Antisense Oligonucleotides Against Inducible Nitric Oxide Synthase Reduce NO Production in RAW 264.7 Cells

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1. Introduction

Nitric oxide (NO), a vascular and neuronal messenger and a cytotoxic and cytostatic agent, is synthesized from L-arginine by the enzyme *nitric oxide synthase* (NOS). Two major forms of NOS have been characterized, and these are distinguished by their Ca^{++}/calmodulin dependence and by whether the enzyme is expressed constitutively (cNOS) or is induced following the exposure to cytokins and endotoxin (iNOS).

cNOS, found in neurons and endothelial cells, transiently produces small amounts of NO, important in both inter- and intracellular signalling (1). iNOS is induced in many cell types including macrophages, hepatocytes, vascular smooth muscle cells, astrocytes and synoviocytes in response to inflammatory and immunologic stimuli. iNOS generates much larger quantities of NO over longer periods of time. The NO released from macrophages, activated by cytokines and endotoxin, contributes to their cytotoxic and cytostatic properties against target cells. Although NO generated by cNOS appears to be beneficial in many physiological processes, the excess NO generated by iNOS has been implicated in the pathogenesis of various inflammatory and immunologically mediated diseases, such as graft vs. host disease, diabetes, viral infections and arthritis. A cytokine-inducible form of NOS is present in rodent macrophages. Macrophages are important effector cells but they also play a pivotal role in the induction and amplification of the immune response. Inhibition of iNOS may suppress the development of

numerous inflammatory and autoimmunological diseases (2). Because NO cannot be stored or inactivated (after release) by conventional mechanisms, it has to be regulated at the level of biosynthesis.

Nitric oxide synthases are inhibited by a wide variety of drugs which interfere with one of the multiple catalytic functions of these enzymes. However, these drugs cannot be regarded as specific iNOS inhibitors because they block other enzymes as well (3). Since antisense oligodeoxyribonucleotides (AS-ODNs) have a potential to inhibit the expression of certain genes within cells in a sequence-specific manner, we decided to investigate whether it was possible to use this approach in order to prevent iNOS translation.

2. Materials and methods

2.1. Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 medium, supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI 1640 medium).

RAW 264.7 cells, preincubated for 4 hrs in the presence of S-ODNs, were exposed to interferon-γ (IFNγ-10 U/ml), tumor necrosis factor-α (TNFα-50 ng/ml) and lipopolisaccharide (LPS-250 μg/ml) to induce NO release. After the desired incubation time, 100 μl aliquots of the culture media were frozen for NO_2 monitoring. The cells were stored at -70°C for RNA extraction.

2.2. Antisense designing

We chose iNOS mRNA fragment containing a five-base loop and a nearby four-base bulge as antisense target. Both the bulge and loop are known to destabilize the structure thus minimizing thermodynamic penalty paid by disruption of the existing structure.

A PCR primers search algorithm was subsequently used to design optimal AS-ODN sequences. This algorithm allows to avoid the sequence regions capable of forming dimers or foldback structures, owing to self-complementarity and/or inverted repeats.

In an average cell the total mRNA complexity is *ca* 4×10^7 nucleotides. It is far lower than that of 4^{20} (*ca* 1×10^{12}) combinations possible with four different nucleotides within oligonucleotide having a length of 20 bases. Under ideal hybridization conditions, with no mismatches tolerated within a heteroduplex, ODNs of 20 nucleotides in length are regarded to be highly selective for the respective target mRNA (4).

2.3. Oligonucleotides

Phosphorothioate ODNs used in this work were prepared by modified phosphoramidite method (5) with S-TETRA sulphurization (6) and after HPLC purification were transformed into sodium salts. Their nucleotide sequences are given below. The position of complementary mRNA regions (for antisense ODNs) are given in parantheses.

