Nitric oxide prevents phosphorylation of neuronal nitric oxide synthase at Serine1412 by inhibiting the Akt/PKB and CaM-K II signaling pathways

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Abstract. Neuronal nitric oxide synthase (nNOS) is an important regulatory enzyme in the central nervous system catalyzing the production of NO, which regulates multiple biological processes in the central nervous system. However, the mechanisms by which nNOS activity is regulated are not completely understood. In the present study, the effects of protein kinases on the phosphorylation of nNOS in GH3 rat pituitary tumor cells were evaluated. We show that phosphorylation of nNOS at Ser1412 could be induced by the phosphatidylinositol 3-kinase/protein kinase B (Akt/PKB) agonist insulin, the calcium/calmodulin-dependent protein kinase II (CaM-K II) agonist A23187 or the cAMP-dependent protein kinase A (PKA) agonist IBMX, respectively. The phosphorylation levels of nNOS at Ser1412, induced by activation of Akt/PKB or CaM-K II, but not by PKA signaling, were reduced by pre-treatment with the NO donor diethylamine-NONOate. This inhibitory effect could be reversed by addition of a reducing reagent, dithiothreitol. Furthermore, the levels of phosphorylation of nNOS at Ser1412, induced by Akt/PKB or CaM-K II but not by PKA signaling, were enhanced by inhibition of nNOS activity with 7-nitroindazole. These findings suggest that the activation of nNOS can be catalyzed by at least three protein kinases, Akt/PKB, CaM-K II or PKA. NO generated from nNOS feedback prevents the activation of nNOS by inhibiting either Akt/PKB or CaM-K II but not PKA signaling.

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Introduction

Nitric oxide (NO), generated by the family of NO synthases (NOS), is a signaling molecule that participates in multiple biological processes in the central nervous system, including neurotransmitter release, plasticity and apoptosis (1). NO exerts its effects by regulating soluble guanylyl cyclase (sGC) to mediate cyclic guanosine monophosphate (cGMP) synthesis (2). In addition to the activation of sGC, NO also exerts cGMP-independent signaling, albeit at much higher levels than needed for sGC activation (2). These include modification of protein function by S-nitrosylation, now recognized as a prototype redox-dependent signaling component that mediates a variety of actions of endogenous NO (3). Under pathological circumstances, overproduction of NO can cause the formation of peroxynitrite, which is associated with excitotoxicity after stroke (4). S-nitrosylation and nitration of proteins by peroxynitrite also exacerbate central nervous system disorders such as dementia (5), Parkinson's disease (6), and Alzheimer's disease (7).

Neuronal NOS (nNOS) is a critical regulatory enzyme in the central nervous system catalyzing the production of NO from arginine. It has been reported that the control of NO production by the NMDA receptor at neural synapses is associated with regulatory phosphorylations of nNOS (8). However, the mechanisms by which these phosphorylations are induced in neurons and by which they regulate nNOS activity are not known. We and others reported that activity of nNOS was regulated by Ca^{2+}-dependent binding of calmodulin (CaM) and multiple protein kinase-dependent phosphorylation events, involving Ca^{2+}/calmodulin-dependent protein kinase (CaM-K) I and II (9,10) and phosphatidylinositol 3-kinase/protein kinase B (Akt/PKB) (11). Since the NO donors induce inactivation of Akt/PKB and CaM-K II enzyme through an S-nitrosylation-dependent pathway (12,13), other nNOS-kinases may also be downregulated by nNOS-mediated NO production.

Here, we examined whether protein kinases Akt/PKB, CaM-K II and protein kinase A (PKA) mediate the phosphorylation of nNOS at Ser1412 and the regulatory role of NO derived

from nNOS in rat pituitary tumor GH3 cells, which has been shown to express constitutive nNOS and produce NO (14).

