The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases

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Abstract Neuronal NO synthase (nNOS) was discovered recently to interact specifically with the protein PIN (protein inhibitor of nNOS) [Jaffrey, S.R. and Snyder, S.H. (1996) Science 274, 774–777]. We have studied the effects on pure NOS enzymes of the same GST-tagged PIN used in the original paper. Unexpectedly, all NOS isoenzymes were inhibited. The IC_{50} for nNOS was 18 ± 6 μM GST-PIN with 63 nM nNOS after 30 min at 37°C. Uncoupled NADPH oxidation was inhibited similarly, whereas cytochrome *c* reductase activity, the K_M for L-arginine, and dimerization were unaffected. We reconsider the physiological role of PIN in the light of these results.

Key words: Nitric oxide; Nitric oxide synthase; Dimerization; Isoenzyme; Inhibitor protein

1. Introduction

In mammalian systems, NO has important functions as a signal molecule (primarily as an activator of soluble guanylate cyclase) and in immune defence [1]. Its synthesis from L-Arg and O_2 is catalyzed by the NO synthases (EC 1.14.13.39; NOS) [1,2]. Three isoenzymes of NOS are known – neuronal, endothelial and inducible – which share the same basic structure and catalytic mechanism. NO is formed at the *P*-450 heme-containing, H_4biopterin-dependent oxygenase active site in the N-terminal half of the protein. The C-terminal reductase domain binds FMN and FAD and passes reducing equivalents from NADPH to the heme; this activity requires the binding of CaM. All three isoenzymes are homodimeric, with the interface between subunits being formed by the oxygenase domain. On binding H_4biopterin, the dimers become unusually resistant to dissociation by SDS [3–6].

The differences between the isoenzymes concern their tissue distribution and regulation. Binding of CaM to nNOS and eNOS is sensitive to Ca^{2+} concentration, coupling their activity to Ca^{2+} influx through the cell membrane [7–9], whereas iNOS binds CaM Ca^{2+}-independently and is regulated mainly at the level of gene transcription. Each isoenzyme has a unique N-terminal sequence that is not strictly necessary for catalytic activity. For eNOS and nNOS there is evidence that these N-terminal domains function in intracellular localization of the enzymes. eNOS can be myristoylated and palmitoylated [10]. It also binds to the structural protein caveolin; this interaction is antagonized by Ca^{2+}/CaM, leading to the proposal of a 'caveolin cycle' of activation/inactivation and relocation of the enzyme [11]. The N-terminus of nNOS contains a 'PDZ domain', which interacts with the postsynaptic density protein PSD-95, and with α_1-syntrophin, a component of the dystrophin complex in skeletal muscle [12,13].

Recently, part of the N-terminal domain of nNOS was used as a probe in the yeast two-hybrid system to discover a 10-kDa protein that inhibited nNOS and that was named accordingly protein inhibitor of nNOS (PIN) [14]. Of the three NOS isoenzymes, only nNOS was retained by an affinity matrix carrying a GST-tagged version of PIN. In HEK 293 cells cotransfected with PIN and nNOS, less cGMP was formed than in cells transfected with nNOS alone. Inhibition of NOS was studied by adding GST-PIN or PIN (purified after thrombin cleavage of the fusion protein) to lysates of HEK 293 cells expressing nNOS and preincubating for 1 h at 37°C before measuring NOS activity. 50% inhibition, relative to controls preincubated without PIN, was observed with 1 μM GST-PIN or 5 μM PIN in this system. Using low-temperature SDS-PAGE with detection by immunoblotting, GST-PIN was found to disrupt the SDS-resistant dimer of nNOS in the same cell extracts. However, the concentration of nNOS in the extracts was not known, so that the stoichiometry of these effects remained unclear. PIN has also been discovered in another context, as a light chain of myosin and dynein, with a highly conserved sequence over a wide spectrum of different organisms [15]. Phenotypes of PIN-deficient *Drosophila* mutants showed that the protein had an essential physiological function [16,17].

PIN has attracted significant attention in view of its potential importance as a regulator of nNOS [18,19], but a fundamental study of the interaction between PIN and purified NOS enzymes is still outstanding. Our aims in the present work were therefore to quantify the potency of the inhibition of nNOS; to identify which step of the NOS reaction was affected; and to check whether PIN could also regulate the activity of eNOS and iNOS.