ASINOS1 (pos. 251-270):
5' CCA GGG GCA AGC CAT GTC TG

ASINOS2 (pos. 244-263):
5' CAA GCC ATG TCT GAG ACT TT

RANDOM INOS1 (random sequence):
5' GAC GTG CGA GTC AGC ACT GC

ASINOS15' (pos. 254-266):
5' GGG CAA GCC ATG

ASINOS13' (pos. 259-270)
5' AAG CCA TGT CTG

ASINOS1G (pos. 253-259)
5' AGG GGC A

SENS INOS1 (sense sequence)
5' CAG ACA TGG CTT GCC CCT GG

ASCREB:
5' TGG TCA TCT AGT CAC CGG TG

ASPENK:
5' ATG GGG CTG CCG TTG TTC AG

ASCFOS:
5' GAA CAT CAT GGT CGT

2.4. Nitric oxide synthase activity

NOS activity was assessed by measuring the level of nitrogen dioxide (NO_2) in cell culture media. Its formation results from spontaneous oxidation of primary metabolite, NO, with oxygen present in the environment. NO_2 is further hydrolyzed in the medium to yield a mixture of nitrate and nitrite salts. Resulting nitrite ions are quantified using fluorometric assay. The

method is based on a reaction of nitrite anions with 2,3 diaminenaphthalene (DAN) to form the fluorescent product 1(H)naphthotiazole.

A 100 µl of a sample was brought to 1ml with double-deionized water. To this mixture 50 µl of the freshly prepared DAN solution (0.025 mg/ml in 0.62 M HCl) was added. After 10 min incubation at room temperature, the reaction was terminated by addition of 25 µl of 2.8 N NaOH. Formation of 2,3-diaminonaphthotriazole was measured with a Hitachi spectrofluorimeter with excitation at 365 nm and emission read at 450 nm. This assay can detect 10 µM concentration of nitrite and therefore is 50-100 times more sensitive than the well known Griess assay (7).

2.5. Lipofection

Lipofectin Reagent™ was used, the liposome formulation of DOPE (dioleylphosphatidiloetanolamine) and DOTMA (dioleyloxypropyltrimethylammonium chloride) in water, which is suitable for the transfer of S-ODNs into cells in culture. For each lipofection 2 µg/ml of Lipofectin Reagent™ and 4 hrs incubation were used.

2.6. Reverse transcription and polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA was extracted from cultured RAW 264.7 cells following the method of Chomczynski and Sacchi (8) and subjected to semiquantitative RT-PCR. Briefly, 1 µg of total cytoplasmic RNA was converted to cDNA using reverse transcriptase and the specific primer (5'TGGCTTGCCCTTGGAAGTTTCTC) complementary to the region of the "reading frame" of the mouse iNOS mRNA. The resulting cDNA was amplified using the same reverse primer and a forward primer (5'TGTCTCTGGGTCCTCTGGTCAAA) to yield a 375-bp PCR product. Semiquantification was achieved by limiting the cycle number down to 25 in the PCR. The signal was approximately linear with the input RNA within the range of 10 ng to 1 µg. Samples were reverse transcribed and amplified using Tth DNA polymerase. The final analysis of the RT-PCR products was accomplished by electrophoresis on a 2% agarose gel and UV detection after ethidium bromide staining. Relative quantities of cDNAs were evaluated using MCID (The Microcomputer Imaging Device) system and an M4 software (the image was reversed to resemble an X-ray film image).

3. Results

A combination of LPS and IFNγ resulted in the expression of the inducible NOS in RAW 264.7 macrophages, as determined by nitrite accumulation in the cell supernatant. Whereas unstimulated cells produced < 2.5 nmol of $NO/10^6$ cells within 24 hrs (a "negative" control), nitrite accumulation in LPS/IFNγ-treated samples amounted to 100(±)7 nmol of $NO/10^6$ cells in 24 hrs (a "positive" control — five experiments). The effect of S-ODNs on

iNOS activity is presented as the ratio (percentage) of stimulation of NO production in RAW 264.7 cells treated with LPS and INF-γ (which was a maximal stimulation). In the presence of antisense oligonucleotide ASINOS1 the level of the cytokine-induced iNOS activity after 24 hrs of incubation was reduced to 10% of that measured for control samples.

Additional controls included:

— SENSE INOS1: sense control which has a nucleotide sequence identical to target mRNA fragment (pos. 251-270). Sense control maintains structural features (eg. palindromes, stem loops, class) but not the composition;

— RANDOM INOS1: oligonucleotide with a nucleotide composition identical to AS-ODN but with a random sequence of nucleotides;

— ASCFOS, ASCREB, ASPENK: antisense oligodeoxynucleotides targeted at other genes (c-fos, cAMP responsive element binding protein and proenkefalin), which were effective in other *in vitro* and *in vivo* models (data not shown).

Even at higher doses (10-20 μM), none of the aforementioned control ODNs significantly reduced or stimulated iNOS activity (Fig. 1).

