Specific iNOS-targeted antisense knockdown in endothelial cells

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Hemmrich, Karsten, Christoph V. Suschek, Guido Lerzynski, Oliver Schnorr, and Victoria Kolb-Bachofen. Specific iNOS-targeted antisense knockdown in endothelial cells. Am J Physiol Cell Physiol 285: C489–C498, 2003.—The inhibition of inducible nitric oxide synthase (iNOS) expression via antisense oligonucleotides (AS-ODN) may represent a highly specific tool. Endothelial cells (EC) represent prime candidate cells for in vivo application, and we therefore aimed at optimizing this technique for effectiveness and specificity in primary nontransformed rat EC. EC or L929 fibroblasts were incubated with iNOS-specific ODN optimizing all experimental steps. We find that ODN uptake, as analyzed by fluorescence microscopy and labeled ODN, was absolutely dependent on vehicle presence, and among the vehicles tested, Lipofectin displayed negligible toxicity and good uptake. In addition, omission of serum was also essential, a factor that might limit its use in vivo. Moreover, intranuclear accumulation of AS-ODN appeared crucial for successive inhibition. The impact of ODN on iNOS mRNA, protein, and enzyme activity was specific and resulted in >95% inhibition of protein formation. In conclusion, in this article we provide a protocol for an optimized AS-mediated knockdown, representing a specific and efficient instrument for blocking of iNOS formation and allowing for studying the impact of iNOS expression on endothelial function. We also expose application problems of this technique when working in inflammatory conditions.

endothelium; inflammation; nitric oxide; inducible nitric oxide synthase

INHIBITION OF PROTEIN FORMATION by adding antisense oligonucleotides (AS-ODN) potentially represents a highly specific technical approach for gene knockdown. Theoretically, the AS method is highly specific because added ODN are supposed to interact only with their mRNA-sense sequences during transcription. However, there are still many unsolved questions about ODN uptake, intracellular ODN accumulation, and the mechanism of how AS-ODN inhibit protein expression. Specific interference with the ribosomal translation of the encoded protein at the mRNA level is thought to be the most prominent effect of ODN. However, two other mechanisms of AS inhibition may also occur: ODN directed to pre-mRNA may inhibit polyadenylation or transport of mRNA into the cytoplasm, thereby interfering with RNA maturation; or they may activate RNase H, causing specific cleavage (6). The power of AS-ODN as specific inhibitors of gene expression is strongly limited by their low spontaneous cellular uptake (15). Furthermore, ODN are rapidly degraded by nucleases if not chemically modified and stabilized (17, 19, 27). To circumvent the obstacle of degradation, a variety of improvements, for instance phosphate-modified analogs, have been developed (20). The problem of effective cellular ODN uptake will be solved by using uptake enhancers like cationic lipids (4). These substances ensure nucleotide uptake and release from endosomal compartments (10). Here, medium constituents and incubation times have a marked influence on micelle formation, as well as incorporation and intracellular localization of ODN (25, 26). Furthermore, it is still controversial whether ODN have to accumulate in the nucleus or whether cytoplasmic accumulation will lead to the desired inhibition (24). Because there are numerous parameters requiring consideration, precise experimental protocols for all the factors involved are necessary for successful AS inhibition. In addition, an inflammatory surrounding as created by cytokines may influence the effects of ODN or lipid vehicles, thereby affecting the outcome.

Chronic inflammatory conditions are usually associated with an overexpression of the inducible nitric oxide synthase (iNOS) as a result of proinflammatory cytokines. The resulting high-output production of nitric oxide (NO) is often associated with cell damage or tissue destruction (1, 3, 19). Because such unregulated iNOS activity and high concentrations of its product, NO, have been found to be associated with many diseases (13), the search for a highly specific method for iNOS inhibition is continuing. Substrate deprivation, competitive inhibition by using substrate analogs (8), manipulation of cofactor availability, and the manipulation of the expression or functionality of iNOS-relevant transcription factors like NF-κB or IkB (12) represent some of the experimental approaches for iNOS inhibition. However, all of these techniques raise problems due to toxicity or lack of specificity. It has been tried only once to inhibit iNOS protein expression in...
endothelium (22). Similar to other approaches, unsatisfactory results concerning the level of iNOS inhibition were reported. It is surprising that endothelial iNOS has only rarely been chosen for iNOS-directed AS treatment, because endothelial cells (EC) represent a prime target for in vivo intervention and are inevitably affected by AS-ODN when applied via the blood. Furthermore, iNOS and its product, NO, represent a system of major importance in a wide range of endothelial functions, e.g., oxidative stress, inflammation, antimicrobial reactions, and regulation of gene expression (2, 14, 16, 18). In addition, excessive NO formation is also held responsible for the circulation failure in patients with septic shock.

