Synthesis and characterization of a specific peptide nucleic acid that inhibits expression of inducible NO synthase

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Received 18 February 1998

Abstract Inducible nitric oxide synthase (iNOS) is modulated at the transcriptional level. Overexpression of this protein may result in high levels of nitric oxide leading to tissue damage and immunosuppression. In order to reduce the pathological effects of NO overproduction many efforts have been devoted to the identification of specific inhibitors of iNOS. The discovery of peptide nucleic acids (PNA), a novel class of molecules able to selectively interact with nucleic acids, prompted us to attempt a new way for the regulation of NO production. Here we describe the synthesis, characterization and in vitro effects of a PNA molecule bearing a homopyrimidine sequence complementary to the 5′ coding region of murine iNOS mRNA. This PNA shows specific interactions with iNOS mRNA in RNase protection assays and is able to block the synthesis of iNOS protein selectively in a rabbit reticulocyte lysate system. These results strengthen the view of a possible pharmacological application of PNA as a compound able to interfere with a specific enzymatic activity even at low concentrations.

Key words: Peptide nucleic acid; Inducible nitric oxide synthase; RAW 264.7 cell; Rabbit reticulocyte lysate system

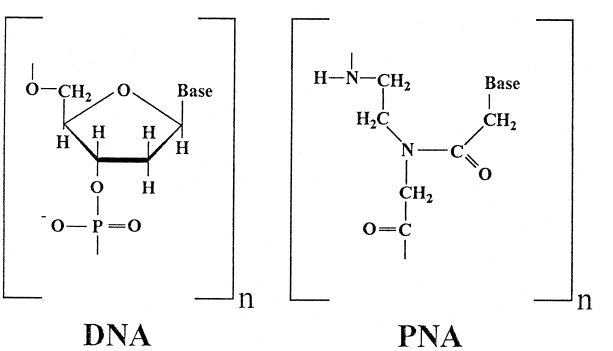
1. Introduction

In recent years nitric oxide (NO) has been increasingly demonstrated to play a fundamental role in cellular functions and in intercellular communications, while its concentration proved to be critical both in physiological and in pathological events [1,2]. In particular, effects of NO in the regulation of vascular homeostasis, as well as its involvement in neurotransmission and in the defense against infectious agents, have been demonstrated [2–4]. However, it has also been reported that imbalances in NO intracellular levels may be responsible for various pathological alterations, such as septic shock, hypertension, stroke and neurodegenerative diseases. [5–7].

This signal metabolite is synthesized by three distinct isoforms of NO synthase (NOS) [2,8,9]: the brain and endothelial enzymes are constitutively expressed and their enzymatic activity is calcium-dependent; in contrast, the inducible NOS (iNOS) is regulated at the transcriptional level by endotoxin and cytokines and is not dependent on calcium concentrations in the physiological range. This latter isoenzyme is responsible for the high production of NO by macrophages in response to various inflammatory stimuli [4]. In these circumstances, NO mediates antimicrobial and antitumor activities of the immune system [4], but, under certain conditions, high levels of NO cause tissue damage and autoimmune diseases [1,2]. These opposite effects suggest that iNOS is under particularly refined mechanisms of regulation and the search for specific modulators of its enzyme activity is actively pursued.

Most of the currently used pharmacological inhibitors of iNOS may also affect the other two isoforms of NOS and to some degree interfere with unrelated metabolic pathways of cells [2,4]. Therefore, an antisense strategy might result in a more selective pharmacological control of iNOS. Natural oligonucleotides are potentially useful tools although their fast in vivo degradation suggests the use of nuclease-resistant analogues. Peptide nucleic acids (PNA) [10] are structural homologues of RNA and DNA in which the entire deoxyribose phosphate moiety is replaced by an uncharged pseudo-peptide backbone consisting of *N*-(2-aminoethyl)glycine units linked to the purine and pyrimidine bases of natural nucleic acids (Scheme 1). This potent DNA mimic was found to form very stable complexes with specific DNA or RNA sequences [10–13] and to be resistant to enzymatic degradation in biological fluids and tissues [14]. Because of these very promising properties PNA can be considered new potential drugs able to interfere with gene expression at the transcriptional or translational level [15,16].