Materials and methods

Materials. The cDNA for rat brain nNOS was a generous gift from Dr Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD) (15). Recombinant rat CaM was expressed in *Escherichia coli* BL21 (DE3) using pET-CM, kindly provided by Dr Nobuhiro Hayashi (Fujita Health University, Toyoake, Japan) (16). Recombinant CaM-K I, CaMK II and CaM-K IV were generated as described previously (17) and the plasmid pME18s-nNOS as detailed earlier (10). The nNOS mutant, Ser1412A, (a mutant bearing Ala in place of Ser1412) was subcloned into pME18s. The nucleotide sequences of each plasmid were confirmed. Active Akt/PKB was obtained were from Millipore (Billerica, MA). Rabbit anti-Akt/PKB polyclonal and mouse anti-phospho-Akt/PKB at Ser473 monoclonal antibodies from Cell Signaling Technology (Danvers, MA). A mouse anti-nNOS monoclonal antibody, A23187, IBMX, insulin and calmidazolium were purchased from Sigma (St. Louis, MO), Wortmannin and 7-nitroindazole (7NI) from Calbiochem (La Jolla, CA), H89 from Seikagaku Corp. (Tokyo, Japan) and DEA-NONOate from Alexis Corp. (San Diego, CA). ECL immunoblotting detection reagents were from GE Healthcare (Piscataway, NJ), λ protein phosphatase (λPPase) was from New England Biolabs (Ipswich, MA) and restriction and DNA-modifying enzymes were from Takara Shuzo (Osaka, Japan). Electrophoresis reagents were products of Bio-Rad. All other materials and reagents were of the highest quality available from commercial suppliers.

Anti-phosphopeptide-specific antibodies. A rabbit polyclonal antibody (pAb) raised against a phosphopeptide based on the amino acid sequence of rat nNOS RLRSESpIAFIEESK (NP1412) was purified from immunized rabbit sera by tandem column chromatography using phosphopeptide and dephosphopeptide-coupled Cellulofine (Seikagaku Corp.).

Purification of expressed proteins. Recombinant rat nNOS was expressed in *Escherichia coli* and purified by 2'-5'-ADP-agarose (Sigma), as described previously (18). Protein concentrations were determined by the method of Bradford using BSA as the standard (19).

Dephosphorylation of nNOS at Ser1412 by protein λ protein phosphatase (λPPase). Partially purified rat brain nNOS (0.5 μg) was incubated with or without 400 units λPPase in 1X λPPase Reaction Buffer (New England Biolabs) with 2 mM $MnCl_2$ in a final volume of 20 μl at 30˚C for 30 min. The reaction was terminated by boiling with SDS-PAGE sample buffer, followed by SDS-PAGE and western blot analysis with phospho-Ser1412-nNOS (NP1412) antibodies.

Cell culture and stimulation. Rat pituitary tumor GH3 cells were maintained in Ham's F10 medium (Sigma) supplemented with 15% horse and 2.5% fetal calf serum and subcultured in 6-cm dishes for 48 h. The cells were serum-starved for 16-18 h prior to addition of activators or inhibitors, either in the presence or absence of 7NI.

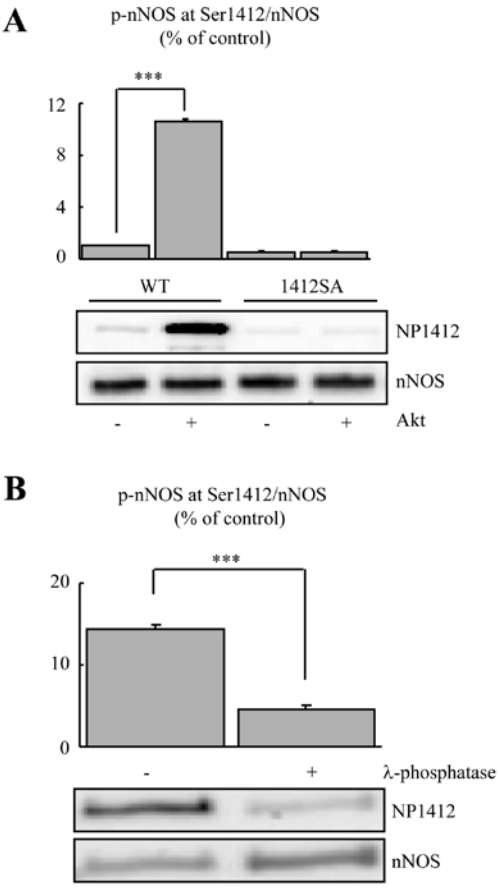

Figure 1. Phosphorylation of nNOS at Ser1412 in rat brain. (A) Equal amounts (0.5 μg) of purified nNOS-WT or nNOS-S1412A mutant were incubated either with active Akt/PKB (50 nM) plus 1 mM ATP or without any enzyme at 30˚C for 10 min, then the reaction was stopped by boiling with SDS-PAGE sample buffer. Western blotting was performed with phospho-specific antibody, NP1412 (NP1412). The immunoblot membranes were then stripped of NP1412 and reprobed with anti-nNOS antibody (nNOS). (B) Partially purified rat brain nNOS was incubated either with or without 400 units λPPase as described in Materials and methods, then the reaction was stopped by addition of sample buffer and western blotting was performed as described above. The histogram shows the amount of p-nNOS at Ser1412 relative to that of nNOS in the membrane. Values are expressed as mean ± SEM (n=3 for each group). ***P<0.001; p-nNOS, phosphorylation of nNOS.

