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ANTISENSE INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION - DOES IT MAKE SENSE?

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CONTENTS

ABBREVIATIONS

AABS	activator binding site
ADMA	asymmetric dimethyl arginine
AGE	advanced glycosylation endpro-duct
AS	antisense
AS-ODN	antisense oligodesoxynucleotides
BCG	Bacille-Calmette-Guérin
BH_4	tetrahydrobiopterin
BHT	butylated hydroxy toluene
γ-IRE	interferon gamma response elements
CAT	cationic amino acid transporter
cGMP	cyclic guanidine monophosphate
cNOS	constitutive nitric oxide synthase
Control-ODN, C-ODN	control oligodesoxynucleotides
DAB	3,3'-diaminobenzidine
DCLHb	diaspirin cross-linked hemoglobin
DOPE	dioleoylphosphatidylethanolamine
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium
ds RNA	double-stranded RNA
EAE	experimental allergic encephalo-myelitis
EC	endothelial cells
ECGS	endothelial cell growth supple-ment
EIU	endotoxin-induced uveitis
ERK	extracellular signal-regulated kinase
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
FMN	flavin mononucleotide
HO-1	heme oxygenase-1
IBD	inflammatory bowels disease
IFN-γ	interferon-gamma
IL-1β	interleukin-1-beta
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
IRP-1	iron regulatory protein-1
LAK	lymphokine activated killer cell
L-NIO	L-N-(1-iminoethyl)-ornithine
L-NMMA	N^G-mono-methyl-L-arginine
LPS	lipopolysaccharides
MKP-3	MAP kinase phosphatase-3
MDA	malondialdehyde
MS	multiple sclerosis
Myr	myristoylation
NF	nuclear factor
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
ODN	oligonucleotide(s)
PAF	platelet activating factor
PGE/PGI	prostaglandin E/I
PHP	pyridoxalated hemoglobin poloxyethylene
PI	propidium iodide
PI-3-K	phosphatidyl-inositol-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMNL	peripheral mono nuclear lympho-cytes
PNA	peptide nucleic acids
PS-ODN	phosphorothioate ODN
RA	rheumatoid arthritis
rAEC	rat aorta endothelial cells
SSRE	shear stress responsive element
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-alpha
VEC	vascular endothelial cells
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells
vWF	von Willebrand factor

INTRODUCTION

ENDOTHELIUM IS MORE THAN A LAYER OF NUCLEATED CELLOPHANE

Although endothelial cells (EC) have long been considered little more than a “layer of nucleated cellophane”, it is now evident that the endothelial layer has a large variety of functions, including hemostasis and hematopoiesis, inflammatory reactions, antigen presentation, immunity, and lipoprotein metabolism which all involve close interactions between immunocompetent cells and vascular endothelium (320, 414).

Endothelial cells are derived from mesoderm and form a simple squamous epithelium that lines the inner lumen of the cardiovascular and lymphatic system (274). Significant physiological differences exist among endothelia from different species and tissues, and between the endothelial cells in the microvasculature and those in large arteries and veins (321). Electron microscopy has revealed that the capillary EC themselves differ from organ to organ and there are almost as many varieties of capillary EC as there are organs and tissues. The most obvious difference is the continuity of capillary endothelial cells. Differences in intercellular endothelial connections form the distinction among three types of capillaries: continuous, discontinuous, and fenestrated. The capillary endothelia are also functionally heterogeneous (452). Recent data provide evidence that the pattern of production of extracellular matrix components by microvascular EC is different from that produced by macrovascular EC such as human umbilical vein endothelial cells (413).

