Tetrahydrobiopterin-dependent stabilization of neuronal nitric oxide synthase dimer reduces susceptibility to phosphorylation by protein kinase C in vitro

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Abstract Binding of (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) stabilizes the homodimeric structure of neuronal nitric oxide synthase (nNOS). In the present study, low-temperature sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed differential susceptibility of stabilized and non-stabilized dimers to in vitro phosphorylation by protein kinase C. Protein kinase C preferentially phosphorylated the non-stabilized dimer. Although a low extent of phosphorylation was detected in the stabilized dimer, most of it was estimated to be due to phosphorylation of the dimer before its stabilization. Phosphorylation did not affect the stabilizing effect of H_4B. These results indicate that H_4B-dependent dimer stabilization prevents nNOS from protein kinase C-dependent phosphorylation in vitro.

Key words: Tetrahydrobiopterin; Nitric oxide synthase; Protein kinase C; Phosphorylation

1. Introduction

The catalytic activity of neuronal nitric oxide synthase (EC 1.14.13.39, nNOS), triggered by calcium-calmodulin [1], requires the binding of many other cofactors, suggesting multiple regulatory mechanisms [2]. The C-terminal reductase domain of nNOS binds to the electron donor, NADPH, and the electron shuttles, FAD and FMN. The N-terminal PDZ domain binds to scaffolding proteins such as PSD95 [3] and CAPON [4] which control the subcellular localization and sensitivity to calcium-dependent activation of nNOS in some neurons. The N-terminal heme domain contains binding sites for heme, L-arginine (Arg) and (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B). H_4B is known as a cofactor critical for electron shuttling in aromatic amino acid hydroxylases [5]. Recent studies revealed that, in addition to redox regulation, H_4B also regulates the homodimeric conformation of all three isoforms of NOS. Each nNOS dimer has two identical, anti-cooperative, binding sites for both Arg and H_4B [6]. Incubation with saturating concentrations of H_4B induces substantial conformational changes in the homodimeric structure of nNOS, yielding a stabilized nNOS dimer with full NO-producing activity [6,7]. Stabilized nNOS dimer is resistant to 2% sodium dodecyl sulfate (SDS) at temperatures below 30°C, enabling separation of stabilized and non-stabilized nNOS dimers by means of low-temperature SDS-polyacrylamide gel electrophoresis (LT-SDS-PAGE) [7].

As another mechanism regulating nNOS activity, phosphorylation has been studied extensively. nNOS is a target of phosphorylation by protein kinase C (PKC) in vitro [8] and in HEK293 cells stably expressing nNOS [9]. Activation of PKC in HEK293 cells resulted in reversible inhibition of nNOS catalytic activity [9,10]. In contrast, PKC-dependent nNOS activation has been reported in cerebellar slices [11–13], striatal [14] and mesencephalic neurons [15], as well as pinealocytes [16] in culture. This discrepancy is likely to be due to the differential susceptibility of nNOS to PKC-dependent phosphorylation, because nNOS was not phosphorylated in cerebellar slices in which PKC activation was confirmed by phosphorylation of GAP43 protein [13]. These results indicated that susceptibility of nNOS to phosphorylation is also a subject of regulation, just like regulation of sensitivity to calcium-calmodulin-dependent activation of nNOS by the nNOS binding proteins mentioned above. The aim of the present study is to clarify the effects of H_4B binding on the susceptibility of nNOS to phosphorylation. LT-SDS-PAGE analysis revealed that H_4B-dependent stabilization prevented PKC-dependent phosphorylation of the nNOS dimer.

2. Materials and methods

nNOS was partially purified from adult rat cerebella as described previously [17]. nNOS was eluted from a 2′,5′-ADP-agarose column (Sigma) with 50 mM Tris-acetate buffer at pH 7.4, containing 1 mM EDTA, 1 mM EGTA, and 10 mM NADPH, and used immediately. Conversion of [^{3}H]Arg to [^{3}H]citrulline, initiated by the addition of calmodulin, was measured according to a previously reported method [17].