Preparation of lysates, purification of nNOS and immunoblotting. For preparation of lysates, cells were homogenized by sonication with TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, and 1% Nodiet P-40). After centrifugation at 15,000 x g for 15 min, a 10 μl aliquot of ADP-agarose (50% slurry) (Sigma) was added to the supernatant, and the mixture was incubated for 1h at 4˚C. After precipitation by centrifugation and removal of the supernatant, the resin was washed 3 times with 300 μl of TNE buffer and boiled with 50 μl of SDS-PAGE sample buffer, then analyzed by SDS-PAGE and transferred to PVDF membranes (Bio-Rad), which were then blocked and probed with appropriate antibodies. Finally, the membranes were developed with the ECL Plus system, and band intensities were quantified by densitometric scanning (LumiVision PRO; Aisin Seiki, Aichi, Japan) using the LumiVision Imager program (Aisin Seiki).

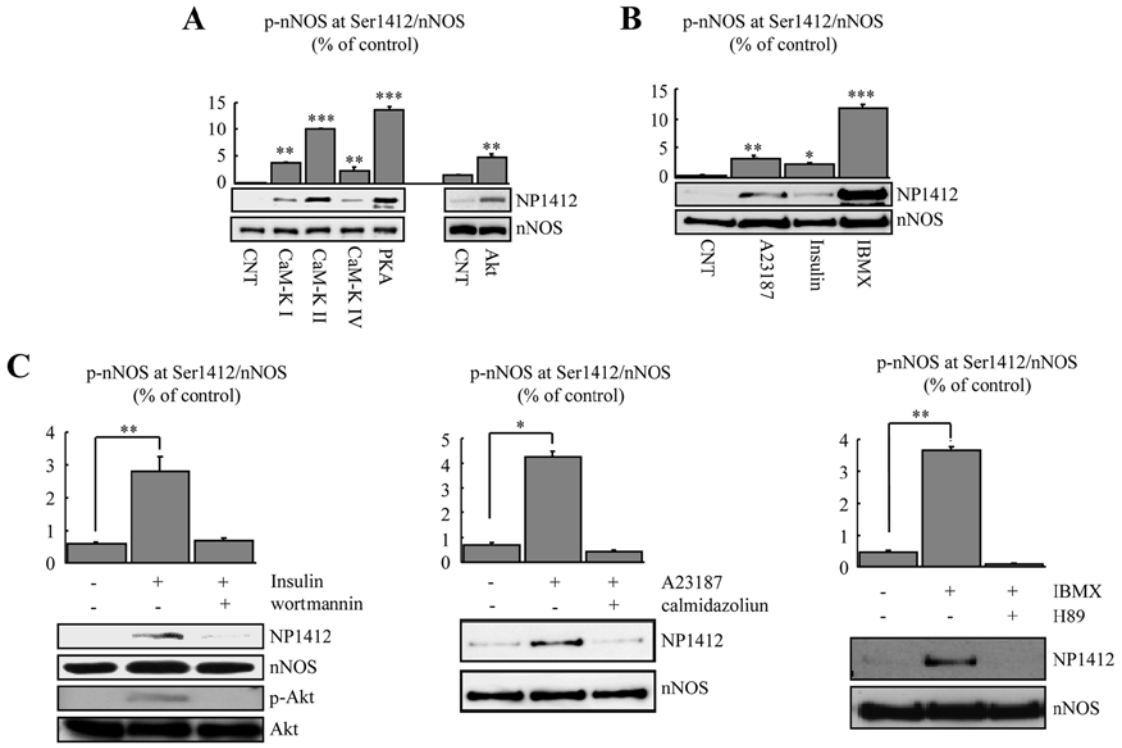

Figure 2. Phosphorylation of nNOS at Ser1412 by Akt/PKB, CaM-K II and PKA. (A) Recombinant nNOS (1 μM) was incubated in a protein kinase reaction buffer with activated CaM-K I, CaM-K II, activated CaM-K IV, PKA or active Akt/PKB with a concentration of 50 nM each at 30˚C for 10 min as described in Materials and methods. Then the reactions were stopped and phosphorylated nNOS was analyzed by western blotting with the phospho-specific antibody, NP1412. The immunoblot membranes were then stripped of NP1412 and reprobed with anti-nNOS antibody (nNOS). (B) GH3 cells were incubated with buffer alone (CNT), A23187 (10 μM, 5 min), insulin (100 μM, 10 min), IBMX (1 mM, 5 min) as indicated. nNOS was isolated by ADP-agarose precipitation, and its phosphorylation was monitored as described above. (C) GH3 cells were untreated or pre-treated with wortmannin (200 nM), calmidazolium (5 μM) or H89 (10 μM) for 30 min and then treated with buffer alone or insulin, A23187 and IBMX as indicated. Then phosphorylated nNOS was monitored as described above. The phospho-Akt/PKB at Ser473 was also examined with anti-p-Akt (p-Akt) in the case of insulin stimulation. The immunoblot membranes were then stripped of anti-p-Ak and reprobed with anti-Akt antibody (Akt). The histogram shows the amount of p-nNOS at Ser1412 relative to that of nNOS in the membrane. Values are expressed as mean ± SEM (n=3 for each group). *P<0.05, **P<0.001, ***P<0.001 vs. control in (A) and (B). p-nNOS, phosphorylation of nNOS.

