Cyclin-Dependent Kinase 5 Phosphorylates Endothelial Nitric Oxide Synthase at Serine 116

Du-Hyong Cho, Jungwon Seo, Jung-Hyun Park, Chulman Jo, Yoon Jung Choi, Jae-Won Soh, Inho Jo

Abstract—Nitric oxide (NO) production in endothelial cells (EC) is regulated by multisite phosphorylation of specific serine and threonine residues in endothelial NO synthase (eNOS). Among these, eNOS-Ser116 is phosphorylated in the basal state, and its phosphorylation contributes to basal NO production. Here, we investigated the mechanism by which eNOS-Ser116 is phosphorylated during the basal state using bovine aortic EC. Although a previous study suggested that protein kinase C was involved in eNOS-Ser116 phosphorylation, overexpression of various protein kinase C isoforms did not affect eNOS-Ser116 phosphorylation. An in silico analysis using a motif scan revealed that the eNOS-Ser116 residue might be a substrate for proline-directed protein kinases. Roscovitine, a specific inhibitor of cyclin-dependent kinase (CDK), 1, 2, and 5, but not an inhibitor of mitogen-activated protein kinase kinase or glycogen synthase kinase 3β, inhibited eNOS-Ser116 phosphorylation dose dependently. Furthermore, purified CDK1, 2, or 5 directly phosphorylated eNOS-Ser116 in vitro. Ectopic expression of the dominant-negative CDK5 but not dominant-negative CDK1 or dominant-negative CDK2 repressed eNOS-Ser116 phosphorylation and increased NO production. In addition, CDK5 activity was detected in bovine aortic EC, and coimmunoprecipitation and confocal microscopy studies revealed a colocalization of eNOS and CDK5. Cotransfection of CDK5 and p25, the specific CDK5 activator, increased eNOS-Ser116 phosphorylation and decreased NO production, but its parent molecule, p35, and p39, another activator, were not detected in bovine aortic EC, which suggests the existence of a novel CDK5 activator. Overall, this is the first study to find that CDK5 is a physiological kinase responsible for eNOS-Ser116 phosphorylation and regulation of NO production. (Hypertension. 2010;55:345-352.)

Key Words: nitric oxide • endothelial nitric oxide synthase • cyclin-dependent kinase 5 • phosphorylation • signal transduction

Endothelial nitric oxide synthase (eNOS) is an essential enzyme responsible for the production of endothelium-derived nitric oxide (NO), which is a key molecule with multiple functions, including vascular homeostasis, angiogenesis, and cell cycle regulation.1,2 The dysregulation of eNOS, therefore, contributes to the pathogenesis of certain diseases, such as atherosclerosis, hypertension, and cancer.3 It is well known that eNOS is regulated at the level of its phosphorylation.3,4 Several specific sites of phosphorylation have been identified, among which eNOS-Ser1179 (bovine sequence) and eNOS-Thr497 have been the most thoroughly evaluated. The phosphorylation of eNOS-Ser1179 increases NO production,5-7 which is mediated by several specific protein kinases, including Akt, AMP-activated protein kinase, calmodulin-dependent kinase II, and protein kinase A (PKA).5-11 Conversely, the phosphorylation of eNOS-Thr497 decreases eNOS activity,8,10 which is mediated by AMP-activated protein kinase8 and protein kinase C (PKC).10,12 This site is also dephosphorylated by phosphatases, such as protein phosphatase 1 and protein phosphatase 2B, which results in an increase in NO production.3,10 Two other sites, eNOS-Ser635 and eNOS-Ser617, have also been identified as phosphorylation targets of PKA and Akt, respectively.13 A recent study14 showed that AMP-activated protein kinase also phosphorylates eNOS-Ser635. Although the phosphorylation of eNOS-Ser635 serves as a positive regulator of NO production,15 the phosphorylation of eNOS-Ser617 does not exert a regulatory role on NO production itself.13 Phosphorylation of eNOS-Ser116 has also been documented. Although the exact function of eNOS-Ser116 phosphorylation is still the subject of debate, we demonstrated that its dephosphorylation was associated with eNOS activation and NO production by the oral antidiabetic drug troglitazone.16 Consistent with these findings, it was found that eNOS-Ser116, like eNOS-Thr497, was phosphorylated in cells in the basal state and that dephosphorylation by protein