LT-SDS-PAGE was performed on ice as described previously [7]. nNOS was incubated with 100 μM H_4B (RBI, USA) for 1–45 min at 15°C, followed by the addition of an equal volume of fresh, chilled 2×SDS sample buffer. The mixture was incubated at 0°C for 30 min before LT-SDS-PAGE using a 6% separating gel. To identify the contents of the SDS-resistant (stabilized) and SDS-dissociating (non-stabilized) nNOS dimers, nNOS immunoreactivities were detected on Western blots using an ECL kit (Amersham) digitized by a 12-bit scanner (Umax, Powerlook II) and analyzed on a personal computer (Apple, PowerMac 8500/150) with NIH-image software. A polyclonal antibody specific to nNOS (Transduction Laboratories) and anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) were used as the primary and secondary antibodies, respectively. Only non-stabilized and stabilized nNOS dimers were detected at separate positions within each lane. The optical density of the nearby background region was subtracted from that of each nNOS band. All experiments were carried out under conditions in which the intensities of nNOS immunoreactivity were in proportion to the total amount of protein loaded (up to 800 ng), which were determined by varying the loading amounts and exposure time. Both stabilized and non-stabilized dimers of nNOS exhibited similar reactivities on Western blots because the

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total blotting intensities of the same sample with or without boiling procedure were similar. For example, groups 3 and 4 in Fig. 3A represent identical samples except that the sample of group 4 was boiled. The total immunoreactivity (average of 2 lanes) of group 3 was 332 560 as determined by NIH-image measurements, while that of group 4 (group 3 boiled) was 312 282. H_4B was dissolved at 10 mM in 10 mM dithiothreitol. Omission of dithiothreitol did not alter the results if H_4B was dissolved in water pre-bubbled with nitrogen gas [18]. The use of 50 mM phosphate buffer at pH 7.4 or 50 mM triethanolamine-HCl buffer at pH 7.0 [7] instead of Tris-acetate buffer also did not alter the result.

nNOS phosphorylation in vitro was carried out by adding an equal volume of PKC-reaction mixture, composed of 50 mM Tris-acetate buffer at pH 7.4, 1.8 mM $CaCl_2$, 10 mM $MgSO_4$, 4 mM dithiothreitol, 40 μM ATP, 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, NEN), 100 nM phorbol myristate acetate, 50 μg/ml of phosphatidylserine, and 100 μg/ml of PKC (a mixture of α, βI, βII and γ isoforms; Boehringer-Mannheim), as described previously [13]. The reaction was terminated after incubation at 15°C for 20 min by the addition of 1 mM EGTA. To determine phosphorylation of non-stabilized and stabilized dimers, LT-SDS-PAGE was carried out, and an autoradiogram of the dried gel was analyzed with an image plate (Fuji Film) using a BAS2000 image analyzer (Fuji Film). Under these incubation conditions, nNOS phosphorylation reached a maximal level within 20 min. Histone-3S (Sigma) phosphorylation by PKC was carried out under the same conditions as described above, using 0.2 mg/ml of histone-3S in place of nNOS, in a 96-well plate equipped with a nitrocellulose membrane (Millipore) and the radioactivity was counted by Cerenkov radiation as described previously [13].

Gel filtration was performed using a Superose 6 column (Pharmacia) according to a method previously reported in [7]. The nNOS concentrations in each fraction (pooled in duplicate) were analyzed by dot blotting (Millipore) using the same nNOS antibody as that mentioned above. The statistical results are presented as mean ± standard deviation.

3. Results and discussion

The nNOS in the preparation was entirely dimeric as determined by gel filtration (data not shown). Fig. 1A shows the results of LT-SDS-PAGE revealing that 20 ± 11% ($n = 4$) of nNOS was in the form of SDS-resistant, stable dimers before incubation with any of the test compounds. Brief incubation with H_4B and Arg increased the amounts of stabilized dimers