Phosphorylation of nNOS in vitro. Phosphorylation reactions for nNOS were performed for 10 min at 30˚C in 40 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 400 μg/ml bovine serum albumin, 1 mM ATP, and 1 μM nNOS in the presence of 1 mM $CaCl_2$ and 1 μM CaM for activated CaM-K I (50 nM), CaM-K II (50 nM), activated CaM-K IV (50 nM) (9), or of 1 mM EGTA for PKA (50 nM), active Akt/PKB (50 nM). Reactions were stopped by boiling with SDS-PAGE sample buffer, followed by SDS-PAGE and western blot analysis with the antibody NP1412. To check for effects of NO on activity of Akt/PKB, CaM-K II and PKA, each protein kinase was preincubated with buffer alone or 1 mM DEA-NONOate for 40 min at 25˚C prior to phosphorylation of nNOS.

Statistical analysis. The significance of variability between the results from each group and the corresponding control was determined using an unpaired Student's t-test. The means ± SEM were calculated. A value of P<0.05 was considered statistically significant.

Results

Identification of in vivo phosphorylation of nNOS at Ser1412. To confirm the *in vivo* phosphorylation of nNOS at Ser1412, we generated a phospho-specific antibody, NP1412 and examined its specificity by western blotting. NP1412 reacted with active Akt/PKB-induced phosphorylated wild-type nNOS but not Ser1412A nNOS *in vitro* (Fig. 1A). NP1412 also recognized phospho-nNOS at Ser1412 in rat brain lysates. The immunoreactive band was significantly attenuated after treatment with the λPPase (Fig. 1B).

Phosphorylation of nNOS at Ser1412 by Akt/PKB, CaM-K II and PKA in vitro and in GH3 cells. *In vitro* kinase assays were employed using recombinant CaM-K I, CaM-K II, CaM-K IV, PKA and Akt/PKB. NP1412 reacted with nNOS phosphorylated by all kinases. The relative suitability of nNOS as a substrate, based on the intensity of the immunoreactive bands, was in the order CaM-K II, PKA > Akt/PKB > CaM-K I > CaM-K IV (Fig. 2A). To examine which protein kinase phosphorylates nNOS at Ser1412 in GH3 cells, the cells were treated with various agonists followed by western blotting with NP1412. When serum-starved GH3 cells were treated with the phosphodiesterase agonist IBMX, phosphorylation of Ser1412 was increased. Calcium ionophore A23187 or insulin-stimulated phosphorylation of Ser1412 was also detected, but only slightly (Fig. 2B).