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phosphatase 2B or a serine to alanine mutation mimicking dephosphorylation increased eNOS activity. It was also demonstrated that the PKC inhibitor calphostin C reduced phosphorylation of eNOS-Ser116, which suggests that phosphorylation of eNOS-Ser116 would be mediated by PKC. However, because calphostin C does not only inhibit PKC, PKC-mediated eNOS-Ser116 phosphorylation has not been clearly demonstrated. Therefore, studies evaluating the effects of wild-type or dominant-negative (DN) PKC constructs are warranted to provide direct evidence of the involvement of PKC in eNOS-Ser116 phosphorylation. In this study, we demonstrate for the first time that cyclin-dependent kinase (CDK) 5 is a physiological kinase responsible for eNOS-Ser116 phosphorylation and NO production in the basal state.

Methods
An expanded Materials and Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

Materials
PKC inhibitors (Go6976 and Ro318220); mitogen-activated protein kinase kinase inhibitors (PD98059 and U0126); a glycogen synthase kinase 3β inhibitor (LiCl); a CDK1, 2, and 5 inhibitor (roscovitine); a proteasome inhibitor (MG132); and recombinant bovine eNOS were purchased from Calbiochem (Darmstadt, Germany). Another glycogen synthase kinase 3β inhibitor (SB216763) was purchased from Calbiochem (Darmstadt, Germany). Another glycogen synthase kinase 3β inhibitor (SB216763) was purchased from Calbiochem (Darmstadt, Germany). Another glycogen synthase kinase 3β inhibitor (SB216763) was purchased from Calbiochem (Darmstadt, Germany).

In Vitro CDK5 Activity Assay
The in vitro CDK phosphorylation assay was performed as described,19 with minor modifications, and is described in detail in the online Data Supplement.

Cloning of Bovine p25
Polymerase chain reaction was conducted using bovine p25 forward primer, bovine p25 reverse primer, and cDNA from BAEC as a template. The polymerase chain reaction products were subcloned into the EcoRI-Xhol site of the pcDNA3.1 vector (Invitrogen). TaKaRa Ex Taq HS polymerase (Takara Bio Inc., Shiga, Japan) was used for all polymerase chain reactions. DNA sequence analyses were conducted by Solgent Co. Ltd. (Daejeon, South Korea). The following primers were used for cloning: Bovine p25 forward primer, 5′-AAATTTGAATTGATGGGATCC-3′; Bovine p25 reverse primer, 5′-ATATCTCGAGTCACCGGTCCAGCCCGAGGA-3′.

RT-PCR
Measurements of the level of CDK5, p35, and p39 mRNAs are described in detail in the online Data Supplement.

Measurement of NO Release
NO production by transfected BAEC was measured as nitrite (stable metabolite of NO) concentration in cell culture supernatants, as described,20 and is described in detail in the online Data Supplement.

Statistical Analysis
All results are expressed as mean±SD, with n indicating the number of experiments. Statistical significance was determined by a Student t test for 2 points. All differences were considered significant at P<0.05.

Results
PKC Does Not Mediate eNOS-Ser116 Phosphorylation
Because a previous study showed that a PKC inhibitor, calphostin C, inhibited eNOS-Ser116 phosphorylation in basal and vascular endothelial growth factor–treated cells, we examined PKC to determine whether it is involved in eNOS-Ser116 phosphorylation. Experiments evaluating the effects of the other PKC-specific inhibitors, Go6976 and Ros18220, did not lead to changes in eNOS-Ser116 phosphorylation (see Figure S1 in the online Data Supplement).

To further clarify our data, we transfected various hemagglutinin-tagged PKC isoforms, α, βI, βII, δ, ε, and ζ, into BAEC. Consistent with the results from PKC inhibitor experiment, overexpression of the PKC genes did not alter the status of eNOS-Ser116 phosphorylation in BAEC (Figure 1), which suggests that PKCs are not involved in the phosphorylation of eNOS-Ser116.
Roscovitine Represses eNOS-Ser116 Phosphorylation, and CDK1, CDK2, or CDK5 Directly Phosphorylates eNOS-Ser116 In Vitro

