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Inhibition of neuronal nitric-oxide synthase by phosphorylation at Threonine1296 in NG108-15 neuronal cells

Tao Song[a,b], Naoya Hatano[a], Kodai Kume[a], Katsuyoshi Sugimoto[a], Fuminori Yamaguchi[a], Masaaki Tokuda[a], Yasuo Watanabe[a,*]

[a] *Department of Cell Physiology, Kagawa University, Faculty of Medicine, 1750-1 Ikenobe, Miki-cho, Kida-gun, Kagawa 761-0793, Japan*
[b] *Department of Anesthesiology, The First Affiliated Hospital, China Medical University, Shenyang 110001, China*

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Abstract **We demonstrate that neuronal nitric-oxide synthase (nNOS) is directly inhibited through the phosphorylation of Thr[1296] in NG108-15 neuronal cells. Treatment of NG108-15 cells expressing nNOS with calyculin A, an inhibitor of protein phosphatase 1 and 2A, revealed a dose-dependent inhibition of nNOS enzyme activity with concomitant phosphorylation of Thr[1296] residue. Cells expressing a phosphorylation-deficient mutant in which Thr[1296] was changed to Ala proved resistant to phosphorylation and suppression of NOS activity. Mimicking phosphorylation mutant of nNOS in which Thr[1296] is changed to Asp showed a significant decrease in nNOS enzyme activity, being competitive with NADPH, relative to the wild-type enzyme. These data suggest that phosphorylation of nNOS at Thr[1296] may involve the attenuation of nitric oxide production in neuronal cells through the decrease of NADPH-binding to the enzyme.**

Keywords: Neuronal nitric-oxide synthase; Phosphorylation; Protein phosphatase; NG108-15 cells

1. Introduction

Neuronal nitric-oxide synthase (nNOS) is a Ca^{2+}/calmodulin (CaM)-dependent enzyme, catalyzing the oxidation of L-arginine to generate nitric oxide (NO) and L-citrulline [1]. The N-terminal oxygenase domain contains binding sites for heme, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H4B), and L-arginine and is the location where oxidative catalysis takes place. The C-terminal reductase domain contains binding sites for FMN, FAD, and NADPH, as found in NADPH-cytochrome P450 oxidoreductase (CYPOR) [1], and functions to transfer reducing equivalents from NADPH to the oxygenase domain. It is homologous to ferredoxin $NADP^+$ reductase (FNR). Protein kinase-dependent phosphorylation events in nNOS also exert effects, phosphorylation at Ser^{741} or Ser^{847} by CaM-K I or II leading to a reduction in enzyme activity in cells [2–4]. It has been reported that CaM-K II-phosphorylates nNOS at Ser^{847} in rat hippocampus after transient forebrain ischemia [5] and that this is promoted by post-synaptic density 95 [6]. In the present study, we investigated the effects of protein phosphatase (PP) inhibitors on nNOS enzyme activity using NG108-15 cells and found that Thr^{1296} residue of nNOS is a key determinant for transducing the nNOS–NADPH interaction via its phosphorylation.

2. Materials and methods

2.1. Materials

The cDNA for rat brain nNOS, the pME18s-FLAG vector, and NG108-15 neuroblastoma × glioma hybrid cells were generous gifts from Dr. Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD) [1], Dr. Tadashi Yamamoto and Dr. Tohru Tezuka (Department of Oncology, Institute of Medical Science, University of Tokyo, Tokyo, Japan) [7], and Dr. Haruhiro Higashida (Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, Kanazawa, Japan) [8], respectively. Recombinant rat CaM was expressed in *Escherichia coli* BL21 (DE3) using pET-CM, kindly provided by Dr. Nobuhiro Hayashi (Fujita Health University, Toyoake, Japan) [9]. A mouse anti-nNOS monoclonal antibody was obtained from Sigma. L-[^{3}H]arginine, [γ-^{32}P]ATP (6000 Ci/mmol), and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech. Restriction enzymes and DNA-modifying enzymes were obtained from Takara Shuzo. Electrophoresis reagents were products of Bio-Rad. All other materials and reagents were of the highest quality available from commercial suppliers.