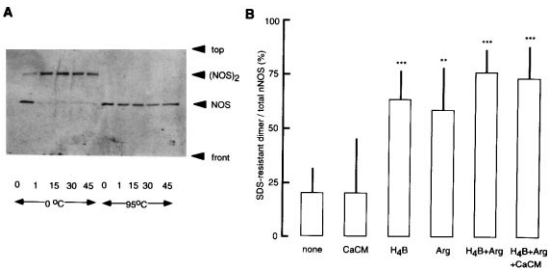

Fig. 1. A: LT-SDS-PAGE showing the separation of stabilized nNOS dimers. Partially purified nNOS (50 ng of total protein/100 μl) was incubated with 100 μM H_4B and 1 mM Arg at 15°C for the indicated time periods (min). For LT-SDS-PAGE, samples were incubated with 2×SDS buffer on ice for 30 min (0°C), while the same samples were boiled at 95°C for 5 min (95°C) as control. $(NOS)_2$ and NOS represent stabilized and non-stabilized nNOS dimers, respectively. A representative result of three similar experiments is shown. B: Effects of H_4B (100 μM), Arg (1 mM), and calcium (1 mM excess) plus calmodulin (300 nM; CaCM) on the formation of the stabilized dimer. Partially purified nNOS was incubated with the compounds indicated at 15°C for 2 min. The ordinate shows the immunoreactivity of stabilized dimer normalized to that of the corresponding boiled samples. Mean and S.D. (vertical bars) of at least four similar experiments are shown. $^{**}P < 0.01$ and $^{***}P < 0.001$.

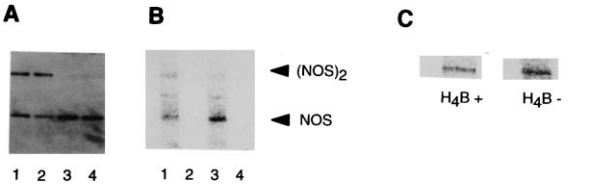

Fig. 2. A: LT-SDS-PAGE of nNOS after incubation with H_4B followed by PKC reaction. B: Autoradiogram of A. Lanes 1 and 2: nNOS was incubated with 100 μM H_4B at 15°C for 5 min; lanes 3 and 4: H_4B was omitted; lanes 1 and 3: after incubation with or without H_4B, nNOS was further incubated with PKC and PKC activators (phosphatidylserine and phorbol myristate acetate) at 15°C for 20 min; lanes 2 and 4: PKC activators were omitted from the incubation. A representative of five similar experiments is shown. C: Autoradiogram of boiled samples. A representative result of three similar experiments is shown.

which were constant up to 45 min. Stabilized dimers were eliminated by boiling at 95°C for 3 min. Fig. 1B shows the effects of H_4B, Arg, and calcium-calmodulin on the formation of stabilized dimers. In the presence of 100 μM H_4B (64 ± 13%, $n = 6$), 1 mM Arg (59 ± 18%, $n = 4$) or both (76 ± 10%, $n = 12$), the amounts of stabilized dimers were consistently increased. An nNOS inhibitor, 7-nitroindazole, which non-competitively reduces its affinity to BH_4 and Arg [3], reduced the dimer-stabilizing effect of BH_4 (26 ± 6%, $n = 2$). These results confirmed that H_4B and Arg stabilized the dimeric conformation of nNOS [7].

The stabilizing effect of H_4B was also confirmed by its effect on the thermal inactivation of partially purified nNOS. nNOS was incubated for 20 min in 50 mM Tris-acetate buffer at pH 7.4, containing 1 mM EDTA and 1 mM EGTA. The residual activity to convert [^{3}H]Arg to [^{3}H]citrulline was then measured in the presence of calcium-calmodulin, Arg, and H_4B. The residual activity after incubation at 0°C was 0.35 ± 0.01 ($n = 3$) μmol citrulline/min/mg protein. When this value was taken as 100%, only 57 ± 5% ($n = 3$) of residual activity was observed after similar incubation at 25°C. Thermal inactivation was enhanced by introducing 1.8 mM calcium chloride (30 ± 3%, $n = 3$) as reported previously [10]. The calcium-induced reduction in nNOS activity was not restored by PKC-dependent phosphorylation, proteinase inhibitors (aprotinin, leupeptin, pepstatin and PMSF) or superoxide dismutase together with catalase. In contrast, Arg and H_4B increased, but not fully restored, the residual activity to 70 ± 3% and 44 ± 1% in the absence and presence of calcium ions, respectively ($n = 3$).

In contrast, calcium-calmodulin did not alter the amounts of stabilized dimers irrespective of the presence of H_4B and Arg (Fig. 1B). Addition of calcium-calmodulin alone did not increase the amount of stabilized dimers (20 ± 25%, $n = 4$). In the presence of H_4B and Arg, further addition of calcium-calmodulin did not alter the amount of stabilized dimers (73 ± 14%, $n = 9$). These results indicated that calcium-calmodulin, the physiological trigger of nNOS activation in neurons, does not participate in the formation and maintenance of stabilized dimers of nNOS.