2. Materials and methods

2.1. Materials

The vector pGEX-PIN carrying the cDNA for GST-PIN was from Dr. Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, USA. Mouse iNOS, expressed in *Escherichia coli* and purified as described [20] was from Dr. Dennis J. Stuehr, Department of Immunology, Cleveland Clinic Research Institute,

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Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase (type I); iNOS, inducible nitric oxide synthase (type II); eNOS, endothelial nitric-oxide synthase (type III); H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin = (6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1-,2-dihydroxypropyl)pteridine; PIN, protein inhibitor of nNOS; GST-PIN, fusion protein of PIN with glutathione *S*-transferase

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Cleveland, OH, USA. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was from MedPro (Amersham), Vienna, Austria. [^{3}H]Arginine was further purified as described earlier [21]. CaM was overexpressed in *E. coli* and purified by phenyl Sepharose chromatography. Other chemicals were from Sigma.

2.2. Preparation of nNOS and eNOS

Rat nNOS, rat nNOSΔ1–98 and bovine eNOS were prepared by expression in baculovirus-infected Sf9 cells and affinity purification on 2′,5′-ADP-Sepharose and then CaM-Sepharose, as previously described [5,22,23].

2.3. Preparation of GST-PIN

The pGEX-PIN vector was transfected into *E. coli* BL21-DE3. GST-PIN was purified essentially as described by Jaffrey and Snyder [14], by affinity chromatography on GSH-agarose. The buffers used all contained 1% (w/v) Triton X-100, except that the protein specifically bound to the column was washed briefly without detergent and then eluted without detergent. As soon as each fraction was collected, a sample was immediately taken for protein estimation, and Triton X-100 was added to the remainder to a concentration of 1% (w/v). This was done in order to allow accurate estimation of the GST-PIN concentration, because the combination of 1% (w/v) Triton and 10 mM GSH interfered with all protein assays tested. Protein was estimated by the brilliant blue G binding assay of Bradford [24] using bovine serum albumin as standard. Purified GST-PIN was stored at concentrations of 0.2–1 mM at −70°C.

2.4. NOS enzyme assay

NOS activity was measured by the conversion of L-[2,3,4,5-^{3}H]Arg to L-[2,3,4,5-^{3}H]citrulline as described previously [25].

2.5. Uncoupled NADPH oxidation

Uncoupled NADPH oxidation by NOS was measured photometrically as already described [26]. NOS was preincubated in a total volume of 180 μl with GST-PIN in 55 mM triethanolamine-HCl, pH 7.0/0.55 mM $CaCl_2$/0.22 mM CHAPS with NADPH (220 μM) for 30 min at 37°C. Reactions were started by adding 20 μl CaM (0.1 mg/ml).

2.6. Effect of GST-PIN on the stability of nNOS dimers

To examine the effect of PIN on the formation of the SDS-resistant dimer of nNOS, 0.63 μM nNOS was preincubated with 100 μM GST-PIN in 30 mM Tris-HCl, pH 7.5/90 mM NaCl/0.6 mM DTT/6 mM GSH/0.6% Triton X-100 for 30 min at 37°C in the presence or absence of 10 μM H_4biopterin and 100 μM L-Arg, before adding sample buffer and analyzing dimerization by low-temperature SDS-PAGE as described [3].

3. Results and discussion

We wished to study inhibition of nNOS by GST-PIN under conditions comparable to those used by Jaffrey and Snyder [14], but with pure nNOS in place of a cell extract. When we incubated nNOS at 37°C without PIN in 50 mM triethanolamine-HCl, pH 7.4, the enzyme was inactivated by more than 90% after 30 min. To reduce this 'PIN-independent' inactivation we performed further incubations in the presence of L-Arg, NADPH, FMN, FAD and H_4biopterin. Under these conditions, the residual activity after 30 min incubation without PIN was 542 ± 27 nmol/min/mg nNOS, or 49 ± 2% (mean ± S.E.M. of three experiments). These observations agree with an earlier study of nNOS stability by Gorren et al. [27]. There was no significant effect of GST-PIN (10 μM) on nNOS activity when the NOS assay was started immediately after adding PIN (96 ± 3%, mean ± S.E.M. of three experiments). Therefore we chose to study the effects of GST-PIN using a 30-min preincubation in the presence of substrate and cofactors. At a nNOS concentration of 10 μg/ml (63 nM), we observed concentration-dependent inhibition of nNOS by GST-PIN so that 50% inhibition was reached at 18 ± 6 μM GST-PIN (mean ± S.E.M. of three experiments), i.e. at a 290-fold molar excess of GST-PIN over nNOS (Fig. 1A). We also examined the effect of varying nNOS concentration at a fixed concentration of 3 μM GST-PIN. Significant inhibition of nNOS was only observed at concentrations of nNOS of 125 nM or less (Fig. 1B).