One of the most prominent homeostatic activities of the endothelium is the regulation of exchanges between blood/lymphatic fluids on one side and tissue on the other. Intercellular junctions participate in these activities in a dynamic way in concert with the functional requirements to these activities. Most of the biological stimuli known to be modulators of endothelial permeability are produced in acute or chronic inflammatory situations. Most substances like thrombin or histamine induce a rapid and short-lived increase in vascular permeability. Others such as the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β) and/or interferon-gamma (IFN-γ) as well as hypoxia and advanced glycosylation endproduct (AGE)-modified proteins induce a sustained response after a delay of hours or days. Other biological modulators of vascular permeability that enhance permeability are vascular endothelial growth factor (VEGF), bradykinin, lipopolysaccharide (LPS), leukotriene (LTB_4), and platelet activating factor (PAF), whereas prostaglandins (PGE_1, PGE_2, PGI_2) decrease it. The agonists described regulate the increase or decrease of second messengers by regulating the status of the intercellular junctions. It has been suggested that endothelial junctions can be closed by stimulating adenylate cyclase and opened by stimulating protein kinase C but little is known in terms of their molecular targets.

ENDOTHELIUM AND INFLAMMATION

The endothelium plays an important role in the development of an inflammatory process, induced by foreign agents or injury. This process is accompanied by endothelial cell activation, loss of endothelial integrity, plasma leakage, and accumulation of leukocytes in extravascular tissue. Activation of the vascular endothelium, resulting in expression of its proinflammatory properties, has also been associated with acute leukocyte-mediated injury occurring during ischemia reperfusion (22). The proinflammatory properties include the production of inflammatory cytokines, chemokines, coagulation factors, and vasoactive agents as well as the expression of surface adhesion molecules that promote leukocyte adhesion (334). The type of leukocyte that binds to the endothelial cell surface and thus accumulates in inflamed tissue depends on the nature of the inflammatory stimulus, its persistence, and the type of inflammatory reaction elicited. Almost immediately after the onset of most types of acute inflammation, neutrophils and monocytes adhere to the vascular endothelium and accumulate at the involved site, monocytes accumulating slightly later and in lower number than neutrophils. After 6 to 24 hours, monocytes become the predominant cell type because they continue to migrate into inflammatory tissue while neutrophil migration has virtually stopped (212, 213). This shift in the type of infiltrating leukocyte correlates with an alteration of the endothelial cell phenotype, referred to as the endothelial cell activation state (187, 316, 353).

ACTIVATION OF ENDOTHELIAL CELLS BY PROINFLAMMATORY MEDIATORS

Under normal steady state conditions, the nonthrombogenic and nonhemostatic surface properties of the endothelium, various adhesion molecules, and the narrow spaces (varying from 10 to 200 nm) between adjacent endothelial cells do not promote leukocyte adherence or transendothelial migration. The increased interaction between leukocytes and endothelial cells which is elicited during inflammation or tissue injury is mainly due to locally generated inflammatory mediators which induce or increase the expression of adhesion molecules on endothelial cells (23, 395). These changes in surface expression of adhesion molecules correlate with a shift in extravasation from granulocytes to monocytes and lymphocytes such as is observed *in vivo* between 6 and 24 hours after onset of inflammation (18, 187, 212, 213, 316, 353, 354). In this respect, the response to cultured endothelial cells to various inflammatory mediators can be divided into at least three different stages: an immediate response within minutes after stimulation, an early response after several hours, and a late response after 1 to 3 days.

What is most notable is that this wide array of inflammatory responses as mentioned above leading to cell adhesion molecule expression appears to funnel through a single signal transduction pathway using the transcription factor NFκB (223, 312). NFκB is an ubiquitous tran-

scription factor involved in the regulation of genes that respond to various forms of external stimulation (468) (Figure 1). Once activated, NFκB translocates to the nucleus where it binds with specific DNA sequences, altering conformation of the basal transcriptional apparatus, results in the transcription of various activation genes. Under non inflammatory conditions, NFκB is bound to IκB, the cytosolic inhibitory protein that keeps NFκB inactive (83). When activated by cytokines, lipopolysaccharide, or hypoxia-reoxygenation, the NFκB-IκB complex is phosphorylated and becomes dissociated. Once dissociated, IκB is degraded rapidly, and in parallel, there is an accumulation of NFκB in the nucleus. This accumulation initiates the transcription of activation genes. In addition, when endothelial cells are activated, transcription of IκB is promoted, leading to a feedback inhibition loop (83). Thus, the release of IκB is the central event required for the activation of NFκB, and ultimately, for gene activation and synthesis of new proteins in response to extracellular stimuli.