2.2. Plasmid construction

The pME18s-nNOS and pME18s-FLAG-tagged nNOS were generated as described previously [3,6]. The nNOS mutants, 1296TA and 1296TD (i.e., a mutant bearing Ala in place of Thr^{1296}) were subcloned into pME18s. The nucleotide sequences of each mutant were confirmed.

2.3. Anti-phosphopeptide-specific antibodies

A rabbit polyclonal antibody (pAb) raised against phosphopeptide based on the amino acid sequence of rat nNOS Cys-Ile-Tyr-Arg-Glu-Glu-phospho-Thr^{1296}-Leu-Gln-Ala-Lys (NP1296) was purified from immunized rabbit sera by tandem column chromatography using phosphopeptide and dephosphopeptide-coupled Cellulofine (seikagaku Corp.).

2.4. Cell culture, transfection

Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum and NG108-15 cells in DMEM containing 10% fetal calf serum and HAT (100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymi-

*Corresponding author. Fax: +81 87 891 2096.
E-mail address: yasuwata@med.kagawa-u.ac.jp (Y. Watanabe).

Abbreviations: CaM, calmodulin; nNOS, neuronal nitric-oxide synthase; NO, nitric oxide; CL-A, calyculin A; OA, okadaic acid; PP, protein phosphatase

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dine). Transient transfection procedures were performed using LipofectAMINE with PLUS reagent (Invitrogen), according to the manufacture's instructions.

2.5. Preparation of lysates and purification of expressed nNOS

For preparation of lysates, cells were sonicated with 0.3 ml of 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 P40). After centrifugation at 15000 × *g* for 15 min, a 15 μl anti-FLAG M2-agarose or ADP-agarose gel (50% slurry) was added to the supernatant, and the mixture was incubated for 1 h 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, and then analyzed by SDS–PAGE followed by Western blot analyses.

2.6. NOS activities assay

NOS activity in vitro was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as described previously [10,11], and nNOS activity in cultured cells was quantified as the formation of L-[^{3}H]arginine to L-[^{3}H]citrulline [12]. Briefly, transfected NG108-15 cells in a 60 mm dish were incubated in 1 ml of buffer containing 25 mM HEPES, 109 mM NaCl, 5.4 mM KCl, 0.9 mM CaCl2, 1 mM MgSO4, and 25 mM glucose (pH 7.3) for 1 h at 37 °C. nNOS activity was assayed by adding a mixture of unlabeled L-arginine (10 μM), L-[^{3}H]arginine (5 μCi/ml) and A23187 (10 μM) or vehicle to the culture. Following incubation at 37 °C for 10 min, cells were washed with ice-cold phosphate-buffered saline and scraped into 2 ml of solution containing 20 mM sodium acetate, 2 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA (pH 5.5) followed by sonication. An aliquot was withdrawn for determination of total protein, expressed nNOS contents and total cellular ^{3}H incorporation, and the remaining sample was applied to Dowex 50W-X8 resine (Bio-Rad) to separate L-[^{3}H]citrulline. A23187-stimulated L-[^{3}H]citrulline formation in cells was expressed as the increase in L-[^{3}H]citrulline formation following subtraction of the levels in non-stimulated cells.

2.7. Statistical analysis

The significance of variability between the results from each group and the corresponding control was determined by unpaired *t* test. The means ± S.E. were calculated. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of CL-A and OA on nNOS activity

The initial objective was to determine whether PP inhibitors could alter nNOS activity in cells. We transfected NG108-15 cells with nNOS and assayed for NOS enzyme activity either with or without PP inhibitors pretreatment using cell lysates. Treatment of calyculin A (CL-A) or okadaic acid (OA) resulted in decreased NOS enzyme activity. The effects of CL-A and OA on the catalytic activity of nNOS are compared in Fig. 1. There were obvious differences between the dose–response effects of CL-A and OA with an approximately 100-fold difference in inhibitory sensitivity. Immunoblots analysis revealed that PP inhibitors pretreatment does not alter the amounts of total nNOS expression in cells. From these data, we conclude that PP inhibitors-induced phosphorylation of nNOS results in attenuation of its catalytic activity in cells. Interestingly, the attenuation of NOS enzyme activity was not observed when purified nNOS using ADP-agarose affinity chromatography was used for the NOS activity assay (data not shown). The residue of Thr^{1296} is within the NADPH-binding domain of nNOS and a consensus phosphorylation site for protein kinases [13–15]. Therefore, we suspected that this residue might be the PP inhibitors-induced phosphorylation site in cells.