It is the aim of the present study to provide an optimized experimental protocol for AS experiments in primary, nontransformed EC, combining low toxicity with strong and specific AS-mediated inhibition.

To examine the effectiveness of transmembrane carriers and to improve the rate of ODN incorporation, we investigated the three widely used transfection reagents FuGENE 6, LipofectAMINE, and Lipofectin and their usefulness under various conditions concerning choice of medium and incubation times. By means of fluorescence microscopy, we explored how different conditions and methods of preparing AS-ODN-lipid complexes determine the intracellular localization of ODN. In parallel, we investigated the inhibitory effects of AS-ODN on cytokine-mediated endothelial iNOS mRNA formation and iNOS protein expression, as well as the specificity of the inhibition achieved by monitoring the induction and expression of interleukin-1β (IL-1β), driven by the same cytokines.

MATERIALS AND METHODS

Reagents. Recombinant human IL-1β was from HBT (Leiden, Netherlands). Recombinant murine interferon-γ (IFN-γ) and recombinant murine tumor necrosis factor (TNF-α) were from Genzyme (Cambridge, MA). Endothelial cell growth supplement (ECGS), LPS (from Salmonella typhimurium), neutral red (3% solution), type I collagen, collagenase (from Clostridium histolyticum), rabbit anti-human von Willebrand Factor (vWF) antiserum, 2-mercaptoethanol, and anti-α-tubulin-antibody were from Sigma (Deisenhofen, Germany). Rat endothelium specific monoclonal antibody Ox43 was from Serotec (Camon, Wiesbaden, Germany), monoclonal anti-mouse endothelial nitric oxide synthase (eNOS) and anti-mouse iNOS antibodies were from Transduction Laboratories (Lexington, KT), and peroxidase-conjugated porcine anti-rabbit IgG was from DAKO (Hamburg, Germany). Rat endothelium specific monoclonal antibody Ox43 was from Serotec (Camon, Wiesbaden, Germany), monoclonal anti-mouse endothelial nitric oxide synthase (eNOS) and anti-mouse iNOS antibodies were from Transduction Laboratories (Lexington, KT), and peroxidase-conjugated porcine anti-rabbit IgG was from DAKO (Hamburg, Germany). Peroxidase-conjugated goat anti-mouse IgG was from Zymed (San Francisco, CA), trypsin, EDTA, and fetal calf serum (FCS, endotoxin free) were from Boehringer Mannheim (Mannheim, Germany), Omniscript RT kit and Taq Core PCR kit were from Qiagen (Hilden, Germany), FuGENE 6 was from Roche (Mannheim, Germany), and RPMI 1640 (endotoxin free), oligo(dT)17-primer, Lipofectin, LipofectAMINE, and Opti-MEM serum-reduced medium were from Life Technologies (Eggenstein, Germany). 3,3′-Diaminobenzidine (DAB) was from Serva (Heidelberg, Germany). Antisense oligonucleotides and controls directed to iNOS have been designed and manufactured by Biognostik (Göttingen, Germany). Chosen for inhibition of iNOS were antisense oligodeoxynucleotides with or without FITC label (5′-TTTGCCCTTACAGTTCT-3′). As controls, we used two oligodeoxynucleotides with identical purine and pyrimidine content (5′-ACTACTACTAGTACTAC-3′ and 5′-ATATCCCTCCAGTACAG-3′), the second one of which was also FITC labeled.