In this study we have explored the ability of a homopyrimidine PNA probe to bind specifically murine iNOS mRNA and consequently to inhibit its translation. RNase protection assays were performed to verify the specific hybridization of PNA to iNOS mRNA, while the inhibitory effect of PNA on iNOS expression was ascertained in a rabbit reticulocyte lysate system.



Scheme 1.

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Abbreviations: PNA, peptide nucleic acid; iNOS, inducible nitric oxide synthase; NO, nitric oxide; Boc, *tert*-butoxycarbonyl; dNTP, deoxynucleotide triphosphate; LPS, lipopolysaccharide; IFNγ, interferon γ; PCA, phenol:chloroform:isoamyl alcohol 25:24:1; dsDNA, double strand DNA; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption-time of flight; ES, electrospray

PII S0014-5793(98)00302-0

2. Materials and methods

2.1. Synthesis of PNAs

PNAs were manually synthesized through the standard method of solid phase peptide synthesis using the Boc strategy [17,18] with minor modifications as described in [19].

PNAs sequences were as follows: PNA1 Gly-Lys-CTTTCT-CCTTTTCCNH$_2$, complementary to the homopurine region 238–251 of the iNOS mouse cDNA (accession number M84373), and PNA2 (scrambled) Gly-Lys-CTTTTTCCCTCTTCNH$_2$, used as control.

The products were analyzed by coupling the RP-HPLC to an ES mass spectrometer using the same conditions described in a previous work [19].

2.2. cDNA of iNOS

iNOS cDNA (inserted in pUC19 vector) was kindly provided by Dr. Luigi Varesio (Istituto Gaslini, Genoa, Italy). cDNA was amplified under the following PCR conditions: 94°C for 10 s, 68°C for 3 min for 20 cycles; the 100 μl reaction volume contained Thermo Buffer (Promega, Madison, WI, USA), 0.2 mM dNTP (Pharmacia, Uppsala, Sweden), 10 pmol primers, 1 mM $MgCl_2$ and 2.5 U Taq polymerase (Promega). Oligonucleotide primers were obtained from TIBMOLBIOL, Genoa, Italy and were: 5′-AATTAGATCTCA-CCTTGGTGAAGGGACTGAGC-3′ (sense) and 5′-AATTAA-GCTTTGCAGCTAAGTATTAGAGCGGCG-3′ (antisense), corresponding to regions 44–65 and 3940–3962 of mouse iNOS. The primers also contained specific sequences for the *Bgl*II (sense) or *Hin*dIII (antisense) restriction enzymes. The product of amplification of 3918 bp contained the complete coding region.

The purified amplification product was digested with *Bgl*II and *Hin*dIII restriction enzymes (Amersham, Milan, Italy) and ligated into *Bam*HI and *Hin*dIII sites of pSP65 plasmid (Promega), which contains SP6 RNA polymerase promoter, using standard protocols [20].

2.3. SP6 RNA polymerase transcription

pSP65 plasmid containing iNOS cDNA was linearized by digestion with *Hin*dIII [20]. Radiolabelled iNOS mRNA transcription, to be used in the RNase protection assay, was obtained in a 20 μl sample containing 1×SP6 buffer (Amersham), 2 μg of template, 30 U of SP6 RNA Polymerase (Amersham), 10 mM DTT (Sigma, St. Louis, MO, USA), 0.5 mM ATP-CTP-UTP mix (Pharmacia, Uppsala, Sweden), 12.5 μM GTP (Pharmacia), 60 μCi [α-^{32}P]GTP (3000 Ci/mmol, Amersham), 20 U RNase inhibitor (Amersham), 0.01% BSA (Amersham). The mixture was incubated for 2 h at 40°C, then 20 U of RNase-free DNase (Boehringer Mannheim Italia, Monza, Italy) was added and the solution was maintained at 37°C for 20 min. Finally, radiolabelled iNOS mRNA was extracted with PCA, ethanol-precipitated [20] and resuspended in 20 μl RNase-free water. The solution was heated at 65°C for 10 min to denature mRNA.

In order to obtain the non-radiolabelled iNOS mRNA to be used in the rabbit reticulocyte lysate experiments 11 μg of cDNA template was incubated with 150 U SP6 RNA polymerase as described above, using 0.5 mM dNTP.