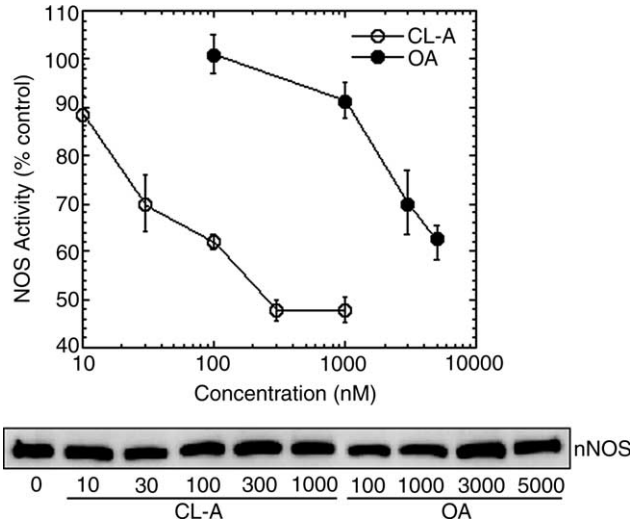

Fig. 1. Dose-dependent effects of CL-A and OA on nNOS enzyme activity in NG108-15 cells. NG108-15 cells expressing nNOS were pretreated with indicated concentrations of CL-A or OA for 20 min. Proportional amounts (70 μl of lysates) were subjected to NOS assay. Data were normalized to the control value, defined as the NOS activity obtained without PP inhibitors. The means ± S.E. of three experiments are shown. The lower panel is a representative immunoblot prepared from cells used for the activity assays.

3.2. Effects of mimicking phosphorylation of nNOS at Thr^{1296} on nNOS catalytic activity

We tested whether Thr^{1296} phosphorylation exerts similar effects on NOS activity as observed in PP inhibitors treatment cells using phosphorylation-mimicking and -deficient mutants. We made single mutants by introducing Ala or Asp residue in place of Thr^{1296} (1296TA or 1296TD). Wild-type and mutant enzymes were expressed using the *E. coli* system and purified on ADP-agarose as described under Section 2. The recombinant nNOSs were at least 90% pure as analyzed by densitometric scanning and gave a major band at 160 kDa on SDS–PAGE with Coomassie Brilliant Blue staining (Fig. 2A). The activities of equal quantities of equally purified wild-type and mutants of nNOS were then determined by monitoring the rate of conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. As shown in Fig. 2A, mimicking the phosphorylation at Thr^{1296} resulted in a large reduction in nNOS activity. 1296TA nNOS, in contrast, did not significantly affect nNOS activity under the same conditions. The inhibition of enzyme activity with 1296TD nNOS was competitive with NADPH but not with the substrate L-arginine (Fig. 2B). In vitro activity assays measuring the conversion of arginine to citrulline in the presence of optimal concentration of Ca^{2+}/CaM and cofactors do not always accurately reflect the NOS catalytic activity in endogenous nNOS-containing living cells such as NG108-15 neuronal cells. Therefore, we examined the effect of mutating Thr^{1296} to Ala or Asp on the ability of nNOS to catalyze formation of L-[^{3}H]citrulline from L-[^{3}H]arginine in NG108-15 cells transfected with the wild-type, 1296TA, or 1296TD nNOS. Immunoblots analysis revealed that wild-type and the mutant constructs to yield similar amounts of total