Measured effect was time-dependent (Fig. 2) and since 8 hrs of incubation with 5 μM AS INOS1 resulted in an over 60% decrease in NO release, that time point was chosen for further experiments.

Both ASINOS1 and ASINOS2 ODNs decreased the measured NO_2 accumulation in a concentration-dependent manner; however at each time point ASINOS1 was more effective when compared to ASINOS2. That phenomenon was more evident especially at higher doses of ODNs (Fig. 3).

The effect of different, shorter fragments of ASINOS1 was analyzed. All

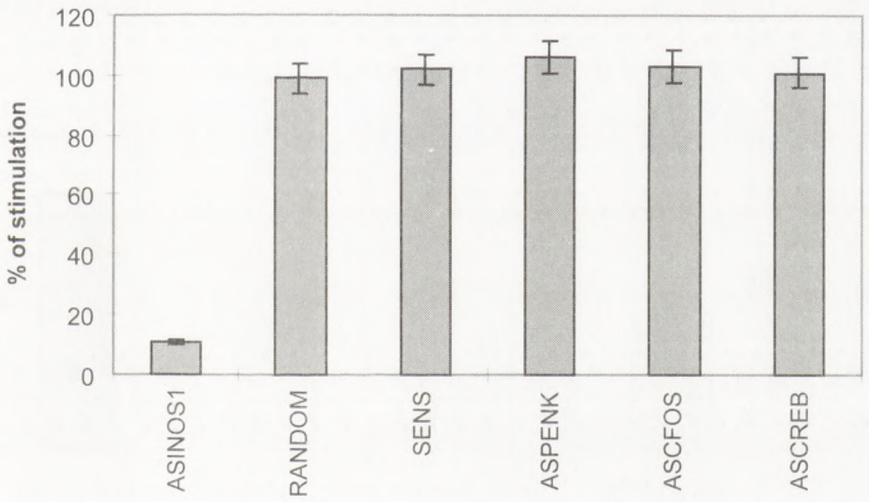

Fig. 1. Effect of different AS-ODNs (10 μM, 24 h) on NO release in RAW 264.7 cells.

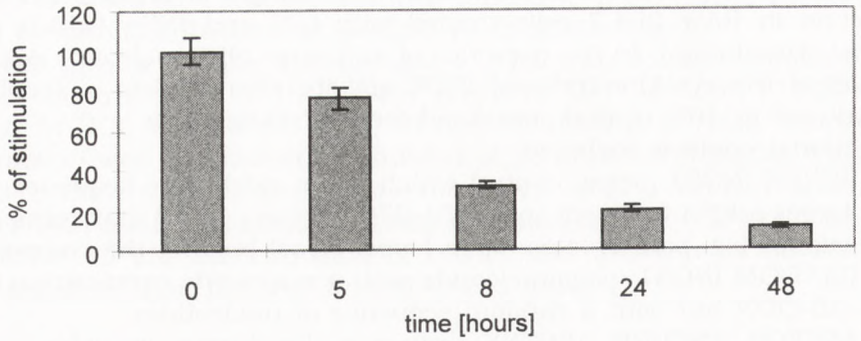

Fig. 2. Effect of ASINOS1 on NO release at different time points.

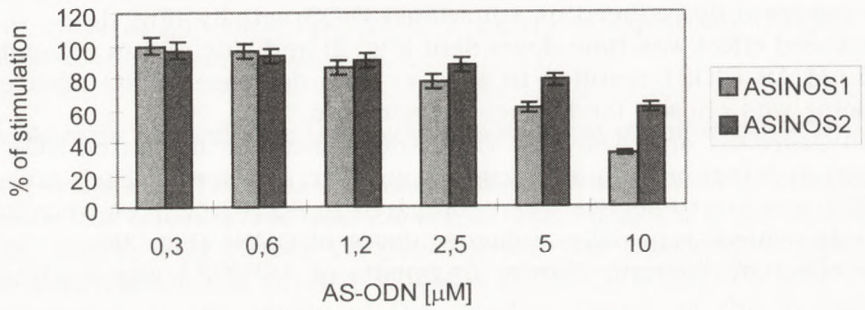

Fig. 3. Effect of ASINOS1 and ASINOS2 (time of incubation 8 h) on NO release in RAW 264.7 cells.