Next, we attempted to identify other protein kinase(s) that might be involved in eNOS-Ser116 phosphorylation. In silico analysis using the motif scan program (Scansite, http://scansite.mit.edu) revealed a PXSP/PSP motif around the eNOS-Ser116 sequence (see the amino acid sequences of eNOS from 101 to 130: 101-lgslvlprk lqtrp Spgpp paeqllsqar-130), which represents a putative substrate sequence for CMGC kinases, such as CDK, mitogen-activated protein kinase, glycogen synthase kinase, and CDK-like kinase. Treatment with U0126 and PD98059, which are specific inhibitors of mitogen-activated protein kinase kinase, had no effect on the phosphorylation of eNOS-Ser116 (Figure S2). Furthermore, treatment with the glycogen synthase kinase 3β inhibitors SB216763 and LiCl also had no effect on eNOS-Ser116 phosphorylation (Figure S2). However, we found that roscovitine, which is a specific inhibitor of CDK1, 2, and 5, dramatically decreased eNOS-Ser116 phosphorylation in a dose-dependent manner (Figure 2A). To further confirm whether CDK1, 2, or 5 directly phosphorylates eNOS-Ser116, we performed an in vitro phosphorylation assay using recombinant CDK1/Cyclin B, CDK2/Cyclin A, or CDK5/p25 in the presence or absence of roscovitine. The results showed that roscovitine repressed eNOS-Ser116 phosphorylation in a dose-dependent manner (Figure 2B).
Ser116, we performed an in vitro phosphorylation assay. Compared with the control, a purified CDK1/cyclin B, CDK 2/cyclinA, or CDK5/p25 complex significantly phosphorylated eNOS-Ser116 (Figure 2B). Furthermore, this phosphorylation was almost completely blocked by roscovitine, which suggests that the eNOS-Ser116 phosphorylation is specifically mediated by CDK1, 2, or 5. Although the CDK5/p25 complex phosphorylated eNOS-Ser116, we failed to find that the CDK5/p35 complex phosphorylated it (Figure S3). These results suggest that CDKs are involved in eNOS-Ser116 phosphorylation in the basal state and that a CDK5 activator plays a critical role in determining the substrate specificity.

Ectopic Expression of DN-CDK5 Inhibits eNOS-Ser116 Phosphorylation and Increases NO Production in BAEC

We next determined which CDK isoform was a kinase responsible for phosphorylation of eNOS-Ser116 in cells. As shown in Figure 3A, ectopic expression of DN-CDK5 alone (but not of DN-CDK1 or DN-CDK2) dramatically repressed eNOS-Ser116 phosphorylation. Furthermore, ectopic expression of DN-CDK5 significantly increased basal NO production in BAEC (Figure 3B).

CDK5 Is Enzymatically Active and Interacts with eNOS in Basal BAEC

In basal BAEC, we clearly detected CDK5 activity (Figure 4A). Furthermore, CDK5 was coimmunoprecipitated with eNOS, suggesting that there was a physical interaction between CDK5 and eNOS in cells (Figure 4B). Confocal microscopy also showed colocalization of eNOS and CDK5 in the perinuclear region (Figure 4C). Taken together, these results showed that CDK5 is a physiological kinase that mediates eNOS-Ser116 phosphorylation within the cell.

Overexpression of both CDK5 and p25 Increases eNOS-Ser116 Phosphorylation and Decreases NO Production

In an attempt to examine the regulatory mechanism by which CDK5 induces eNOS-Ser116 phosphorylation in cells, we transfected either CDK5, p25, or both constructs in BAEC. We found that overexpression of CDK5 alone had no effect on eNOS-Ser116 phosphorylation (Figure 5A). However, transfection with p25 alone led to a significant increase in eNOS-Ser116 phosphorylation, which increased further when both CDK5 and p25 were cotransfected in the cells. Furthermore, a significant decrease of NO release (to 72.5 ± 7.4% of the control) was also observed when BAEC were transfected with both CDK5 and p25 (Figure 5B), suggesting a physiological role for CDK5 activity in NO production.

p25 or p35 Protein Is Not Detected in BAEC

Because it has been well established that p35 and p39 are CDK5 activators in neurons, we evaluated these activators to determine whether they are also expressed and play a role in regulation of CDK5 activity in BAEC. RT-PCR clearly demonstrated that p35 mRNA was expressed in several types of EC, including BAEC (Figure 6A). However, the use of commercially available anti-p35 antibody, which is also known to recognize the proteolytic peptide p25, did not detect p35 or p25 protein expression in any of the EC tested, whereas it was clearly expressed in neuronal cells (Figure 6B). These results suggest that posttranscriptional or posttranslational regulation of neuronal p35 occurs in EC. Furthermore, another neuronal CDK5 activator, p39, was not detected in EC, even at the mRNA level (Figure 6A).