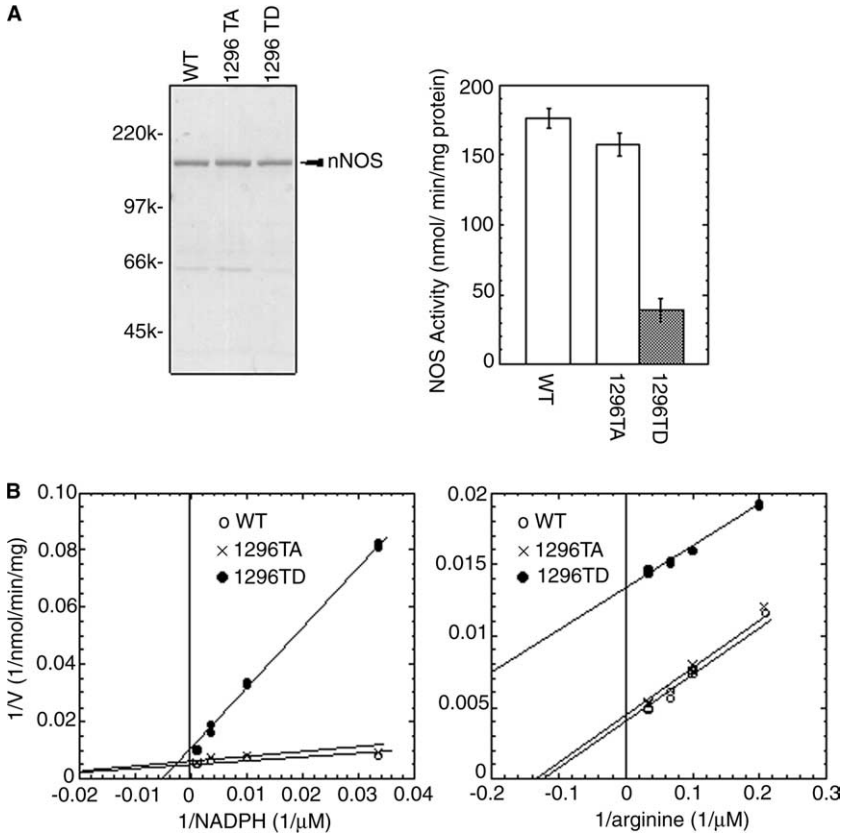

Fig. 2. Effects of Thr^{1296} phosphorylation-mimicking or phosphorylation-deficient mutant of nNOS on enzyme activity in vitro. (A) equal amounts (0.5 μg) of wild-type nNOS (WT) and the indicated mutants in *E. coli* were separated by 7.5% SDS–PAGE and stained by Coomassie Brilliant (left panel). Equivalent amounts (50 nM) of purified recombinant nNOSs were used for the NOS enzyme activity assay, measuring the conversion of arginine-to-citrulline in the presence of 1 mM $CaCl_2$, 1 μM CaM, 30 μM L-[^{3}H]arginine, 0.1 mM NADPH, 20 μM BH_4, 4 μM FAD, and 4 μM FMN (right panel). The means ± S.E. of three experiments are shown. (B) kinetic analysis of wild-type (○), 1296TA (×), or 1296TD (●) nNOS. NOS enzyme activity was assayed under the same conditions as described in panel A except for the concentration of arginine and NADPH. For titration of NADPH (left panel), 30 μM L-[^{3}H]arginine and 30–1000 μM NADPH were used. For titration of arginine (right panel), 1000 μM NADPH and 5–30 μM arginine were used. The assays were performed in duplicate for each point and results are presented as double-reciprocal plots (Lineweaver–Burk).

nNOS (Fig. 3). Cells were then incubated with L-[^{3}H]arginine and treated with calcium ionophore A23187 or vehicle for 10 min, harvested, and lysates were analyzed for L-[^{3}H]citrulline formation as described in Section 2. When cells were transfected with wild-type or 1296TA nNOS, A23187 treatment led to a similar increase in nNOS activity (Fig. 3). However, A23187 induction of 1296TD nNOS enzyme activity was significantly lower than that with the other nNOSs.

The above results demonstrated that mimicking phosphorylation of nNOS at Thr^{1296} resulted in attenuation of NOS enzyme activity in vitro as well as in transfected cells through the decrease of NADPH-binding to the enzyme. Therefore, we focused on whether mimicking phosphorylation of nNOS at Thr^{1296} might alter the ADP-agarose association relative to the wild-type enzyme. When human embryonic kidney HEK-293 cells were transfected with the wild-type, 1296TA, or 1296TD nNOS, immunoblots revealed similar amounts of total nNOS expression (Fig. 4). However, mutation of Thr^{1296} to Asp resulted in decrease of ADP-agarose binding ability relative to wild-type or 1296TA nNOS by measuring the expressed nNOSs on partial purification using ADP agarose, elution with NADPH and analysis by Western blotting using anti-nNOS antibody (Fig. 4).