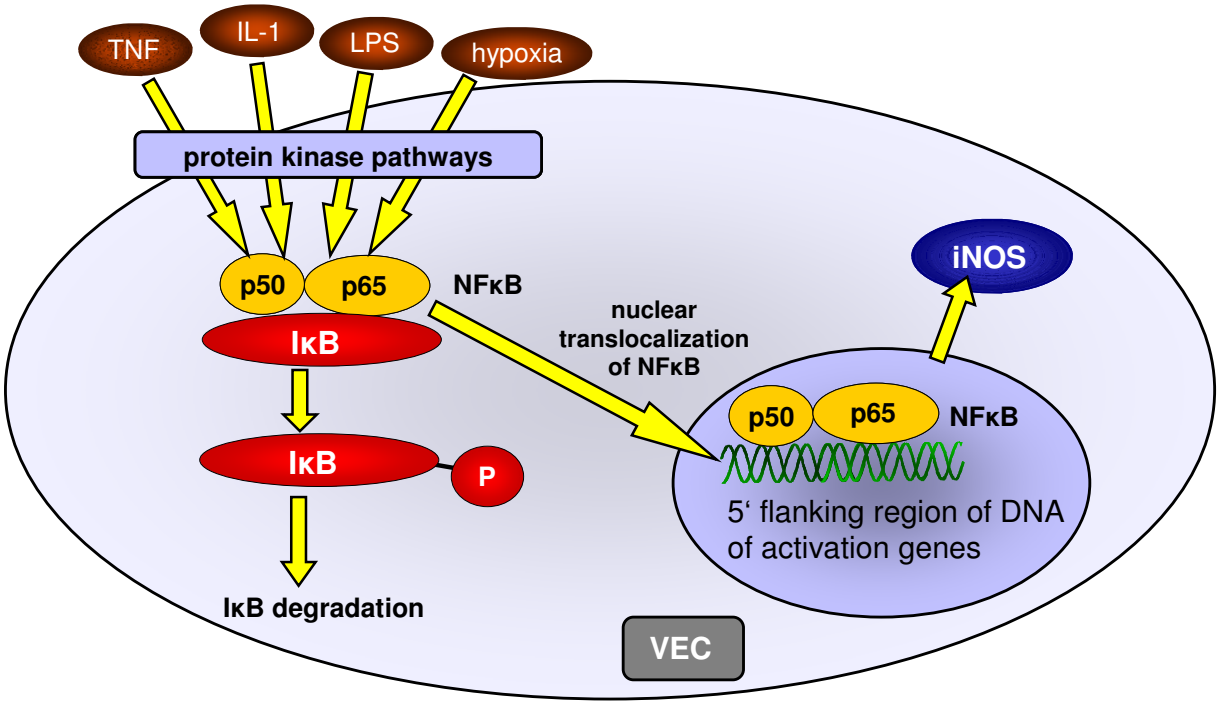


FIGURE 1. Signal transduction through NFκB. *NFκB is an ubiquitous transcription factor involved in the regulation of genes that respond to various forms of external stimulation. Usually NFκB is bound to IκB that keeps NFκB inactive. After activation the NFκB-IκB complex is phosphorylated, and the complex becomes dissociated. IκB is degraded rapidly, and NFκB translocates to the nucleus and binds with specific DNA sequences resulting in the initiation of transcription of the activation genes.*