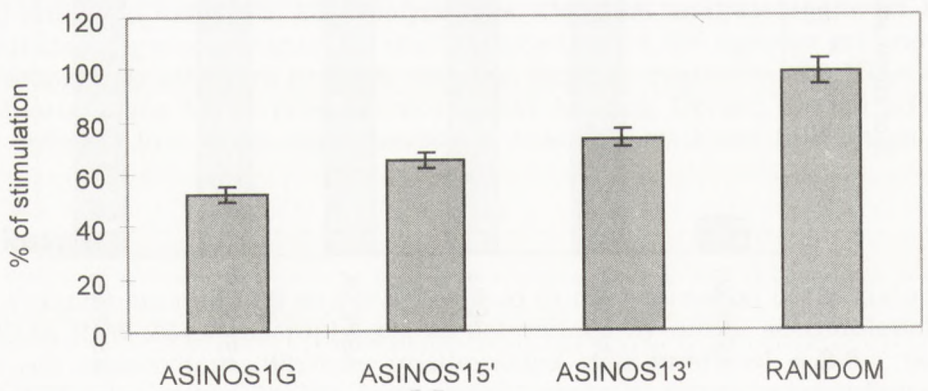

Fig. 4. Effect of three variants (10 μM, 8 h) of ASINOS1 on NO release in RAW 264.7 cells.

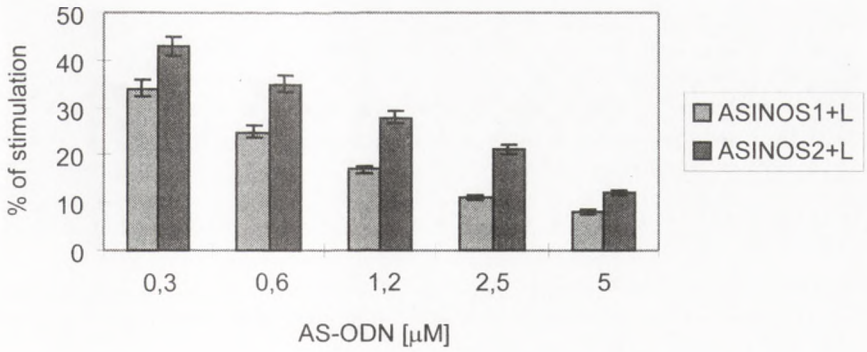

Fig. 5. Effect of Lipofectin on ASINOS1 and ASINOS2 activity (time of incubation: 8 hours).

three ODNs: ASINOS15', ASINOS13' and ASINOS1G, inhibited iNOS yet to a lower extent than ASINOS1 or ASINOS2. Although the differences between the activities of the three variants were rather small, the more upstream part of the loop was targeted, the greater supression of NO release was observed. The effect of ASINOS1G, containing "G-quartet", was the lowest of all AS-ODNs studied (Fig. 4).

A major problem concerning AS ODNs is their inability to efficiently penetrate cellular membranes. When added to cell cultures the ODNs have a tendency to accumulate in the cytoplasmic granules (endosomes and lysosomes). The application of Lipofectin Reagent™ in order to improve the uptake of oligonucleotides greatly enhanced the effect of AS-ODNs on the iNOS activity. That effect was dependent on the concentration of AS-ODNs. The suppression of NO release was enhanced threefold at lower doses (0.3 μM) up to sevenfold at higher ones (2.5-5 μM) of ASINOS1, whereas for ASINOS2 a two- and sixfold enhancement, respectively, was observed. The presence of vehicle alone (up to 5 μg/ml of Lipofectin Reagent™) had no significant effect on the accumulated NO_2 levels (Fig. 5).

Semiquantitative RT-PCR was performed for comparison of mRNA levels in samples resulting from different experiments. Primers for PCR were chosen in order to flank a portion of mRNA which served as a target for AS-ODNs. Because RNases H have been implicated in mediation of the AS-ODNs effect via degradation of the RNA portion of DNA-RNA hybrids, such fragmentation of RNA should have resulted in lowering the quantity of cDNA obtained in RT-PCR. Indeed, addition of 10 μM ASINOS1 lowered the amount of iNOS mRNA by 40%. On the other hand, incubation with 10 μM random ODN did not decrease the level of iNOS mRNA (Fig. 6).