3.3. Detection of Thr^{1296}-phosphorylated nNOS in NG108-15 cells

To directly demonstrate that nNOS can be phosphorylated at Thr^{1296} in cells, we transfected NG108-15 cells with the FLAG-tagged nNOS and partially purified expressed nNOS using either ADP-agarose or anti-FLAG M2-agarose chromatography and quantified the phosphorylation state at Thr^{1296} with or without CL-A (1 μM) for 20 min (Fig. 5A). In the absence of treatment, phosphorylation at Thr^{1296} residue was only faint, or undetectable. CL-A-induced phosphorylation of nNOS at Thr^{1296} was only observed when nNOS was purified using anti-FLAG M2-agarose chromatography. We next examined whether phosphorylation of nNOS at Thr^{1296} on nNOS could directly influence the NOS enzyme activity in NG108-15 cells, transfected with FLAG-tagged wild-type, 1296TA, or 1296TD nNOS and assayed for NOS enzyme activity either with or without CL-A pretreatment (Fig. 5B). Treatment of CL-A resulted in decreased NOS enzyme activity when wild-type nNOS was transfected as observed in Fig. 1. Cells expressing a phosphorylation-deficient mutant, 1296TA nNOS, proved to resistant to the decrease in NOS enzyme activity. Mimicking the phosphorylation at Thr^{1296} resulted in a large reduction in nNOS activity either with or without CL-A treatment.

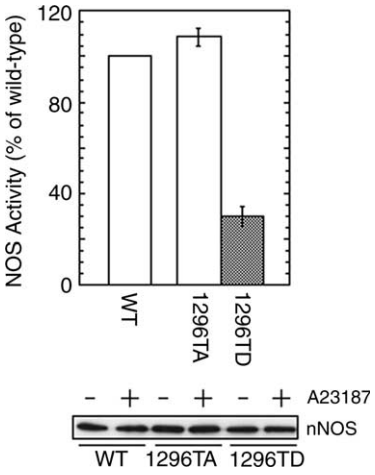

Fig. 3. nNOS enzyme activity in NG108-15 cells transfected with wild-type, 1296TA, or 1296TD mutant nNOS. A. NG108-15 cells were transfected with wild-type nNOS (WT) or the indicated mutants. 48 h after transfection, L-[^{3}H]arginine was added with or without A23187 (10 μM) and after 10 min cells were harvested and lysed, and nNOS enzyme activity was measured with reference to the formation of L-[^{3}H]citrulline following subtraction of the levels in un-stimulated cells. Data were normalized to the control value, defined as the NOS activity obtained with transfection of wild-type enzyme. The means ± S.E. of three experiments are shown. The lower panel is a representative immunoblot prepared from cells used for the activity assays, either with (+) or without (−) A23187 treatment.

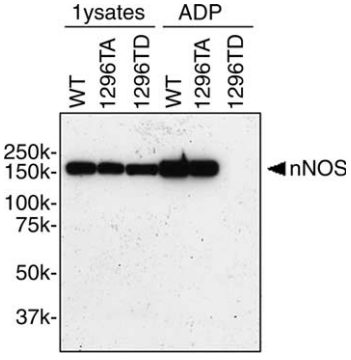

Fig. 4. ADP-agarose binding ability of wild-type, 1296TA, or 1296TD nNOS. HEK-293 cells were transfected with wild-type (WT), 1296TA (1296TA), or 1296TD (1296TD) mutant and 48 h thereafter, cell lysates (lysates) were immunoblotted with anti-nNOS antibody. Expressed nNOS was also affinity-purified from transfected cells using the ADP-agarose chromatography technique (ADP) and similarly subjected to immunoblotting. The data are representative of at least two independent experiments.

Expressed nNOS was partially purified from transfected cells using anti-FLAG M2-agarose chromatography and quantified the phosphorylation at Thr^{1296}. CL-A treatment led to an increase in nNOS phosphorylation when cells were transfected with wild-type but not with 1296TA nNOS. Either with or without CL-A treatment, the NP1296-immunoreactivity against 1296TD nNOS was evident when it was transfected.