Discussion

Previously, we reported that the PPARγ ligand, troglitazone, decreased eNOS-Ser116 phosphorylation in a PPARγ-
independent manner, which led to increased NO production. However, to date, no kinases responsible for eNOS-Ser116 phosphorylation have been identified. This study clearly shows that CDK5 phosphorylates eNOS-Ser116 in BAEC that are in the basal state. We believe that this provides the first solid evidence that CDK5 is a physiological kinase that mediates eNOS-Ser116 phosphorylation and that the modulation of CDK5 activity may play an important role in regulating basal NO production in EC.

There has been only 1 study conducted to date that has demonstrated that pretreatment with the PKC inhibitor calphostin C represses basal eNOS-Ser116 phosphorylation. However, the authors in this previous study did not provide direct evidence that PKC itself was indeed a kinase responsible for eNOS-Ser116 phosphorylation. Calphostin C has a rather broad ability to inhibit kinases in addition to PKC, such as myosin light chain kinase, PKA, and protein kinase G; therefore, it is not surprising that calphostin C inhibited protein kinase activities other than PKC under the experimental conditions in the previously conducted study. On the basis of this notion, we evaluated various isoforms of PKCs to determine whether they directly phosphorylated eNOS-

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Figure 4. CDK5 is enzymatically active and interacts with eNOS in basal BAEC. Cell lysates from BAEC without transfection were immunoprecipitated (IP) using anti-CDK5 antibody, and the immunoprecipitates were incubated with 2 μCi of [γ-32P]ATP and 2 μg of histone H1 as a substrate in the presence or absence of 100 μmol/L roscovitine. After 1 hour of incubation at 30°C, the reaction was terminated by adding Laemmli sample buffer. CDK5 enzymatic activity was assessed by measuring the level of histone H1 phosphorylation by autoradiography. The blot shown is representative of at least 3 experiments. Quantification of histone H1 phosphorylation was analyzed as described in Figure 1. Differences were considered to be statistically significant at *P<0.05 (A). For the coimmunoprecipitation experiments, the cell lysates were transfected with CDK5 and IP using antibody against eNOS or CDK5 as described in Methods. A control using nonimmune IgG was also performed. The immunoprecipitates were then dissolved with Laemmli sample buffer and separated by SDS-gel electrophoresis. The bound proteins in the immunoprecipitates were then subjected to Western blot (WB) analysis using the respective antibodies. The blot shown is representative of at least 3 experiments (B). Cells were grown on coverslips, and subcellular locations of eNOS and CDK5 were detected by confocal microscopy, as described in Methods. The confocal images shown are representative of at least 3 experiments (C).

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Figure 5. Overexpression of CDK5/p25 increases eNOS-Ser116 phosphorylation and decreases NO production in BAEC. BAEC were transfected with cDNA encoding CDK5, p25, or CDK5/p25 or with empty vector. Cell lysates were prepared and p-eNOS-Ser116 and eNOS were analyzed as described in Figure 1. Overexpression of CDK5 and p25 was measured by Western blot analysis using antibodies specific to CDK5 and p35, respectively. The anti-p35 antibody used in this study is known to clearly recognize its proteolytic peptide, p25. The blot shown is representative of at least 5 experiments. Quantification of eNOS-Ser116 phosphorylation was analyzed as described in Figure 1 (A). NO released by BAEC transfected with empty vector or cDNA encoding CDK5/p25 was measured by the Griess method, with minor modifications. Each bar represents the mean NO production (after normalization to total cellular protein) relative to control ± SD from at least 3 experiments (B). Differences were statistically significant at *P<0.05 and **P<0.01.
Ser116 and which isoforms, if any, were responsible for the phosphorylation. Evaluation of the effects of more specific inhibitors (Figure S1) and isoform-specific PKC gene transfection (Figure 1) revealed that the PKC isoforms did not play a significant role in eNOS-Ser116 phosphorylation. These findings indicate that PKCs are not kinases responsible for eNOS-Ser116 phosphorylation. Furthermore, in silico analysis using the motif scan program (Scansite) available at http://scansite.mit.edu revealed that the eNOS-Ser116 site is a putative substrate for the CMGC kinase superfamily, but not for the AGC kinase superfamily, such as PKA, protein kinase G, and PKC.21