NITRIC OXIDE SYNTHASES

In the presence of molecular oxygen and catalyzed by the enzyme nitric oxide synthase, the guanidino nitrogen of L-arginine undergoes a five-electron oxidation to yield the gaseous free radical, nitric oxide and citrulline in equimolar concentrations. Flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), protoporphyrin IX heme, and tetrahydrobiopterin (BH_4) are

essential cofactors for this reaction. The flavins apparently store and donate electrons to the heme group, which then catalyzes the oxidation process. Tetrahydrobiopterin appears to be important in maintaining the enzyme in its active dimeric form (263, 360). NO has a half-life ($t_{1/2}$) of seconds and usually is oxidized to the stable, inactive endproducts nitrite and nitrate (NO_2 and NO_3^-). N^G-substituted-L-arginine derivatives competitively inhibit the NO synthases (345).

Three distinct isoforms of the NOS enzyme have been isolated and represent the products of three different genes (Figure 2 and Table 1). It is unknown whether additional NOS isoforms exist. However, it is now nearly five years since all known NOS isoforms were cloned which makes it increasingly unlikely that novel highly homologous isoforms will be discovered. Two of the NOS enzymes are continuously present and are thus termed constitutive NOS (cNOS). One cNOS enzyme was first identified in endothelial cells (endothelial cNOS, eNOS) and its active form is localized to plasma membrane caveolae in these cells (149). The second cNOS was initially localized to neurons (neuronal cNOS, nNOS), but has also been identified in other tissues such as skeletal muscle or keratinocytes (358).

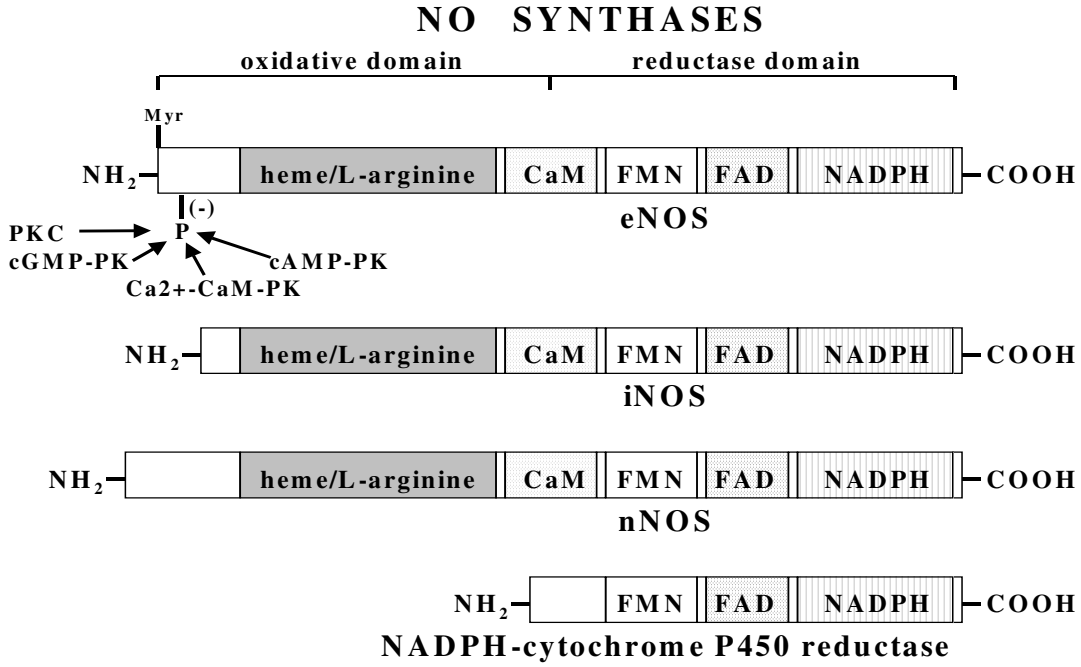


FIGURE 2. Schematic structure of eNOS, iNOS and nNOS, and the cytochrome P450 reductase. *Indicated are the common binding sites for NADPH, FAD, and FMN in the carboxyl-terminal domains. The binding-sites for calmodulin (CaM) and heme/L-arginine in the NH_2-terminal, as well as the sites for protein phosphorylation (P) and myristoylation (Myr) are also shown. Phosphorylation is produced by PKC and Ca^{2+}-, -CaM-, cGMP-, and cAMP-dependent kinases.*