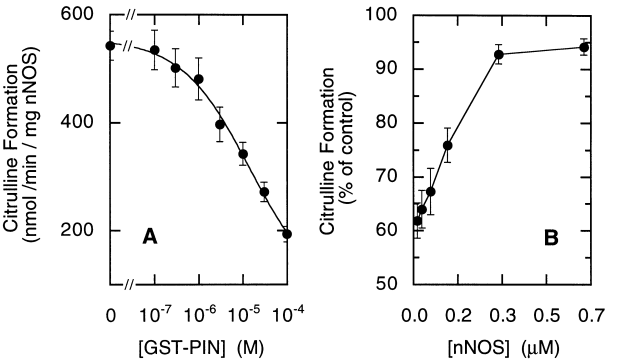

Fig. 1. Inhibition of rat nNOS by GST-PIN. NOS was preincubated with GST-PIN for 30 min at 37°C, in 55 mM triethanolamine-HCl, pH 7.0/0.55 mM $CaCl_2$/0.22 mM CHAPS with L-Arg (110 μM; including L-[2,3,4,5-^{3}H]Arg), H_4biopterin (11 μM), NADPH (220 μM), FMN (5.5 μM), and FAD (5.5 μM). NOS reactions were started by adding 10 μl CaM (0.1 mg/ml). Error bars denote S.E.M. of three experiments. A: Dependence of inhibition on PIN concentration at fixed nNOS concentration of 63 nM. The data were fitted to a Hill-type equation. B: Dependence of inhibition on nNOS concentration at fixed PIN concentration (3 μM).

The specificity of PIN for nNOS as opposed to iNOS or eNOS was proposed on the basis of results with a PIN affinity matrix and the yeast two-hybrid system rather than enzyme assays. We therefore checked the effects of GST-PIN on the enzyme activity of all three isoenzymes as well as of nNOSΔ1–98, a truncated nNOS lacking the PDZ domain (Fig. 2). GST-PIN significantly inhibited all forms of NOS examined. This suggests that the inhibitory effects of PIN are not absolutely specific for a particular isoenzyme and may be unrelated to the interaction with the N-terminal domain of nNOS observed in the yeast two-hybrid system.

This potency of PIN is lower than that reported by Jaffrey and Snyder [14]. A variety of reasons for this apparent discrepancy may be considered. Firstly, in the original study the amount of nNOS protein in the cell extracts was unknown, whereas our experiments were done at known concentrations of pure nNOS. Secondly, a difficulty arises because the effect of PIN on nNOS is rather slow to develop and could only be observed so far under conditions where even the control samples without PIN suffered considerable inactivation. The concern may therefore remain that part of the inhibition by PIN represents a potentiation of a preexisting inactivation process. Indeed, for the comparison of the different isoenzymes (Fig. 2), preincubations were done without substrate or cofactors,

Table 1
Effects of PIN on uncoupled NADPH oxidation by nNOS

[GST-PIN] (μM)	NADPH oxidation (μmol/min/mg nNOS)
0	0.6 ± 0.02
3	0.4 ± 0.03
15	0.17 ± 0.01

Values shown are means ± S.E.M. of three experiments.