Evaluation of several members of the CMGC kinase superfamily revealed that CDK5 was a physiological kinase capable of phosphorylation of the eNOS-Ser116 in basal EC. Unlike other types of CDKs, CDK5 exhibits functions that are not involved in the cell cycle.22 CDK5 activity is critical for neuronal functions such as axonal guidance, neuronal migration, membrane transport, dopamine signaling, and cytoskeletal dynamics.23 Recently, several studies have shown that CDK5 plays an important role in the exocytosis, differentiation, and senescence of nonneuronal cells such as pancreatic $\beta$ cells,24,25 monocytes,26 muscle cells,27 and testis.28 For example, CDK5 regulates glucose-induced insulin exocytosis by phosphorylating Munc1825 or the L-type voltage-dependent calcium channel24 in pancreatic $\beta$ cells. In muscle cells, CDK5 phosphorylates nestin, the intermediate filament protein, which plays an important role in muscle development and regeneration.27 In EC, there has been only 1 report demonstrating that roscovitine, a selective inhibitor of CDK5, inhibited cell proliferation and induced apoptosis by blocking CDK5 expression, suggesting that CDK5 plays a role in the regulation of apoptosis.29 Although the molecular mechanism by which CDK5 mediates the growth inhibition of EC has not been fully defined, it is likely that it can be attributed to CDK5-mediated phosphorylation in the substrate(s) responsible for EC proliferation and apoptosis. In this regard, the present study shows that eNOS-Ser116 is a physiological substrate in nonneuronal EC. Because NO plays an important role in cell proliferation and apoptosis,30 the results of this study suggest that CDK5 is involved in the proliferation of EC through the suppression of NO production in the basal state. However, further study is needed to elucidate the relationship between increased eNOS-Ser116 phosphorylation, reduced NO production, and EC proliferation.

It is well known that the CDK5 monomer has no kinase activity and that its binding with specific activators is necessary and sufficient for the maximal enzyme activity to occur. We found that there is CDK5 activity in EC (Figure 4A) but that ectopic expression of wild-type CDK5 did not further increase eNOS-Ser116 phosphorylation (Figure 5A). These results suggest that the level of CDK5 activator limits the kinase function of CDK5 on eNOS-Ser116 in basal EC. Although overexpression of p25 alone or of both CDK5 and p25 led to a significant increase in eNOS-Ser116 phosphorylation (Figure 5A), the neuronal p35 protein, a parent molecule of p25, was not expressed (Figure 6). Nevertheless, the mRNA of p35 was clearly detected in EC. These results suggest that posttranscriptional or posttranslational regulation of p35, such as mRNA stability, translational efficacy, stability of the protein product, or other posttranslational modifications, occurred. Because it was previously reported that p35 and p25, as well as cyclins and other CDK activators, underwent ubiquitination–proteasome degradation,31 we evaluated the effects of treatment with 10 $\mu$mol/L of MG132, a proteasome inhibitor, for 6 hours on the protein expression of p35 in BAEC. However, we were still unable to detect p35 protein in MG132-treated EC (Figure S4), which suggests that the absence of p35 protein in BAEC does not result from protein degradation via the ubiquitin-proteasome pathway. Furthermore, another neuronal CDK5 activator, p39, was not found in EC at the mRNA level, which suggests that neither p39 nor its metabolite, p29, is involved in regulation of CDK5 in these cells. Taken together, these results suggest that there is an unknown CDK5 activator(s) in EC other than the previously established neuronal p35 or p39. In this regard, a novel gene named IC53, as an isoform of the C53 gene, which encodes a CDK5-binding protein, was cloned32 and was later found to be expressed mainly in EC.33 Most recently, Zhuo et al reported a significantly decreased eNOS activity in aorta and serum NO production from transgenic mice in which IC53 was specifically overexpressed in EC.34 In the same study, they also found that knockdown of IC53 mRNA by small hairpin RNA in human umbilical vein
endothelial cells increased eNOS activity by 126.3% without an alteration of eNOS mRNA level. Taken together, it is likely that the IC53 protein may be a potential candidate for the CDK activator responsible for eNOS-Ser116 phosphorylation. However, further study is needed to clarify this issue.