To compare the susceptibility of stabilized and non-stabilized nNOS dimers to phosphorylation, nNOS was incubated with 100 μM H_4B for 5 min, followed by PKC reaction for 20 min. The Western blots in Fig. 2A show that 60% of nNOS was stabilized after incubation with H_4B, which was not affected by phosphorylation (lanes 1 and 2). If both stabilized

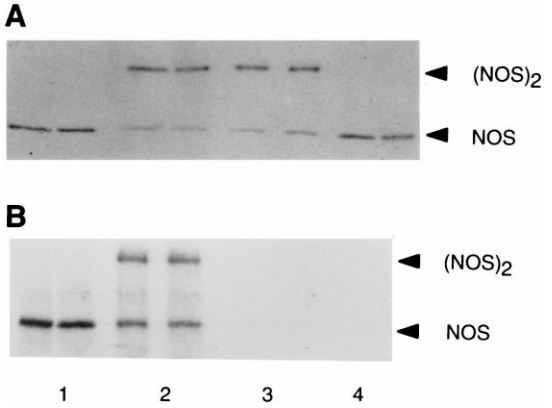

Fig. 3. A: Western blots of LT-SDS-PAGE of nNOS after incubation with PKC followed by 100 μM H_4B for 5 min. B: Autoradiogram of A. Groups 1 and 2: phosphorylated by PKC; groups 3 and 4: PKC activators were omitted; groups 2 and 3: LT-SDS-PAGE; groups 1 and 4: boiled samples. Two lanes within each group contained identical samples. A representative result of three similar experiments is shown.

and non-stabilized dimers were equally susceptible to phosphorylation, stabilized dimers should carry 60% of the total radioactivity. However, the autoradiograms shown in Fig. 2B demonstrate that only faint phosphorylation was associated with stabilized dimers, whereas non-stabilized nNOS was heavily phosphorylated. The stabilized dimer contained only $27 \pm 1\%$ ($n = 4$) of the total nNOS phosphorylation signals. nNOS phosphorylation found in boiled samples, representing total nNOS phosphorylation, was reduced by H_4B to $69 \pm 6\%$ ($n = 3$) of the control (Fig. 2C), showing that the total degree of nNOS phosphorylation was reduced by the formation of stabilized dimers.

The low extent of phosphorylation in stabilized dimers (Fig. 2B) could be generated either by phosphorylation of stabilized dimers or by stabilization of phosphorylated dimers. When nNOS was first phosphorylated by PKC and then incubated with H_4B and Arg, $72 \pm 2\%$ ($n = 3$) of the total nNOS was stabilized as shown in Fig. 3A. This result, together with that shown in Fig. 1B representing that 76% of the total nNOS was stabilized in the absence of PKC treatment, indicates that phosphorylation did not affect stabilization (unpaired *t*-test), and suggests that stabilization of pre-existing phosphorylated dimers contributes to some degree to the extent of phosphorylation. Since maximal phosphorylation of nNOS was obtained after a 20-min reaction with PKC, stabilization of phosphorylated nNOS is expected to contribute predominantly to the extent of phosphorylation. Actually, the phosphorylation signals carried by the stabilized dimers after this order of incubations was $51 \pm 3\%$ ($n = 3$) of the total (Fig. 3B). When the phosphorylation signals in stabilized dimers were assumed to be generated exclusively by phosphorylation of stabilized dimers, stabilized dimers is estimated to contain only 6% of the total phosphorylation signals after this order of incubations. Therefore, stabilization of phosphorylated non-stabilized dimers explains most (88%) of the phosphorylation associated with stabilized dimers.

The effect of H_4B was not through inhibition of PKC. Between nNOS bands corresponding to stabilized and non-stabilized dimers, an unknown phosphoprotein was consistently observed in the autoradiograms (Fig. 2B). This phosphoprotein was not detected by the nNOS-specific antibody (Fig. 2A), and did not disappear on boiling (Fig. 3B), indicating that it was not nNOS. The phosphorylation signals on this contaminant were not altered by H_4B, indicating that PKC activity was not altered by H_4B. To confirm this, the effects of H_4B on the phosphorylation of histone-3S, a well-established substrate of PKC, were tested. Histone-3S phosphorylation increased proportionally with reaction time, and H_4B did not significantly alter the slopes of the linear regression curves (1593 ± 199 and 1569 ± 161 cpm/min/4 μg histone-3S in the presence and absence of H_4B, respectively, $n = 3$, paired sample *t*-test), indicating that H_4B did not affect PKC activity.