Preincubation of GH3 cells with the PI3 kinase inhibitor wortmannin, the CaM antagonist calmidazolium or the PKA inhibitor H89, almost completely blocked each agonist-induced

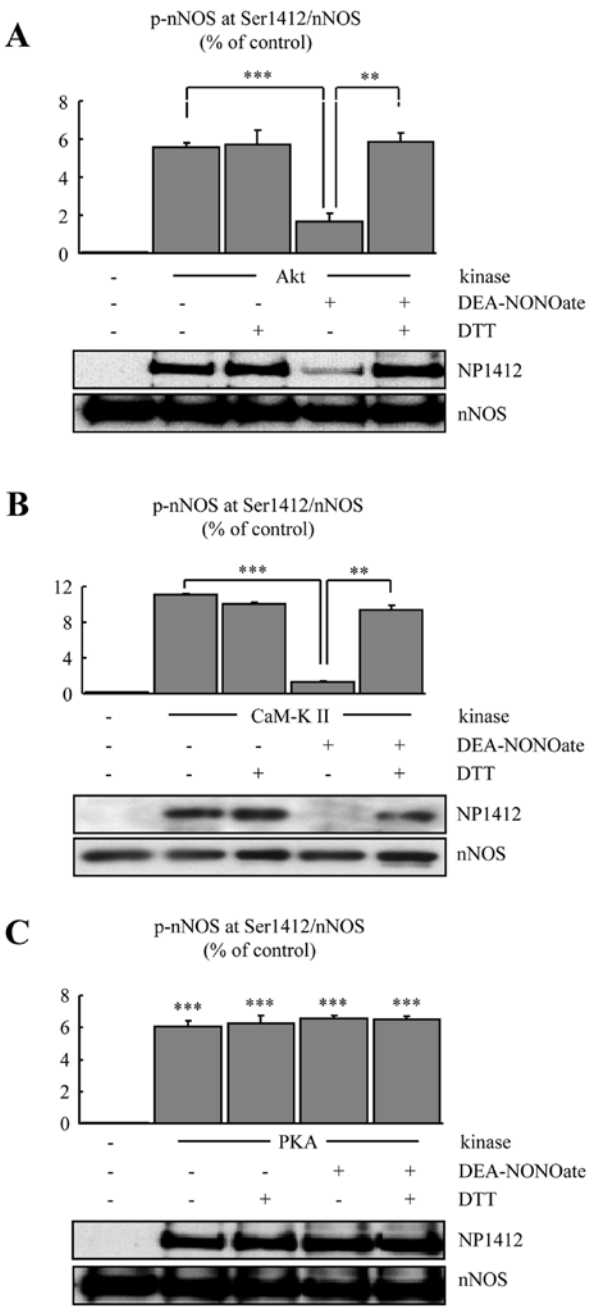

Figure 3. Effects of a NO donor on the phosphorylation of nNOS at Ser1412 by Akt/PKB, CaM-K II and PKA *in vitro*. Active (A) Akt/PKB, (B) CaM-K II or (C) PKA was preincubated with buffer alone or 1 mM DEA-NONOate for 40 min at 25˚C in 250 mM HEPES-NaOH (pH 7.7) and 0.5 mM EDTA. Then each kinase activity was assessed by *in vitro* nNOS phosphorylation assay as described for Fig. 2A. Incubation with dithiothreitol (DTT) (10 mM) restored the activity of DEA-NONOate-treated Akt/PKB and CaM-K II but not PKA. The histogram shows the amount of p-nNOS at Ser1412 relative to that of nNOS in the membrane. Values are expressed as mean ± SEM (n=3 for each group). **P<0.001, ***P<0.001 vs. control in (C). p-nNOS, phosphorylation of nNOS.