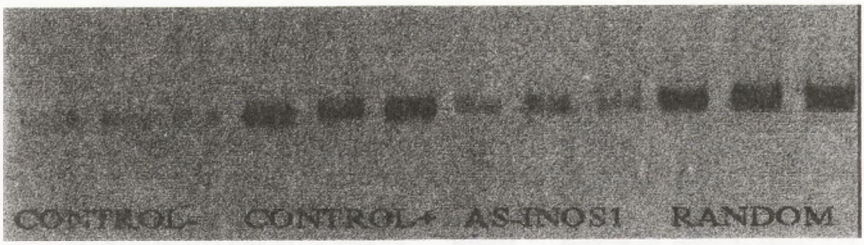

Fig. 6. Semiquantitative RT-PCR of iNOS mRNA. Blank were non-stimulated cells, the "positive" control were cells stimulated with LPS, IFNγ and TNFα. RANDOM was 10 μM RANDOM INOS1. ASINOS1 was at 10 μM concentration.

4. Discussion

Phosphorothioate analogues of AS-ODNs, which are of great interest due to their nuclease resistance and capacity of eliciting the RNase H activity, have been widely used in recent years as potent antisense inhibitors of gene expression (9). In this study we described the inhibition of NO production by phosphorothioate AS-ODNs using the iNOS mRNA as a target.

We clearly demonstrated that different AS-ODNs, complementary to different sites of the same target, were able to reduce NO production, whereas control ODNs were incapable of affecting iNOS activity in RAW 264.7 cells. We chose five ODNs of three types of control, which maintained structure characteristics, composition and hybridization selectivity. Since the controls were negative, it is likely that the observed phenomena resulted from a true antisense mechanism. Apart from control experiments, we validated the measured effect by using different AS-ODNs against the same region of mRNA. Despite some differences, all the tested AS-ODNs inhibited iNOS activity at comparable concentrations and within comparable time periods. It is well known that the affinity of oligonucleotides for their complementary sequences increases with the increase of the length of the oligonucleotide (4). Three shorter AS-ODNs did not inhibit NO production so efficiently as ASINOS1 and ASINOS2 with a length of 20 nucleotides. ODNs containing four consecutive guanine residues (so called "G quartet") were reported either to induce an immunological response or to inhibit some genes in a non-specific manner (10). ASINOS1G, which also contains the "G quartet", inhibited iNOS activity to a lesser extent than other AS-ODNs.

The decreased level of the target mRNA, as demonstrated by RT-PCR, strongly suggests that the RNase H mechanism may, indeed, contribute to the antisense effect. However, since ASINOS1 caused up to 90% decrease in NO production and lowered the level of mRNA by 40%, it cannot be accepted as sole evidence of the mechanism. Thus the mechanism involving blockade of ribosomal readthrough cannot be excluded.

Several reports (4,11-13) also emphasize basic significance of the plasma membrane as a barrier to oligodeoxyribonucleotide-directed antisense effect in intact cells. Lipofectin Reagent™ is largely employed for plasmid DNA transfections. Since single-stranded, low molecular weight ODNs are endowed with the same overall chemical features as plasmid double-stranded DNA, Lipofectin Reagent™ was expected to be a good ODN carrier. This cationic lipid/AS-ODN combination could improve the cellular uptake of oligomer and partly overcome the AS-ODN degradation by serum and cytoplasmic nucleases. The application of Lipofectin Reagent™ greatly enhanced the level of inhibition of iNOS activity.

ANTINOS1 appears to be a potent and highly specific inhibitor of iNOS translation from murine RAW 264.7 macrophages. Since the macrophage produced NO with its cytotoxic activity against pathogens also affects other cells, including macrophages, the application of ANTINOS1 may down-regulate the macrophage activity at sites of prolonged elevated NO release, thus protecting cells against damages. ANTINOS1 can be applied in murine models of inflammatory and immunological damages, such as arthritis and induced peripherial neuritis. In all of these diseases macrophages appear to play a crucial role.

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Summary

Several oligodeoxyribonucleotides in a nuclease-resistant phosphorothioate form (S-ODNs) targeted to iNOS mRNA were tested in RAW 264.7 cells as potential antisense inhibitors. Antisense S-ODNs inhibited iNOS activity in a time- and dose-dependent manner. Maximal inhibitory effect was >90% while control S-ODNs were ineffective. Application of Lipofectin Reagent, which itself had no significant effect, greatly increased antisense activity. Decreased levels of the target mRNA, as demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR), suggest the RNase H mediated mechanism.

Key words
nitric oxide synthase, antisense oligonucleotides, phosphorothioate oligonucleotides.

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