2.4. RNase protection

Samples (20 μl) containing iNOS radiolabelled mRNA (500 000 cpm), 0.3 M NaCl, 10 mM Tris pH 7.4, 4 mM EDTA, 0.02% tRNA (all from Sigma) and 1 μM PNA1 or 1 μM PNA2, were incubated at 42°C for 30 min, then 20 U RNase T1, 2.5 μg RNase A and 1.5 μg BSA were added and the solution was incubated for 1 h at 37°C [21]. RNA fragments were analyzed by 7 M urea/20% polyacrylamide gel electrophoresis followed by autoradiography as described [20].

2.5. Cell cultures

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was cultured in DMEM containing 4 mM glutamine and supplemented with 10% FCS [22]. Cell stimulation was performed by adding 10 U/ml mouse recombinant IFNγ (Sigma) and 50 ng/ml LPS (Sigma).

2.6. Purification of RAW 264.7 poly(A)$^+$

Total RNA was purified from unstimulated RAW 264.7 and cells stimulated with LPS and IFNγ for 6 h using described protocols [23,24]. Poly(A)$^+$ was obtained from total RNA using oligo(dT)-cellulose type 77F and following the supplier's protocol (Pharmacia Biotech, Cologno Monzese, MI, Italy).

2.7. Rabbit reticulocyte lysate system

Aliquots (50 μl) containing 20 μl rabbit reticulocyte lysate (Amersham), translation mix (Amersham), 0.1 M potassium acetate, 0.5 mM magnesium acetate, 60 μCi [^{35}S]methionine (specific activity 1000 Ci/mmol, Amersham), 4 μg iNOS mRNA or RAW 264.7 polyA, were incubated at 30°C for 90 min. Different concentrations of PNA (1, 0.1, 0.01 μM of PNA1 and 1 μM PNA2) were simultaneously added to different samples at the beginning of incubation. Analysis of translation products was performed with a 10% SDS-PAGE [25]. After electrophoresis, the gels were fixed with 7% acetic acid for 1 h at room temperature, then they were dried for 1 h and exposed overnight to Hyperfilm β-max autoradiography film (Amersham).

3. Results

3.1. PNA synthesis

The PNA1 sequence complementary to the 238–251 region of iNOS cDNA was deliberately chosen to match in proximity of the beginning of the coding region and in a homopurine sequence (Section 2.1). The second PNA sequence, used as control, was obtained by scrambling the bases used for the first PNA molecule and verifying its non-complementarity to mouse genomic DNA.

Following their chemical synthesis, both types of PNAs were purified as described in [19], with a final yield of 52%. The analyses performed as described in Section 2.1 showed a molecular ion at *m/z* 3838.23 for both molecules, consistent with the $[M+H]^+$ ion of the expected PNAs. The molecular weights were also confirmed by MALDI-TOF spectrometer analyses (data not shown).

3.2. PNA binding to iNOS mRNA

The radiolabelled iNOS mRNA obtained by SP6 RNA polymerase transcription was incubated with 1 μM PNA1

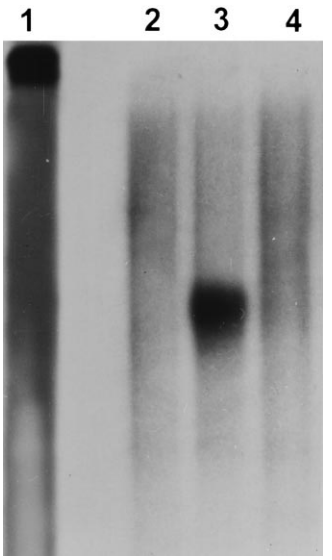

Fig. 1. RNase protection assay demonstrates PNA1 binding to iNOS mRNA. Lane 1 shows the undigested radiolabelled iNOS mRNA. Following RNase treatment, an undigested fragment appeared in the PNA1 sample (lane 3), while complete digestion was observed in the untreated (lane 2) and in the PNA2-treated (lane 4) samples.

and 1 μM PNA2 and then digested with RNase (Section 2.4). Results are shown in Fig. 1. Specifically, lane 1 represents the undigested radiolabelled iNOS mRNA, lane 2 the same RNase-digested sample, lane 3, corresponding to the PNA1-treated sample, shows the presence of a RNase-resistant fragment, and lane 4 represents the PNA2-treated sample in which absence of non-specific binding is demonstrated by complete mRNA degradation.