Following a rise in intracellular Ca^{2+}, calcium-calmodulin binding results in the activation of cNOS leading to the transient synthesis of small amounts of NO. The low level of NO generated by a cNOS acts as a messenger molecule by activating soluble guanylate cyclase and increasing intracellular cGMP (346). Endothelial cell derived NO plays a central role in the

regulation of vascular tone (480). Neuronal NO acts as a neurotransmitter also by activating guanylate cyclase (51) with important functions in the central nervous system including a role in the formation of memory (427). In the autonomic nervous system, NO functions as an inhibitory neurotransmitter producing relaxation in the gastrointestinal tract (54, 102) and the corpus cavernosum, thereby mediating penile erection (55).

The two cNOS enzymes are contrasted by the third, the inducible NOS isoform (iNOS), which is typically not expressed in resting cells and must first be induced by certain cytokines, microbial products, or lipopolysaccharides. iNOS expression then generates large amounts of NO, a production that can be sustained over days. Induced NO synthesis was

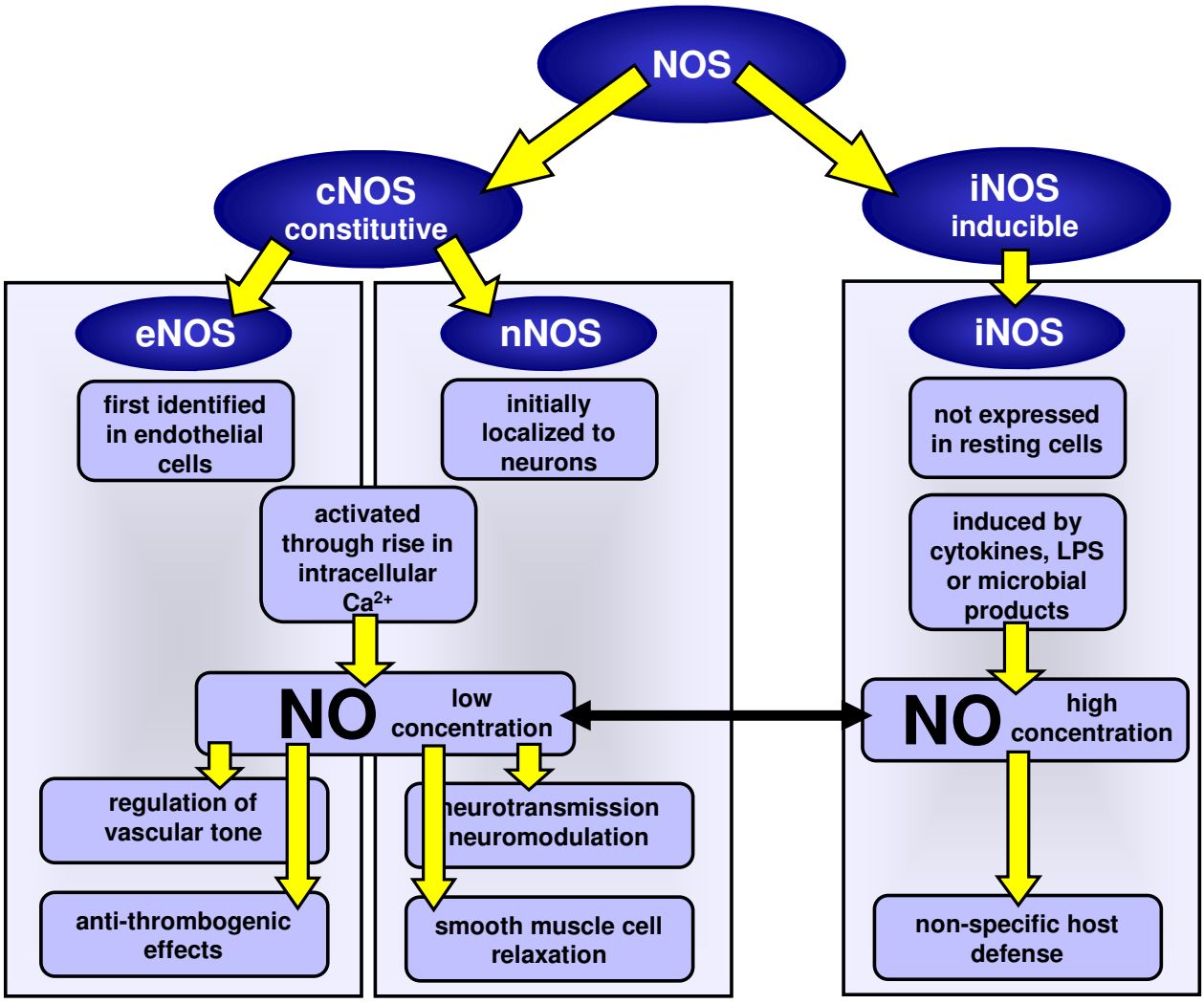


FIGURE 3. The isoforms of nitric oxide synthases. *Two cNOS enzymes (eNOS, nNOS) are contrasted by a third, inducible NOS isoform (iNOS), which is typically not expressed in resting cells and must first be induced by certain cytokines, microbial products, or lipopolysaccharides. Constitutively expressed eNOS and nNOS produce small amounts of NO whereas iNOS expression generates large amounts of NO.*

first described in murine macrophages stimulated by LPS (453), and the murine iNOS cDNA was later cloned by three independent laboratories (303, 306, 513). Many other cell types