Cell cultures and cellular characterization. Rat aorta EC were isolated by outgrowth from rat aortic rings exactly as described (21). Briefly, aortic segments were placed on top of a collagen gel (1.8 mg collagen/ml) in 24-well tissue culture plates and incubated in RPMI 1640 with 20% FCS and 100 μg/ml ECGS for 4 to 6 days, depending on the degree of cellular outgrowth. Aortic explants were then removed, and cells were detached with 0.25% collagenase in HBSS and replated onto plastic culture dishes in RPMI 1640/20% FCS. Cells were subcultured for up to 10 passages, and removal from culture dishes for each passage was performed by treatment with 0.05%/trypsin/0.02% EDTA in isotonic NaCl for 3 min.

EC were characterized by using a crossreacting rabbit-anti-human vWF antiserum, the rat vascular endothelium specific monoclonal antibody Ox43, the monoclonal mouse-anti-eNOS antibody, and the respective secondary peroxidase-conjugated porcine anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG antisera at conditions exactly as described previously (21). EC showed the antigen phenotype vWF high Ox43 high eNOS high exactly as published (21). The labeling also proved the purity of these cultures, since the respective staining patterns were found in virtually all cells.

Control experiments concerning ODN uptake and intracellular accumulation were also performed with the mouse fibroblast cell line L929, usually maintained in RPMI 1640/10% FCS or as indicated.

Experimental design. All experiments with EC were performed with cells from passages 2 to 8. EC or L929 were cultured in 6- (2 × 10⁵ cells) or 12-well (0.8–1 × 10⁵ cells) tissue culture plates in RPMI 1640 (20% FCS for EC, 10% FCS for L929) and incubated overnight to allow attachment before superenatant was replaced by 1) fresh Opti-MEM serum-reduced medium, 2) RPMI 1640/10% or 20% FCS, or 3) RPMI 1640 without FCS. Cells were either resident or activated by cytokines (IL-1β, TNF-α, and IFN-γ, each at 1,000 U/ml) before analysis by fluorescence microscopy.

Analysis of oligonucleotide uptake without lipid encapsulation. After overnight attachment in 6- or 12-well plates, culture supernatants were replaced by 1,000 μl (6-well) or 600 μl (12-well) of fresh medium containing the phosphorothioate AS-ODN at concentrations indicated for 2 to 72 h and monitored for positive uptake. Cells were then activated by cytokines, and mRNA formation, protein expression, and nitrite accumulation were measured.

Analysis of ODN uptake with lipid encapsulation. To examine the impact of transmembrane vehicles on ODN incorporation, FuGENE 6, LipofectAMINE, and Lipofectin were investigated as lipid vehicles.

FuGENE 6 was used at amounts of 1.5–15 μl in 600 μl of Opti-MEM serum-reduced medium on 12-well culture dishes. Diluted ODN, used at concentrations from 0.13 to 0.49 μM, were added to the prediluted FuGENE 6 reagent.

LipofectAMINE was used according to the manufacturer’s recommendations at concentrations as indicated. A solution of 1 μM/lipofectin has a final concentration of 1.05 μM 2,3-dioleoyloxy-N-[2(5-carboxamidomethyl)aminopropyl]-N,N-dimethyl-1-propanaminium trifluoroacetate and 0.673 μM dioleoylphosphatidyl ethanolamine (DOPE). Vehicles and ODN were used at concentrations as indicated in 12-well plates with 600 μl of supernatant.
Lipofectin was prepared according to the recommendations of the supplier and used at concentrations as given. A solution of 1 μM/l has a final concentration of 0.75 μM N-[1-(2,3-dioleyloxypropyl)N,N,N-trimethylammonium chloride and 0.68 μM DOPE. Lipofectin was applied in six-well culture dishes with 1 ml of supernatant.

When using LipofectAMINE or Lipofectin, ODN and lipid were diluted separately in Opti-MEM or RPMI 1640/20% FCS, 10% FCS, or 0% FCS, incubated at room temperature for 15 min (LipofectAMINE) or 45 min (Lipofectin), and then combined and incubated at room temperature for 15 min to allow for micelle formation.