These data indicate that PNA1 binds its complementary region on iNOS mRNA, thereby preventing RNA digestion, while a non-specific sequence (PNA2) is unable to inhibit enzymatic degradation.

3.3. PNA inhibition of iNOS synthesis in the rabbit reticulocyte lysate system

In order to investigate the selective inhibition of PNA1 on iNOS translation, two kinds of experiments were performed.

First, iNOS cDNA was transferred into pSP65 plasmid containing SP6 RNA polymerase promoter and the mRNA was obtained as in [2,3]. The resulting transcription product was then incubated in the rabbit reticulocyte lysate system, in which it generated a 130 kDa protein, corresponding to the M_r of iNOS (Fig. 2, lane 1). The presence of 1 μM PNA1 cazused the complete disappearance of the 130 kDa protein (lane 2). Finally, no inhibition whatsoever of iNOS mRNA translation was apparent in the sample treated with 1 μM PNA2 (lane 3).

A second approach was designed to check the influence of the specific PNA on protein expression. In these experiments, a purified polyA from RAW 264.7 cells was incubated in the same cell-free translation system, giving rise to a large amount of products. The purified mRNA was obtained both from LPS- and IFNγ-stimulated cells and from untreated ones.

Fig. 3 shows that a 130 kDa protein, consistent with the M_r of iNOS, was produced only by purified poly(A)$^+$ from stimulated RAW 264.7 cells (lane 2), while being undetectable in the experiments containing poly(A)$^+$ from unstimulated cells (lane 1). Previously, the iNOS gene had been found to be transcribed under the same conditions of stimulation of RAW 264.7 cells with LPS and IFNγ [22]. Moreover, selective inhibition of biosynthesis of the 130 kDa protein was afforded

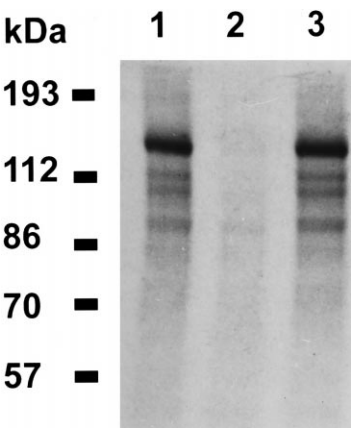

Fig. 2. PNA1 inhibition of iNOS synthesis in a cell-free translation system. Full-length iNOS mRNA in the rabbit reticulocyte lysate system generates a 130 kDa protein corresponding to native iNOS (lane 1). The presence of 1 μM PNA1 inhibits biosynthesis of iNOS (lane 2), while 1 μM PNA2 has no effect on the translation (lane 3).

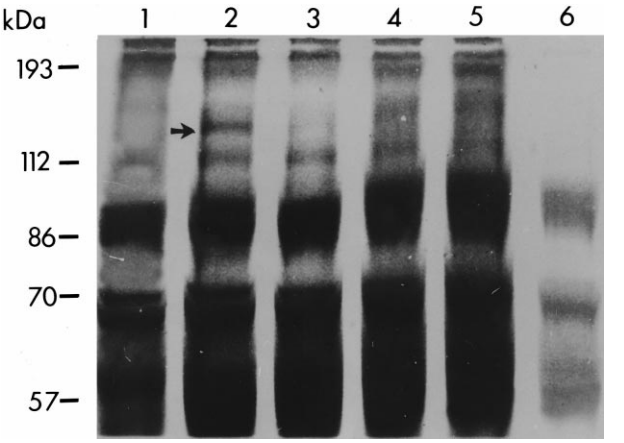

Fig. 3. Translation of poly(A)$^+$ from stimulated and unstimulated RAW 264.7 cells and inhibition by PNA1. Gel electrophoresis shows the patterns of proteins obtained by translation of poly(A)$^+$ from unstimulated (lane 1) or LPS- and IFNγ-stimulated cells (lanes 2–6). The 130 kDa band (arrow) is present in the case of stimulated cells only. The presence of 1 μM, 0.1 μM and 0.01 μM PNA1 (lanes 3, 4 and 5, respectively) inhibited expression of iNOS band without affecting translation of the other proteins. Lane 6 shows the non-specific inhibitory effect of 10 μM PNA1 on the rabbit reticulocyte lysate system activity.

in a dose-dependent manner by PNA up to 1 μM (lanes 3–5), while the highest concentration, i.e. 10 μM PNA1 (lane 6), resulted in a non-specific inhibition of protein synthesis.