To examine the effect of PKC-dependent phosphorylation in vitro on nNOS activity, partially purified nNOS was incubated with or without PKC in the absence of calmodulin to avoid calcium calmodulin-dependent inactivation [19], and the activity was assayed by addition of calmodulin. PKC phosphorylated nNOS and enhanced its catalytic activity 2.2 ± 0.4-fold ($n = 4$), consistent with previously reported results [13].

Thus, the present study revealed a novel regulatory role for H_4B in nNOS phosphorylation. Although H_4B stabilized both phosphorylated and unphosphorylated nNOS dimers similarly, PKC phosphorylated non-stabilized nNOS dimers preferentially. When both stabilized and non-stabilized dimers coexist, the phosphorylation of stabilized dimers was negligible. The rate limiting enzyme of H_4B biosynthesis [5], GTP cyclohydrolase-I [20], and the enzyme catalyzing the second step of H_4B biosynthesis, 6-pyruvoyl tetrahydropterin synthase [21], are expressed in catecholamine-negative neurons in the cerebellum. Reduced nNOS activity was observed in the cerebellum of hph-1 mice lacking 90% of the activity of GTP cyclohydrolase-1 [22]. These observations support the idea that H_4B is colocalized with, and regulates the activity of nNOS in the cerebellum. Since calcium-calmodulin, the physiologically relevant activator of nNOS, had no effect on the stabilization of nNOS dimers ([7] and Fig. 1), H_4B-dependent stabilization of nNOS dimers seems to be a prerequisite to rapid, calmodulin-dependent production of NO in response to stimulation by excitatory neurotransmitters. These considerations suggest that endogenous H_4B stabilizes nNOS in neurons to some extent and reduces its susceptibility to PKC-dependent phosphorylation.

Phosphorylation has been considered as a regulatory mechanism for nNOS catalytic activity. However, clear understanding of this issue has not yet been achieved. In the present report, PKC-dependent phosphorylation in vitro enhanced the catalytic activity of partially purified nNOS consistent with previous reports [8,13]. In neuronal cultures and cerebellar slices, nNOS activity was also enhanced by PKC activation [12–16]. Interestingly, nNOS was not phosphorylated by PKC in these cerebellar slices [13]. These results support the idea that endogenous H_4B inhibits nNOS phosphorylation by PKC in some neurons and suggest other mechanisms for PKC to regulate nNOS activity. In contrast, the catalytic activity of nNOS expressed in HEK293 cells was reduced by PKC-dependent phosphorylation [9,10]. Occurrence of PKC-dependent phosphorylation suggests that only low concentrations of H_4B were present in these cells. nNOS can produce superoxide in the absence of H_4B [23], leading to oxidative stress. Reduced NO production by PKC activation in HEK293 cells might indicate that direct nNOS inhibition by PKC-dependent phosphorylation involved different mechanisms in cells and in vitro, or alternatively, that phosphoryla-

tion might enhance the production of superoxide in the absence of H_4B.

There are several subjects worthy of future research. Although nNOS phosphorylation by protein kinase A [24], calmodulin-dependent protein kinase II [8] and protein kinase G [25] were reported, it is not clear whether nNOS susceptibility to phosphorylation by these kinases is also regulated by H_4B-dependent dimer stabilization. The mechanism for the differential susceptibility is also not clear. It is conceivable that conformational changes associated with stabilization conceal phosphorylation sites from PKC. However, the possibility of interaction of nNOS binding proteins that can block access of PKC to the phosphorylation site is not excluded. nNOS binding proteins such as PIN [26] can bind to nNOS and inhibit nNOS activity through destabilization of the nNOS dimer, and increase its susceptibility to PKC-dependent phosphorylation. Furthermore, although H_4B concentrations in specific neurons are not known, even H_4B at 100 μM did not prevent a small proportion of nNOS dimer dissociation. Therefore, the actual correlation between intracellular H_4B level, content of stabilized nNOS dimers, and susceptibility to PKC-dependent phosphorylation in neurons remains to be examined. Several preliminary trials to reduce intracellular H_4B levels, using an inhibitor of GTP-cyclohydrolase-1, diaminohydroxypyrimidine [5], were attempted. However, H_4B levels in cerebellar slices and rat cerebellum in vivo determined by the method reported in [27] were not easily affected by perfusion or by intraperitoneal injection of the inhibitor, respectively, suggesting poor access of this compound to neurons in these methods. Direct injection of the inhibitor into rat cerebellum in vivo should be tried in the future.

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