Cell treatments. After overnight attachment of freshly seeded cultures in 6- or 12-well plates, supernatants were replaced by 1 ml or 600 μl of fresh Opti-MEM, RPMI 1640/20% or 10% FCS according to the cell type, or RPMI 1640/0% FCS, containing lipid alone or ODN-lipid complexes prepared as described. After incubation periods of 2 to 24 h, cells were activated by cytokine challenge and incubated for an additional 24 h in cytokine-containing Opti-MEM, RPMI 1640/20% or 10% FCS, or RPMI 1640 without FCS. Additionally, Lipofectin and LipofectAMINE were checked for endotoxin content using the Limulus amoebocyte lysate test, and endotoxin levels were always below the detection limit.

Growth rates of cell cultures and viability. Cell growth was determined by neutral red staining. Cells were incubated for 90 min with neutral red (1:100 dilution of a 3% solution), washed twice with PBS, dried, and lysed with isopropanol containing 0.5% 1 N HCl. Supernatants were measured at 530 nm. Additionally, viability of EC and L929 was routinely controlled using trypan blue exclusion.

Nitrite determination. Cellular NO production was measured by quantifying the nitrite accumulation in culture supernatants of EC by using the diazotization reaction as modified by Wood et al. (23) and NaNO₂ as standard.

Reverse transcription and polymerase chain reaction. Total cellular RNA (with 1 μg RNA/probe) was prepared using the Omniscript RT kit, and reverse transcription (RT) was carried out at 37°C for 60 min with oligo (dT) (15 mer) as primer. The cDNA (500 ng each) was used for PCR with primer ODN and amplification protocols as shown in Table 1.

PCR products were subjected to electrophoresis on 1.8% agarose gels. Bands were visualized by ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed by using the KODAK 1D software (KODAK, Stuttgart, Germany).

Western blot analysis of the iNOS protein. Cells, treated as indicated, were washed, scraped from the dishes, lysed, transferred to a microcentrifuge tube, and boiled for 5 min in electrophoresis buffer. Proteins (30 μg per lane) were separated by electrophoresis in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Further incubations were 2 h blocking buffer (2% BSA, 5% nonfat milk powder, 0.1% Tween 20 in PBS buffer), 1 h at 37°C with a 1:2,000 dilution of the monoclonal anti-iNOS antibody, and 1 h with a 1:2,000 dilution of the secondary horseradish peroxidase-conjugated rabbit-anti-mouse-IgG antibody. Finally, blots were incubated for 5 min in ECL reagent (Pierce, Rockford, IL) and exposed to an autoradiographic film. To control for equal loading of total protein in all lanes, blots were also stained with a mouse anti-a-tubulin antibody at a dilution of 1:2,000.

Statistical analysis. Data are given as arithmetical means ± SD. Values were calculated using the Student’s t-test (two-tailed for independent samples).

RESULTS

ODN uptake in the absence or presence of vehicles. Cultured EC or L929 cells were incubated with various concentrations of ODN in RPMI 1640 with FCS (20% for EC, 10% for L929), FCS-free RPMI 1640, or Opti-MEM serum-reduced medium (Opti-MEM).

In the absence of transport vehicles, fluorochrome-labeled ODN were found dispersed in the supernatant or attached to the outer cell membrane, and intracellular accumulation was never observed, irrespective of the various concentrations, different medium conditions, cytokine activation, or incubation times (Fig. 1, A–C). The longer the incubation times and the higher the concentration of FITC-labeled ODN, the brighter was the green fluorescence surrounding the cells and covering the culture dishes.

Using the transmembrane vehicle FuGENE 6, a similar picture was seen as in the absence of uptake enhancers. Although ODN and lipid concentrations, as well as incubation times and culture medium conditions (RPMI, Opti-MEM, FCS), were again varied, no uptake of ODN was observed in any combination (Fig. 1D).