4. Discussion

The combination of beneficial [2–4] and detrimental [5–7] effects afforded by a high release of NO by iNOS justifies the need to properly modulate the macrophagic production of NO. At the same time, it may be critical to avoid any unwanted effects on other NO-responsive cells, tissues and organs. Accordingly, recent research has focused on the development of selective modulators of iNOS, in order to inhibit uncontrolled formation of NO without interfering with the production of NO by endothelial and neuronal isoforms. Conventional inhibitors used so far display their effects on enzyme functions, so they are substrate analogs, flavoprotein binders or heme binders, but none of them proved to be devoid of effects on other NOS isoforms or on other biochemical pathways [2]. Inhibitors of induction of this enzyme, such as corticosteroids, TGF-b, interleukin-4, interleukin-10, and dexamethasone, can also interfere with other targets of cellular metabolism [2,26]. This background suggested the synthesis of a specific anti-iNOS PNA (PNA1) and the in vitro evaluation of its inhibitory effects on iNOS mRNA translation.

The homopyrimidinic PNA sequence used in these experiments was chosen for its complementarity to the 5′ initial coding region of iNOS and because homopyrimidine PNAs had been previously demonstrated to form triplexes with complementary dsDNA (DNA_2-PNA_1) or RNA (RNA_1-PNA_2) [27–29]. These properties should allow the PNA to feature its inhibitory action on the iNOS isoform only.

The RNase protection assay confirmed the ability of PNA1 to bind a specific sequence of iNOS mRNA. The inhibitory effect of PNA1 on iNOS synthesis was then demonstrated using the rabbit reticulocyte lysate, one of the standard systems for the study of translation of exogenous mRNA tem-

plates [30]. Translation of the in vitro produced iNOS mRNA to yield a 130 kDa protein, corresponding to the M_r of iNOS, was completely inhibited by 1 μM PNA1, while control PNA2 did not affect the system at all (Fig. 2).

A different approach was followed to test the ability of PNA1 to exert its specific inhibitory effects in the presence of a large amount of macrophagic mRNAs. Poly(A)$^+$ translation from LPS- and IFNγ-stimulated RAW 264.7 cells was non-specifically inhibited at 10 μM PNA1 (Fig. 3, lane 6). Similar data were obtained in a totally different application by Gambacorti-Passerini et al. [29] who noticed a non-specific inhibition of translation at greater than 2 μM PNA in a similar cell-free system. In our experiments, a 130 kDa protein band was apparent in the polyA translation products from LPS- and IFNγ-stimulated RAW 264.7 cells, which show enhanced transcription of iNOS mRNA over unstimulated cells [22,31,32]. Conversely, the 130 kDa band was not present in the unstimulated cell sample (Fig. 3), providing further evidence for the identity of this protein with iNOS. Moreover, synthesis of this protein was selectively inhibited by concentrations of PNA1 as low as 0.01 μM.

All these data suggest that PNA1 may be a potentially useful tool for the pharmacologic control of the high output NO pathway, although results in intact cells are required to explore the in vivo applications of this study. Specifically, use of suitable carriers to transport PNA1 inside cellular compartments, such as hydrophobic peptides, liposomes or red blood cells [19,33], seems to be the reasonable evolution of this work and is currently pursued in our laboratory.

Acknowledgements: This work was partially supported by CNR Target Project 'Biotechnology' and by AIRC. We are indebted to Prof. A. De Flora for constant support during this work and stimulating discussions and critical reading of the manuscript. We gratefully acknowledge Dr. Francesco Grieco for his helpful advice. We thank Dr. Luigi Varesio for providing us murine iNOS cDNA.

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