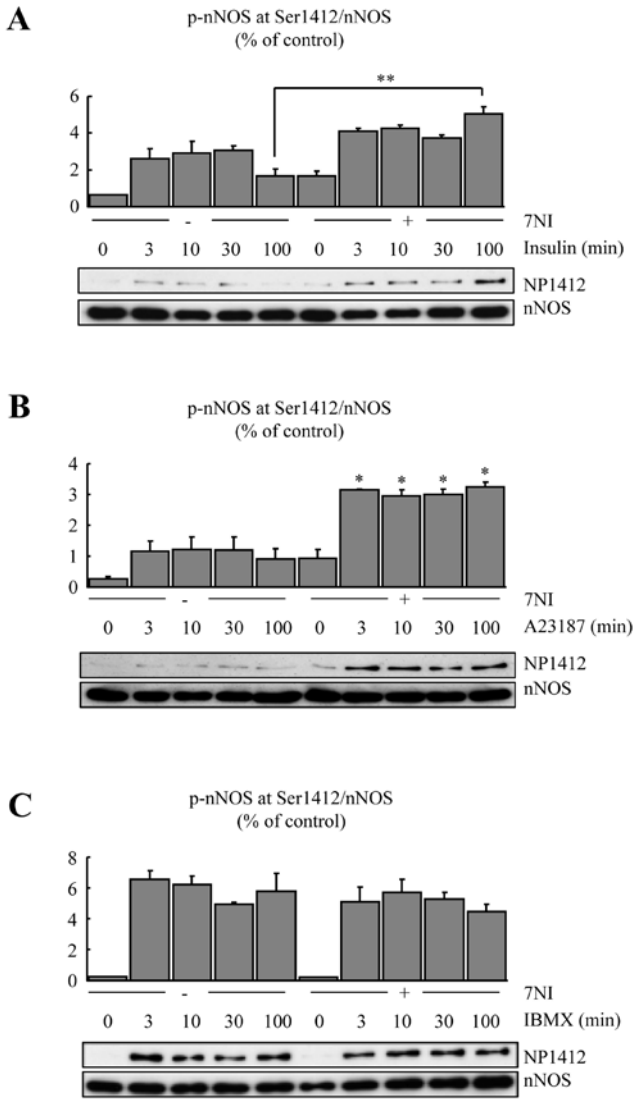

Figure 4. Effects of 7NI on the phosphorylation of nNOS at Ser1412 stimulated by insulin, A23187 and IBMX in GH3 cells. GH3 cells were treated with buffer alone or (A) 100 μM insulin, (B) 10 μM A23187 or (C) 1 mM IBMX and for 0, 3, 10, 30 or 100 min in the presence (+) or absence (-) of 7NI (100 μM) (100 min). Note that 7NI was applied for 100 min in each case. nNOS was isolated by ADP-agarose precipitation, and its phosphorylation was analyzed by western blotting with the phospho-specific antibody, NP1412. The immunoblot membranes were then stripped of NP1412 and reprobed with anti-nNOS antibody (nNOS). The histogram shows the amount of p-nNOS at Ser1412 relative to that of nNOS in the membrane. Values are expressed as mean ± SEM (n=3 for each group). *P<0.05, **P<0.001 vs. each same time point in (A). p-nNOS, phosphorylation of nNOS.

phosphorylation of nNOS at Ser1412 (Fig. 2C). Phospho-Akt/PKB at Ser473 was also detected when the GH3 cells were treated with insulin, but this effect was abolished by preincubation of GH3 cells with the PI3 kinase inhibitor wortmannin (Fig. 2C).

NO reversibly inactivates Akt/PKB and CaM-K II-mediated phosphorylation of nNOS at Ser1412 in vitro. To assess whether NO is involved in regulation of the activity of Akt/PKB, CaM-K II and PKA, *in vitro* kinase assays were performed using nNOS as a substrate. Preincubation with a NO donor, DEA-NONOate almost completely inhibited the phosphorylation of nNOS at Ser1412 mediated by Akt/PKB (Fig. 3A) and CaM-K II (Fig. 3B). This inhibitory effect was reversed by the addition of dithiothreitol, a reducing reagent. In the case of PKA, no changes were noted in the phosphorylation of nNOS at Ser1412 by pretreatment with DEA-NONOate (Fig. 3C).

Inhibiting nNOS activity enhances insulin- and A23187-induced phosphorylation of nNOS at Ser1412 in GH3 cells. We

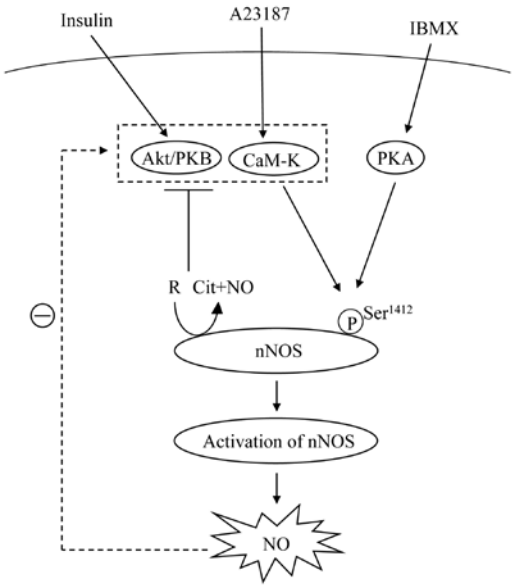

Figure 5. Model for the nitric oxide feedback regulation of neuronal nitric oxide synthase phosphorylation at Ser1412. Activated protein kinases, Akt/PKB, CaM-K II and PKA induce phosphorylation of nNOS at Ser1412 leading to activity of nNOS. Overgeneration of NO by nNOS feedback suppresses activity of nNOS by downregulating activity of both Akt/PKB and CaM-K II but not PKA.