With LipofectAMINE (Fig. 1, E–H), good intracellular labeling of cells was observed with 4 μl of lipid/600 μl of medium and ODN at a concentration of 0.7 μM. Best uptake of ODN was achieved by leaving lipid and ODN complexes on the cells for at least 4 h before cytokine activation. Earlier removal of ODN caused a significantly lower uptake rate and, in addition, fluorescence microscopy showed ODN still attached to the outer cell membrane and not yet incorporated (Fig. 1E). Incubation of cells for at least 4 h led to accu-

<table>
<thead>
<tr>
<th>Species/Product</th>
<th>Sequence</th>
<th>Product Size, bases</th>
<th>Amplification Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat iNOS</td>
<td>S</td>
<td>ATGCCCGATGGCACCATCGA</td>
<td>394</td>
</tr>
<tr>
<td>Rat IL-13</td>
<td>AS</td>
<td>TCTCCAGGCCCATCCCGTC</td>
<td>519</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>S</td>
<td>CAAGCTCATGTTTACTGTTCC</td>
<td>416</td>
</tr>
</tbody>
</table>

PCR was started with 30 s at 94°C, and amplification was always followed by a final incubation step at 72°C for 10 min. S, sense; AS, antisense; iNOS, inducible nitric oxide synthase.
lation of ODN in the nucleus, as evidenced by bright fluorescence signals over the nuclei with hardly any label in the cytoplasm (Fig. 1F). ODN were still found in the nucleus after an incubation period of 20 h (Fig. 1, G and H).

When using Lipofectin, good labeling of cells was observed with 6 μl of lipid in 1 ml Opti-MEM and ODN concentrations of 0.6 μM (Fig. 1, J and K). Again, most of the label accumulated in the nuclei, but here, a significant portion was also seen in cytoplasmic organelles. After 20 h, these cytoplasmic aggregates had disappeared and left a slight homogenous coloring of the cytoplasm, with the nuclei showing intensive fluorescence signals.
Cell viability. Next, we monitored cell viability under various incubation conditions with or without lipid and with or without ODN (Fig. 2). Incubation conditions with negative uptake in the absence of vehicles showed no toxicity, irrespective of ODN concentrations (Fig. 2A). Unloaded LipofectAMINE vesicles or ODN-loaded micelles showed significant, concentration-dependent toxic effects (Fig. 2, B and C, and Table 2).

Table 2. Comparison of LipofectAMINE and Lipofectin and their effectiveness in delivering ODN into endothelial cells and L929 fibroblasts

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Medium During Preparation of Lipid-ODN Complexes</th>
<th>Medium During Incubation of Lipid-ODN Complexes on Cells</th>
<th>ODN Incorporation</th>
<th>Toxicity, %</th>
<th>Inhibition of Nitrite Production, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipofectAMINE</td>
<td>RPMI 1640/FCS</td>
<td>RPMI 1640/FCS</td>
<td>Cytoplasmic</td>
<td>30 ± 13</td>
<td>0</td>
</tr>
<tr>
<td>LipofectAMINE</td>
<td>RPMI 1640/no FCS</td>
<td>RPMI 1640/FCS</td>
<td>Cytoplasmic</td>
<td>28 ± 9</td>
<td>0</td>
</tr>
<tr>
<td>LipofectAMINE</td>
<td>RPMI 1640/FCS</td>
<td>RPMI 1640/no FCS</td>
<td>Cytoplasmic/nuclear</td>
<td>46 ± 8</td>
<td>0</td>
</tr>
<tr>
<td>LipofectAMINE</td>
<td>RPMI 1640/no FCS</td>
<td>RPMI 1640/no FCS</td>
<td>Nuclear</td>
<td>57 ± 10</td>
<td>0</td>
</tr>
<tr>
<td>LipofectAMINE</td>
<td>Opti-MEM/no FCS</td>
<td>Opti-MEM/no FCS</td>
<td>Nuclear</td>
<td>55 ± 11</td>
<td>0</td>
</tr>
<tr>
<td>Lipofectin</td>
<td>RPMI 1640/FCS</td>
<td>RPMI 1640/FCS</td>
<td>Cytoplasmic</td>
<td>1 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>Lipofectin</td>
<td>RPMI 1640/no FCS</td>
<td>RPMI 1640/FCS</td>
<td>Nuclear</td>
<td>7 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>Lipofectin</td>
<td>Opti-MEM/no FCS</td>
<td>Opti-MEM/no FCS</td>
<td>Nuclear</td>
<td>6 ± 6</td>
<td>60 ± 8 (see Fig. 6A)</td>
</tr>
</tbody>
</table>