have since been reported to express an iNOS after cytokine and/or LPS stimulation, and constitutive iNOS expression has been identified in certain epithelial cell types. While constitutive NOS isoforms were relatively easy to demonstrate in humans (216, 358), inducible NO synthesis in a specific cell type was more difficult to find until a strong iNOS expression in cytokine and LPS-stimulated human hepatocytes in culture could be shown (171, 194, 246, 319, 370). Evidence for cytokine-induced NO biosynthesis in humans has been reported in cancer patients receiving interleukin-2 therapy (189, 371) and in patients during sepsis (372).

Since NO production has both beneficial and detrimental consequences, understanding the molecular mechanisms that regulate NOS expression is critical to the control of NO release in homeostatic and pathophysiologic conditions. Furthermore, since endogenous NO production has been demonstrated in nearly every cell type and organ system, it is of little surprise that the regulatory mechanisms controlling NO production are as numerous and as diverse as the roles and functions that NO exhibits (Table 1).

Research to date has shown that regulation of the NOS genes is complex; NOS expression is governed by transcriptional, post-transcriptional/ translational, and post-translational mechanisms. Although the central dogma remains in place that NO production is regulated primarily by ‘constitutive’ expression of nNOS or eNOS, or by ‘inducible’ expression of iNOS, exceptions to this paradigm exist. For example, inducible NOS has been shown to be ‘constitutively’ expressed in certain tissues (171, 194, 246, 319). In a similar manner, although the cNOS protein is constitutively expressed in endothelial cells, full cNOS enzyme activity may depend upon cytokine induction of GTP cyclohydrolase I, the rate-limiting enzyme in the synthesis of the essential NOS cofactor, tetrahydrobiopterin (498). Thus, full activity of cNOS relies upon inducible mechanisms.

