This stimulatory role for CaMKII in the regulation of eNOS differs somewhat from the role played by this kinase in the regulation of neuronal NOS activity, which is decreased after phosphorylation by CaMKII.$^{26,27}$

The formation of a Ca$$^{2+}$$/CaM complex and its subsequent binding to a specific domain within eNOS is generally thought to be a prerequisite for the activation of the enzyme by receptor-dependent agonists.$^{78-30}$ The results reported in the present study demonstrate that dephosphorylation of Thr$$^{495}$$ is important for facilitating the association of CaM. Furthermore, the corresponding Asp$$^{495}$$ mutant binds CaM only weakly. Alterations in the CaM-binding domain change the Ca$$^{2+}$$ sensitivity of the various NOS isoforms, and substitution of eNOS and inducible NOS (iNOS) CaM-binding domains in eNOS/iNOS chimeric proteins produces major alterations in the Ca$$^{2+}$/CaM dependency of the intact enzymes.$^{31}$ Moreover, although eNOS exhibits a basal activity at resting concentrations of intracellular Ca$$^{2+}$$, several groups have proposed that the activity of the enzyme is regulated by at least one autoinhibitory mechanism. Although a polypeptide insert in the FMN-binding domain of eNOS$$^{32,33}$ could mask the CaM-binding domain to account for such an effect, the results of the present investigation indicate that the constitutive phosphorylation of Thr$$^{495}$$ continually damps NO output by hindering the association of CaM with its binding site.

The constitutively active kinase, which maintains the phosphorylation of Thr$$^{495}$$ in unstimulated endothelial cells, appears to be a phosphoryl ester–sensitive isoform of PKC. Our findings are therefore in accordance with some of the earlier work on the phosphorylation of NOS indicating that eNOS is a PKC substrate and that PKC-mediated phosphorylation inhibits eNOS activity.$^{7,34,35}$ The results of the present study go a long way toward elucidating the mechanisms underlying such observations. Indeed, the PKC inhibitor Ro 31-8220 attenuated the basal phosphorylation of eNOS Thr$$^{495}$$, as well as the rephosphorylation of this site in agonist-stimulated cells, and enhanced NO production in both cultured cells and isolated arterial segments.$^{36}$ Moreover, using an in vitro assay of eNOS activity, we found it possible to demonstrate that preventing the dephosphorylation of Thr$$^{495}$$, using calyculin A, rendered eNOS basically inactive, whereas the activity of the dephosphorylated enzyme was markedly enhanced by both Ca$$^{2+}$$ and CaM. More importantly, because calyculin A actually enhanced the phosphorylation of Ser$$^{1177}$$ over the assay period without increasing eNOS activity, it is tempting to suggest that the maximal activation of eNOS at physiological concentrations of Ca$$^{2+}$$ and CaM requires the simultaneous phosphorylation of Ser$$^{1177}$$ and dephosphorylation of Thr$$^{495}$$.

The phosphatase that dephosphorylates Thr$$^{495}$$ in response to an increase in [Ca$$^{2+}$$], remains to be definitively identified, but circumstantial evidence, based on the pharmacological sensitivity of the agonist-induced dephosphorylation to calyculin A and the effect of this inhibitor on the ability of CaM to activate eNOS, indicates that PP1 can dephosphorylate Thr$$^{495}$$ in agonist-stimulated endothelial cells. Experiments investigating a potential association between eNOS and the phosphatases PP1 and PP2A suggested that both are associated with eNOS, but we failed to observe any stimulus-related change in the association of these proteins (authors’ unpublished observation, 2001). At this stage, therefore, it is only possible to speculate about the signaling pathway that could elicit the transient activation of PP1. In addition, in contrast to a recent report,$^{25}$ we found no evidence, either in intact cells or in cell lysates, to suggest that the Ca$$^{2+}$$-dependent phosphatase calcineurin dephosphorylates Thr$$^{495}$$ in bradykinin-stimulated endothelial cells. The reasons underlying these apparently contradictory observations are unclear but may be related to the cell type investigated, i.e., bovine as opposed to porcine and human endothelial cells. In addition, although it would be tempting to speculate that the reported interaction between calcineurin and Hsp90$$^{37}$ may account for the regulatory action of the heat shock protein on the activity of eNOS,$^{38}$ we found no significant effect of geldanamycin on the dephosphorylation of Thr$$^{495}$$ (authors’ unpublished observations, 2001).

In summary, the results of the present study show that bradykinin and histamine elicit the CaMKII-mediated phosphorylation of eNOS Ser$$^{1177}$, which enhances enzyme activity. The constitutive phosphorylation of a second residue, i.e., eNOS Thr$$^{495}$, however, dampens enzyme activity by preventing the binding of CaM. An essential process in the agonist-induced, Ca$$^{2+}$$-dependent activation of eNOS is therefore the stimulation of a phosphatase, most probably PP1, to dephosphorylate Thr$$^{495}$$ and thus facilitate CaM binding and the initial burst of NO production.

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