Inhibition of nitrite formation was only determined in endothelial cells. To gain comparable results for different incubation conditions, 4 μl of LipofectAMINE were diluted in 600 μl of the respective medium. Lipofectin was used at amounts of 6 μl in 1,000 μl medium. Both lipids were combined with fixed amounts of oligonucleotides (ODN) (LipofectAMINE, ODN at 0.7 μM; Lipofectin, ODN at 0.6 μM). Serum was added to the medium as indicated (20% FCS in EC and 10% FCS in L929 fibroblasts). Medium conditions (i.e., FCS content, RPMI 1640, or Opti-MEM) were varied 1) during micelle formation and preparation of lipid-ODN complexes and 2) during incubation of lipid-ODN complexes on cells. ODN incorporation patterns, i.e., aggregation in the nucleus or cytoplasm, were investigated by fluorescence microscopy using FITC-labeled ODN. Numbers of live and dead cells were determined by neutral red and trypan blue exclusion assay.
With Lipofectin as vehicle, we found no toxicity in the absence of ODN (Fig. 2D) and only very little cell loss at Lipofectin concentrations above 6 µL/ml in the presence of ODN (Fig. 2E and Table 2). Interestingly, ODN exerted a significant growth inhibition that was not observed with vehicle alone (Fig. 2E).

Incubation protocols influencing ODN uptake and intracellular ODN localization. Because LipofectAMINE mediated excellent uptake but caused significant toxicity at the same time, we also examined the impact of the presence of FCS on viability and uptake modalities in EC and L929 fibroblasts incubated and cultured under otherwise identical conditions as endothelial cells were examined by fluorescence microscopy 6–8 h after ODN addition. All micrographs at right represent the respective bright-field images. A and B: incubation with LipofectAMINE-encapsulated ODN (4 µL + 0.7 µM) in the permanent presence of FCS (10%) during complex formation and cell incubation. C and D: LipofectAMINE-ODN (4 µL + 0.7 µM) were prepared without FCS and then added to the cells cultured with FCS (10%). E and F: formation of LipofectAMINE-ODN complexes (2 µL + 0.7 µM) was as above, followed by incubation on cells in DMEM 1640/0% FCS. After 4 h, a washing step was followed by further incubation with FCS (10%). G and H: as in E, but using a different complex ratio (4 µL + 0.7 µM ODN). J and K: as in E, but using a different complex ratio (1 µL + 0.7 µM ODN). Bar in K = 30 µm.
iNOS activity was ~10–15% stronger without medium replacement. Addition of cytokines did not affect the process of ODN uptake, their intracellular localization, or the kinetics of ODN incorporation.

Impact of AS-ODN on iNOS mRNA synthesis, protein expression, and enzyme activity. With the conditions described above, we next determined the effect of AS-ODN on iNOS mRNA expression in EC cultures incubated for a 5.5 h-period, followed by cytokine activation (IFN-γ, IL-1β, TNF-α, 1,000 U/ml each) for 24 h. As seen in Fig. 5, A and B, cytokine-mediated activation induced the de novo formation of iNOS mRNA in sham-treated cells as expected. Addition of AS-ODN via Lipofectin as vehicle resulted in some minor decrease in iNOS mRNA expression to 70 ± 10% of the level of sham-treated or control ODN-treated cells (Fig. 5A, lane 1), whereas Lipofectin with either one of two control ODN sequences (see Reagents) did not alter the cytokine-mediated iNOS expression (Fig. 5A, lane 2). Interestingly, the vehicle control, i.e., empty Lipofectin vesicles, led to a significant and dose-dependent increase in iNOS-specific mRNA formation (Fig. 5A, lane 4 and 5B). This lipid-mediated increment in iNOS expression was completely abolished by loading the vehicle with control ODN (Fig. 5A, lane 2). Because endotoxin contamination of all lipid formulations was essentially below the detection limit as measured by Limulus lysate assay, this iNOS upregulation must be regarded as a lipid-specific effect.