4. Discussion

This study provides the first evidence, to our knowledge, that the Thr^{1296} residue of nNOS is an important regulator

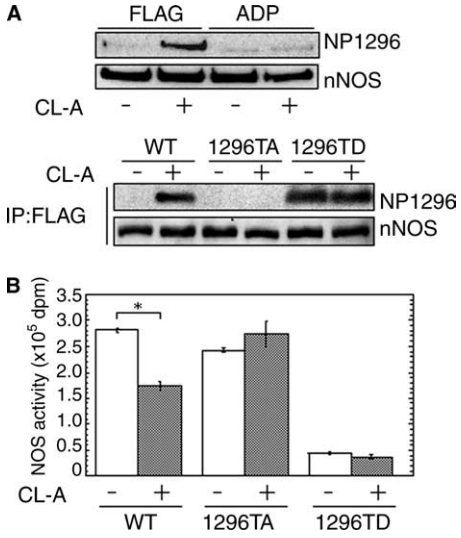

Fig. 5. Effects of CL-A-induced phosphorylation of nNOS at Thr^{1296} on NOS activity in NG108-15 cells. (A) NG108-15 cells expressing FLAG-nNOS were pretreated with (+) or without (−) 1 μM CL-A for 20 min. Expressed nNOS was partially purified from transfected cells using either ADP-agarose (ADP) or anti-FLAG M2-agarose (FLAG) chromatography and subjected to Western blotting with anti-nNOS (nNOS) or anti-phospho Thr^{1296} on nNOS (NP1296). The data are representatives of at least two independent experiments. (B) NG108-15 cells expressing FLAG-wild-type, FLAG-1296TA, or FLAG-1296TD nNOS were treated with (+) or without (−) 1 μCL-A for 20 min. Expressed nNOS was partially purified from transfected cells using anti-FLAG M2-agarose (FLAG) chromatography and similarly subjected to immunoblotting. Proportional amounts (70 μl of lysates) were subjected to NOS assay (lower panel). Note that NP1296-immunoreactivity against 1296TD nNOS was evident when it was transfected. The means ± S.E. of three experiments using three independent transfections are shown; the asterisk represents significant difference ($^*P < 0.01$).

of its enzyme activity via its phosphorylation in neuronal cells. NG108-15 cells possess nNOS as determined by Western blotting (data not shown), but endogenous expression was found to be normally too low for analysis and Thr^{1296}-phosphorylated nNOS showed decrease of ADP-agarose binding ability relative to un-phosphorylated enzyme (Fig. 5A). Therefore, we employed cells transfected with FLAG-tagged nNOS for the study of in situ phosphorylation of the enzyme. An approximately 100-fold difference in inhibitory sensitivity of OA and CL-A in cells (Fig. 1) would mean that OA has a 50–100-fold weaker effect than CL-A on PP1. OA and CL-A are reported to inhibit PP2A with similar potency [16,17].

Phosphorylation of nNOS at Thr^{1296} can be thought of as physiological, given the catalysis by endogenous kinases associated with decrease in nNOS enzyme activity in NG108-15 cells. The phosphorylation of nNOS at Thr^{1296} was evident only after PP inhibitors treatment. Thus, dephosphorylation activity of PP1 at Thr^{1296} on nNOS is thought to be higher than the phosphorylation activity by endogenous kinases in cells. Anti-phospho Thr^{1296} on nNOS antibody recognizes 1296TD nNOS (Fig. 5), indicating that the mutant is structurally resemble to phospho-nNOS at Thr^{1296} residue. Treatment with CL-A or OA also lead to increase in nNOS phosphorylation at Ser^{847} but not at Ser^{741} under the conditions employed here in Fig. 5. Cells expressing 847SA nNOS also exhibited the decrease in NOS enzyme activity when CL-A was treated (data

not shown), indicating a low stoichiometry of nNOS phosphorylation at Ser^{847} in the condition in Fig. 5.

From the sequence alignments and the mutation studies of nNOS, endothelial NOS, inducible NOS, CYPOR, and FNR, it has been concluded that the Phe^{1395} residue in nNOS regulates its catalytic activity by controlling $NADP^+$ [18,19]. The Thr^{1296} residue found in nNOS is absent in endothelial NOS, inducible NOS, CYPOR, and FNR. Thus, the results of the present study shed new light on mechanism for transducing the nNOS–NADPH interaction through the phosphorylation of Thr^{1296} residue in nNOS. A major concern is whether nNOS is actually phosphorylated in vivo in the brain at Thr^{1296} and determination of possible physiological outcomes in neuronal cells, as well as which protein kinases might participate, awaits future elucidation.

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