Considering that eNOS is a physiological substrate of CDK5 in EC, it is likely that the 2 proteins interact with each other in cells at certain levels. In this regard, unlike the nuclear localization of other CDK isoforms, such as CDK1 and 2, CDK5 has been detected in the perinuclear region of neuronal cells.35–37 Furthermore, some studies have shown that eNOS is localized in the perinuclear region of cultured EC and intact human blood vessels.38,39 which indicates that it may be cell cycle–dependent (or cell density–dependent). In the present study, abundant eNOS was found in the perinuclear regions in BAEC (Figure 4C). Moreover, eNOS was clearly colocalized with CDK5. These findings, together with the commounprecipitation of CDK5 with eNOS, further demonstrate that eNOS is a physiological substrate of CDK5.

Perspectives

This study is the first to show that multifunctional CDK5 directly phosphorylates eNOS-Ser116. Previous studies have shown that CDK5 plays a pivotal role in regulation of cortical neuronal cells.35–37 Furthermore, some studies have shown that eNOS is localized in the perinuclear region of cultured EC and intact human blood vessels.38,39 which indicates that it may be cell cycle–dependent (or cell density–dependent). In the present study, abundant eNOS was found in the perinuclear regions in BAEC (Figure 4C). Moreover, eNOS was clearly colocalized with CDK5. These findings, together with the commounprecipitation of CDK5 with eNOS, further demonstrate that eNOS is a physiological substrate of CDK5.

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Disclosures

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Cyclin-dependent Kinase 5 Phosphorylates Endothelial Nitric Oxide Synthase at Serine 116

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Methods

Cell culture, drug treatment, and transfection

Bovine aortic endothelial cells (BAEC) were isolated as previously described\(^1\) and then maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO\(_2\). EC were confirmed based on their typical cobblestone configuration when viewed by light microscopy and by a positive indirect immunofluorescence test for von Willebrand factor VIII. Cells between passages 5 and 9 were used for all experiments. After the BAEC reached confluence, the cells were further maintained for the indicated times in MEM supplemented with 0.5% NCS containing the indicated drugs or chemicals. Human umbilical vein endothelial cells (HUVEC) and human brain microvascular endothelial cells (HBMEC) were purchased from Cambrex Bio Science (Walkersville, MD) and Cell Systems (Kirkland, WA), respectively, and then grown in specific media according to the manufacturer’s instructions as described previously\(^2\). The brain hippocampus and cortex were carefully excised from adult male C57BL/6 mice (Bio Genomics, Inc., Charles River Technology, Gyeonggi-do, South Korea) as previously described\(^3\).

For transfection, BAEC were transferred to 60-mm plates and allowed to grow until they were approximately 60% confluent, at which time they were transfected with 3 \(\mu\)g of cDNAs encoding the wild-type (WT) or dominant negative (DN) genes using lipofectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After transfection for 5 h at 37 °C, cells were further incubated in fresh MEM for 24 h.

Western blot analysis

For western blot analysis, cells were treated with various chemicals, washed with ice-cold DPBS and then lysed in lysis buffer [20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 mmol/L \(\beta\)-glycerophosphate, 1 mmol/L NaF, 1 mmol/L \(\mathrm{Na}_3\mathrm{VO}_4\), and 1 \(\times\) Protease Inhibitor Cocktail \(\text{TM} \) (Roche Molecular Biochemicals, Indianapolis, IN)]. The protein concentrations were then determined using a BCA protein assay kit (Sigma, St. Louis, MO). Equal quantities of protein (20 \(\mu\)g) were separated on sodium dodecyl sulfate–polyacrylamide gel under reducing conditions, after which they were electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibodies followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK).

In vitro phosphorylation assay

To determine if CDK directly phosphorylates eNOS-Ser\(^{116}\) in vitro, 200 ng of recombinant CDK1/cyclin B, CDK2/cyclin A, CDK5/p25, or CDK5/p35 were incubated with 5 \(\mu\)g of recombinant bovine eNOS as a substrate in the presence or absence of 100 \(\mu\)mol/L roscovitine in 25 \(\mu\)L kinase assay buffer (25 mmol/L HEPES [pH 7.5], 1 mmol/L DTT, 10 mmol/L MgCl\(_2\), 0.2 mmol/L EGTA, 200 mmol/L ATP, 1 mmol/L PMSF, 1 mmol/L \(\beta\)-glycerophosphate, 1 mmol/L NaF, 1 mmol/L \(\mathrm{Na}_3\mathrm{VO}_4\), and 1 \(\times\) Protease Inhibitor Cocktail \(\text{TM} \)). After incubation for 1 h at 30 °C, the reaction was terminated by adding Laemmli sample buffer. The samples were then boiled for 5 min, after which they were subjected to western blot analysis using antibodies against eNOS and p-eNOS-Ser\(^{116}\).