Next, we examined the specificity of this AS-mediated effect by monitoring the impact on another gene, also expressed de novo in the presence of proinflammatory cytokines. The gene expression pattern of IL-1β is similar to iNOS expression with comparable induction kinetics. Neither lipid vesicles alone nor the combination with either AS or control ODN showed any impact on the de novo mRNA expression of IL-1β during cytokine activation (Fig. 5A). Therefore, AS-ODN-mediated inhibition of iNOS must be regarded as specific, not interfering with general cytokine inducibility.

Next, the degree of AS inhibition on protein expression was studied using Western blotting (Fig. 5C). We find an almost complete block of iNOS protein formation by AS-ODN (Fig. 5C, lane 1), whereas control ODN had no effect (lane 2). The degree of inhibition for iNOS protein synthesis was better than 95% compared with cytokine treatment only. Again, a significant increase in iNOS protein formation by empty vehicle addition was observed (lane 4). In resident cells, iNOS protein was completely absent (lane 5).

To also assess the effect of AS-ODN on NO formation, nitrite accumulation in culture supernatants was measured. Treatment with AS-ODN caused a significant and dose-dependent decrease in nitrite formation without any effects of the two different control ODN (Fig. 6A). Using empty vehicles, again a highly relevant and dose-dependent increase in nitrite accumulation was found (Fig. 6B). In conclusion, with the protocol used here, we find a significant AS-ODN-mediated inhibition of iNOS on all
levels of enzyme synthesis and activity when using Lipofectin as uptake enhancer.

**DISCUSSION**

Our experiments demonstrate for the first time that the use of AS-ODN packed into lipid vesicles indeed offers a tool to achieve a specific and high degree of iNOS inhibition in EC in an inflammatory setting. This is promising for in vivo trials, but our setting at the same time shows general problems and limitations of this technique.

In agreement with an increasing number of publications (9, 11), we see neither uptake, as monitored by fluorescence microscopy, nor inhibition of nitrite formation in the absence of suitable vehicles. However, Lipofectin in contrast to LipofectAMINE displays negligible toxicity in nontransformed cells and thus represents a good solution for intracellular delivery of ODN. Surprisingly, we always observed that the addition of Lipofectin alone significantly enhances iNOS mRNA and protein expression, as well as NO formation, in activated EC only. Because LPS contamination could not be detected using the Limulus lysate assay, this effect appears to be due to the lipid molecules themselves by incorporation of the charged lipid moieties into the cellular membranes. Our experiments further show that vesicles loaded with ODN do not cause this increase in iNOS expression and NO production, irrespective of the ODN sequence or specificity; therefore, proper loading protocols should solve this problem. Nevertheless, it still has to be taken into consideration that in an inflammatory setting, the application of

**Fig. 5.** Impact of AS ODN and Lipofectin on inducible nitric oxide synthase (iNOS) mRNA and protein formation. Endothelial cells were incubated with Lipofectin-control ODN complexes (C-ODN), Lipofectin-encapsulated AS-ODN, or Lipofectin alone for 5.5 h, followed by cytokine activation (IL-1β, IFN-γ, and TNF-α at 1,000 U/ml each) for 24 h. As reference, cells were sham treated (act.). Nonactivated, resting cells were processed in parallel. The presence of iNOS-specific mRNA was analyzed by RT-PCR, and iNOS protein formation was investigated by Western blotting. A: graph at top gives the relative signal intensities of iNOS-specific amplification in relation to the housekeeping gene amplification in each probe and the graph at bottom shows a representative gel. RT-PCR reveals a small but significant effect of AS ODN on iNOS mRNA formation and a highly significant up-regulation in the presence of empty vehicle. No effects were found on the inducible control gene IL-1β. Results are based on 6 individual experiments. B: empty lipid vesicles (Lipofectin) were used as in A (act. + Lipofectin). In the absence of cytokines, Lipofectin did not induce endothelial iNOS mRNA formation. Shown is the dose-dependent induction of iNOS mRNA expression when Lipofectin is used without ODN in cytokine-activated cells. Presented data are based on 9 individual experiments. C: Western blotting reveals a highly effective block of iNOS protein formation by AS ODN, with no effect of control ODN and a significant increase by empty vehicle. Shown is a representative blot from 3 individual experiments. *P < 0.05 in A, B, and C compared with activated (act.) cells.
lipid-encapsulated AS-ODN might turn an ODN-mediated iNOS knockdown into an upregulation of NO production, probably due to interactions with charged lipids and cytokine-mediated signaling. However, the iNOS-enhancing property of the vehicles used here appears to represent a restrictive activity that does not act on all inflammatory responses, because the cytokine-driven IL-1β expression is not altered by empty vehicles. We have no explanation for this observation, and there are no published data that would help to understand this currently. However, because lipid segregation, i.e., lipid rafting, is known to modulate a number of immune responses, this might be a possible mechanism triggered by introducing loaded lipids into the cell membrane.