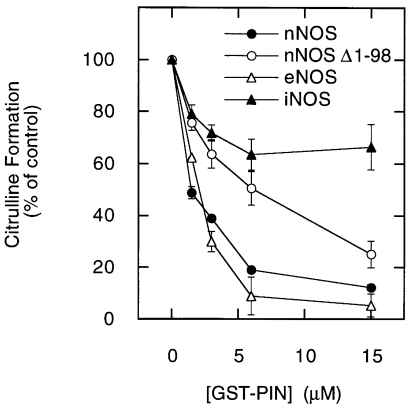

Fig. 2. Effect of GST-PIN on NOS isoenzymes. NOS isoenzymes (0.3 μM) were preincubated with GST-PIN for 30 min at 37°C, in 55 mM triethanolamine-HCl, pH 7.0. Error bars denote S.E.M. of three experiments. Rat nNOS, bovine eNOS and rat nNOSΔ1–98 were expressed in baculovirus-infected Sf9 cells; mouse iNOS was expressed in *E. coli* (see Section 2).

and 50% inhibition of nNOS was observed at around 3–6 μM GST-PIN. Thirdly, it might be suggested that the less potent inhibition we have observed is due to our use of the intact GST-PIN fusion protein rather than PIN itself. However, this seems unlikely because in the original study the intact GST-PIN inhibited nNOS five-fold more potently than PIN. Finally, it could be speculated that perhaps the inactivation by PIN is potentiated by other as yet unidentified factors present in HEK 293 cell extracts. We tested this by adding HEK 293 cell extracts to incubations of nNOS with GST-PIN, but found no effect (data not shown).

To characterize the mechanism of the inhibition of nNOS by GST-PIN, we first examined whether GST-PIN affected the interaction of the enzyme with its substrate L-Arg. The V_{max} of the enzyme was depressed (without PIN, 716 ± 43 nmol/min/mg; with 10 μM GST-PIN, 471 ± 75 nmol/min/mg) but no significant effect on the apparent K_M was observed (without PIN, 14 ± 3.2 μM; with 10 μM GST-PIN, 10 ± 1.7 μM). Thus PIN acts differently to the several L-Arg analogues that are already well-known as competitive NOS inhibitors. To identify the step in the nNOS reaction which was inhibited by GST-PIN, we examined the effects of GST-PIN on the part-reactions of NADPH oxidation and cytochrome *c* reduction. NADPH oxidation was inhibited with a similar potency to the complete NOS reaction (Table 1). Cytochrome *c* reductase activity of nNOS was not significantly inhibited when the enzyme was preincubated with 10 μM GST-PIN in 55 mM triethanolamine-HCl, pH 7.0/0.55 mM $CaCl_2$/0.22 mM CHAPS, with 110 μM NADPH and 200 μM cytochrome *c* for 30 min at 37°C (data not shown). These results suggest that the inhibition may result from an impaired efficiency of oxygen activation at the heme or a disruption of electron transfer between the reductase and oxygenase domains. The reported disruption by PIN of the nNOS dimer, which would presumably involve heme loss [21,28] and therefore the loss of NOS and NADPH oxidase activities, would explain the results. However, a similar mode of inhibition was observed with a peptide corresponding to residues 564–582 of rat nNOS [29], which did not involve disruption of the *P*-450 heme site, as reflected in the binding capacity for H_4biopterin or N^G-nitro-L-arginine. Therefore it seemed pertinent to reexamine the effect of GST-PIN on the formation of SDS-resistant nNOS dimer by SDS-PAGE at 4°C (Table 2). No significant effect of GST-PIN was observed, either in the presence or in the absence of L-Arg and H_4biopterin, after a 30-min preincubation of nNOS with a 160-fold molar excess of PIN.

Table 2
Effects of PIN on the proportion of SDS-resistant dimer formed by nNOS

	Control	With 100 μM GST-PIN
−H_4biopterin and L-Arg	23.5 ± 1	23.8 ± 2
+H_4biopterin and L-Arg	49.8 ± 3	51.5 ± 2

Values shown are means ± S.E.M. of four experiments.

The possible physiological role of PIN as a regulator of NOS must be reconsidered in the light of these results. Firstly, PIN is not an isoenzyme-specific inhibitor. Secondly, because of its low potency, a physiological role in NOS regulation can be limited to situations where there is a large excess of PIN over NOS. This condition may be met in some nerve terminals that contain nNOS [14,19]. However, even here its role as a NOS inhibitor is probably secondary to its other functions in myosin and dynein complexes.

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