next tested whether NO effects are sufficient to dampen Akt/PKB or CaM-K II activity in GH3 cells. After treatment with either A23187 (Fig. 4A) or insulin (Fig. 4B), slight phosphorylation of Ser1412 was observed. Pretreatment with 7NI, an inhibitor of NOS, increased the phosphorylation. Pretreatment with 7NI had no effects on IBMX-induced phosphorylation of Ser1412 (Fig. 4C).

Discussion

This study reports the novel finding that the phosphorylation of nNOS at Ser1412 can be catalyzed by activation of three protein kinases, Akt/PKB, CaM-K II or PKA. Generation of NO prevents phosphorylation of nNOS at Ser1412 by inhibiting activity of either Akt/PKB or CaM-K II but not PKA signaling in rat pituitary tumor GH3 cells. To our knowledge, this is the first study to explore the mechanisms by which NO feedback regulates the activity of nNOS through phosphorylation at Ser1412 in rat pituitary tumor GH3 cells.

Each phosphorylation site of nNOS provides an independent step in the activation and deactivation of nNOS and collectively these steps tightly regulate the production of NO (11). Previous studies from our laboratory demonstrated that the phosphorylation of nNOS at other sites including Ser847, Ser741 and Thr1296 were associated with the reduction of nNOS activity in neuronal cells (9,10,20,21). In contrast, the phosphorylation of nNOS at Ser1412 was necessary for increased activation of nNOS in neuronal cells (11,22). Phosphorylation of nNOS at Ser1412 could be induced by the activity of Akt/PKB signaling, and this action increased the production of NO (11). In the present study, the phosphorylation of nNOS at Ser1412 could be detected when the GH3 cells were stimulated with insulin, A23187 or IBMX, which induced the activity of Akt/PKB, CaM-K II or PKA signaling, respectively. Furthermore, preincubation of GH3 cells with three protein kinase antagonists or inhibitors completely blocked each agonist-induced phosphorylation of nNOS at Ser1412. Taken together, these data confirmed the previous study and extended the results to show that activation of all three protein kinases contribute to phosphorylation of nNOS at Ser1412.

Our present data are consistent with the previous study (22) showing that NO donors inactivated Akt/PKB signaling via S-nitrosylation, which was reversed by a reducing agent. In addition, we found that Akt/PKB signaling in GH3 cells was also sensitive to an inhibitor of nNOS. CaM-K II has been implicated in the phosphorylation of nNOS at Ser847 (11). We here demonstrated that activation of CaM-K II also induced phosphorylation of nNOS at Ser1412, and this effect could be inhibited by NO released from nNOS as well as exogenous NO. PKA was activated or inhibited through cysteine-targeted oxidation of regulatory or catalytic subunits, respectively. We did not assess modification of protein cysteinyl thiols here, but could not detect any effects of NO on PKA-induced phosphorylation of Ser1412 *in vitro* as well as *in situ*. The data indicate that NO selectively mediates protein kinases to control activity of nNOS through regulation of phosphorylation at Ser1412, although the precise mechanisms remain elusive. Based on our results, we illustrate how NO feedback regulates nNOS activity through three protein kinases (Fig. 5). It was shown that phosphorylation at Ser1412 by Akt/PKB is necessary for activation of nNOS by the NMDA receptor in cortical neurons (14). However, it is not clear which signaling is more susceptible to NO-mediated signaling pathways between the Akt/PKB and the CaM-K II under physiological and pathological conditions. Further experiments, including physiological coupling of NO and Akt/PKB or CaM-K II are needed to be performed in the future, along with determination of the NO-sensor residues.

In summary, the present study demonstrates that activation of nNOS can be catalyzed by at least three protein kinases, Akt/PKB, CaM-K II or PKA. NO generated from nNOS feedback regulates the activation of nNOS through either the Akt/PKB or CaM-K II, but not the PKA signaling pathway. Pharmacologic interventions that target these signaling pathways may provide effective neuroprotective therapies that limit NO-induced neurotoxicity in the brain.

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