The exact mechanism of AS-mediated inhibition is still under debate, but in our experimental setup, we found a distinct correlation between intranuclear accumulation of ODN and successful inhibition of enzyme activities because nitrite production was reduced only when ODN had entered the nucleus. In the continuous presence of FCS, FITC-labeled ODN accumulated around the nuclear envelope and in the cytoplasm but not within the nuclear compartment. Even when formation of ODN-lipid complexes was performed in FCS-free medium and FCS was present only during cell culture incubation, ODN were not localized in nuclei. In cell culture systems, it poses no problem for creating a FCS-free or -depleted environment. However, under in vivo conditions with the constant presence of serum, it is questionable whether ODN or other gene transfer applications using vehicle-mediated uptake will still show the same effectiveness observed in cell culture.

With LipofectAMINE, excellent staining of nuclei with hardly any ODN left in the cytoplasm was seen, but, surprisingly, no inhibition of nitrite formation was observed. LipofectAMINE-ODN interactions might be responsible for this result, prohibiting ODN from getting in close contact with their target structures. Concerning the mechanism of AS inhibition, our results underline the finding that AS-ODN exert their main effect not on the level of mRNA but rather on protein formation. Indeed, the decrease in mRNA synthesis as seen with Lipofectin was found to never exceed a level of 40% relative to sham-treated controls, whereas inhibition of protein synthesis was higher than 95%. This finding suggests a direct action mainly on specific protein synthesis. One explanation for the differences in iNOS mRNA vs. protein inhibition might be seen in the blocking of iNOS mRNA transport into the cytoplasm, which would also explain the necessity of nuclear accumulation of ODN for successful action. A similar observation has been made with using iNOS-specific AS inhibition in mouse mixed glial cell cultures (7).

When investigating the impact on enzyme activity as assessed by accumulation of nitrite in the culture supernatants, the degree of inhibition did not completely reflect the nearly complete inhibition of protein formation, again an observation also noted with the mouse glial cultures (7). This may be explained by cytokine-mediated induction of proteins with accessory functions on enzyme activity, for instance, increased arginine transport via cytokine-inducible CAT-2 (5), but lack of feedback inhibition might also be responsible for the few enzyme molecules formed to work at maximal activity. Potentially, increased endothelial NOS3 activity could also contribute. Together, our data demonstrate a complex effect of AS-ODN, affecting every level of iNOS-derived NO synthesis, albeit at different degrees. However, suppression of iNOS expression can be regarded as a specific process because no effect was found on concomitant endothelial IL-1β expression, another inflammation marker also synthesized de novo in the presence of proinflammatory stimuli, following a similar time pattern, and in part using identical activation pathways such as NF-κB.

In conclusion, we show here that AS-ODN targeted to iNOS represent a powerful tool for studying endothelial dysfunctions and inflammatory disorders. Especially when considering the possible protective activity that more recently has been attributed to high-output NO synthesis via iNOS activity, the specific inhibition
achieved through AS-ODN-lipid complexes will allow us to define beneficial vs. detrimental effects mediated by this early inflammatory response.

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DISCLOSURES

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REFERENCES