In vitro CDK5 activity assay

BAEC were prepared and lysed in 1 \(\times\) E1A lysis buffer (50 mmol/L HEPES [pH 7.4], 0.1% Nonidet P-40, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L NaF, 10 mmol/L
β-glycerophosphate, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, and 1 × Protease Inhibitor Cocktail<sup>TM</sup>), after which they were centrifuged at 12,000 g for 10 min. The supernatant (300 μg protein) was then immunoprecipitated using 4 μL of CDK5 antibody. As a mock experiment, 4 μL of control serum (normal rabbit serum) were used instead of CDK5 antibody. The immunoprecipitates were then washed twice with E1A lysis buffer and two times with kinase assay buffer. Next, the immunoprecipitates were resuspended in 25 μL of kinase assay buffer, after which the kinase assay was started by adding 2 μCi of [γ-32P] ATP and 2 μg of histone H1 as the substrate in the presence or absence of 100 μmol/L roscovitine. After incubation for 1 h at 30 °C, the reaction was terminated by adding Laemmli sample buffer. The samples were then boiled for 5 min and subjected to 12% SDS-PAGE, after which the dried gel was exposed to X-ray films at –80 °C. The CDK5 activity was quantified by measuring the densitometry of bands obtained using an image analysis program (ImageJ; NIH, Bethesda, MD).

**Co-immunoprecipitation assay**

BAEC were transfected with WT-CDK5 and lysed in 1 × E1A lysis buffer and after which they were centrifuged at 12,000 g for 10 min. The supernatant (400 μg protein) was then immunoprecipitated using 5 μL of eNOS antibody, CDK5 antibody or mouse IgG for 12 h at 4 °C, followed by incubation for 2 h at 4 °C with 30 μl of a 50% slurry of pre-equilibrated protein G-Sepharose. The immunoprecipitates were then washed 5 times with 1 × E1A lysis buffer. Finally, the bound proteins were eluted with Laemmli sample buffer and subjected to western blot analysis using the appropriate antibodies.

**Reverse Transcription (RT) -PCR**

Total RNA was isolated from several EC, including BAEC and mouse brain tissues, using Trizol Reagent (Invitrogen), and then used for RT reactions. RT reaction was performed using 200 units of SuperScript<sup>TM</sup> RNase H<sup>+</sup> reverse transcriptase (Invitrogen), 10 pmol of oligo-dT, and 2 mmol/L of dNTPs in a 20 μL reaction mix containing 1 μg of RNA for 1.5 h at 42 °C. PCR amplification of cDNA encoding each targeted gene was conducted in a total volume of 20 μL containing 0.5 units of TaKaRa Ex Taq HS polymerase, 10 pmol of each primer, and 1 μL of RT sample. The following PCR primer pairs were designed to detect all human, mouse and bovine genes:


**Measurement of NO release**

NO production by transfected BAEC was measured as nitrite (stable metabolite of NO) concentration in cell culture supernatants, as described in our previous study<sup>4</sup>. Briefly, after cells were transfected with empty vector or CDK5 and p25, the culture medium was changed to Kreb’s solution (pH 7.4) and equilibrated for 1 h at 37 °C. At the end of the incubation, 200 μL of each supernatant was carefully transferred into a 96-well plate, with the subsequent addition of 100 μL of Griess reagent. After color development at room temperature for 15 min, the absorbance was measured on a microplate reader at a wavelength of 520 nm. A calibration curve was plotted using known amount of sodium nitrite solution. The measured amount of NO was normalized to total protein amount of the cells.
References


Figure S1.
Figure S2.

A

[Image of a graph showing relative phosphorylation of p-eNOS-Ser^116 and eNOS under different treatments with DMSO, PD98059, and SB216763. The graph includes a bar chart with data points for each condition.]
Figure S2.
Figure S3.

Mock          CDK5/p25          CDK5/p35
-   +          -   +          -   +          Roscovitine

Roscovitine

p-eNOS-Ser^{116}
eNOS

Relative phosphorylation

Mock          CDK5/p25          CDK5/p35
-   +          -   +          -   +          Roscovitine
Figure S4.

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<th>MG132</th>
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