TABLE 1

THE THREE ISOFORMS OF NITRIC OXIDE SYNTHASES			
SPECIFICITY	ENDOTHELIAL (eNOS)	NEURONAL (nNOS)	INDUCIBLE (iNOS)
Molecular Mass (kD)	135	155	130
Function of NO	anti-aggregant, EDRF	neurotransmitter, neuromodulator, relaxation of smooth muscle	non-specific; host defense
Cofactor Ca^{2+}/calmodulin	dependent	dependent	dependent
Other cofactors: NADPH, FAD, FMN, H_4-biopterin	dependent	dependent	dependent
Stimuli	acetylcholine, bradykinin, serotonin, ATP, shear stress	neuro-excitatory amino acids	proinflammatory cytokines
Mechanism of regulation (+), activation; (-), inhibition)	(+)Ca^{2+}/calmodulin protein interaction, (+)HSP 90, (+)dimerization, (-)phosphorylation	(+)Ca^{2+}/calmodulin protein interaction, (+)dimerization, (±)phosphorylation	(+)dimerization, (+)phosphorylation
Regulation of gene expression (+), activation; (-), inhibition)	(+)shear stress, (+)proliferation, (-)TNF-α (±)estrogens	(+)estrogens	(+)IL-1-β, (+)IFN-γ, (+)TNF-α, (-)TGF-β, (+)AMPc, (+)GMPc, (+)NFκB, (-)NO, (-)glococorticoids
Mechanism of regulation	transcriptional, mRNA stability	transcriptional	transcriptional, mRNA stability
Gene structure	26 exons, 25 introns	29 exons, 28 introns	26 exons, 25 introns
Chromosome (human)	7	12	17
Subcellular site	membrane>>cytosol	cytosol	cytosol>>membrane
NO output	low (pM)	low (pM)	high (μM)

REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS)

The inducible NOS (iNOS) cDNA has been cloned from mouse (303, 306, 513), rat (368, 504), and human cells (68, 153, 196, 437), and is also found in other species. The sequence of the human iNOS reveals a 4145 base pair cDNA containing a 3459 base pair open reading frame which encodes a polypeptide of 1153 amino acids with a calculated molecular mass of 131 kDa. The human iNOS gene is approximately 37 kb in length and is composed

of 26 exons and 25 introns. This genomic structure is similar to that of the human nNOS and eNOS genes suggesting a divergence from a common 'ancestral' NOS gene. The human iNOS gene could be mapped to chromosome 17 at position 17cen-q11.2 (69). As already mentioned, the human nNOS and eNOS genes reside on chromosome 12 and 7, respectively, confirming that the three NOS genes are distinct. The ATG start site at nucleotide 207 conforms to the critical requirements of the Kozak consensus sequence TAGAGATGG which is the usual consensus sequence appearing at the translational start site of most mammalian genes (252). Similar to other NOS isoforms, iNOS contains consensus recognition sites for the cofactors FMN, FAD, and NADPH in the carboxyl half of the protein which have been shown to be important for iNOS enzyme activity. In addition, a consensus calmodulin recognition site is also present.

MAMMALIAN CELLS WHICH EXPRESS THE iNOS	
CELL TYPE	**SPECIES**
Astrocytes	human, mouse
Cardiac myocytes	rabbit, rat, guinea pig
Chondrocytes	human, rabbit
Endothelial cells (aorta, brain, islet, kidney)	human, bovine, pig, rat, mouse
Epithelial cells (gastric mucosa, intestinal, kidney, lung, retina)	human, bovine, pig, rat, mouse
Fibroblasts (skin, mining, lung)	human, rat, mouse
Hepatocytes	human, rat
Islet-β-cells	rat
Keratinocytes	human, mouse
Cupful cells	rat
Macrophages (blood, bone marrow, lung, peritoneum)	human, rat, mouse, bovine
Mesangial cells	human, rat
Neutrophils	human, rat
Ovarian cells	rat
Osteoblasts	human, rat, mouse
Vascular smooth muscle cells	human, rabbit, rat

The calcium-chelating agent EGTA and the calmodulin antagonist trifluoperazine decreased iNOS activity by 50%-65%. These results were especially surprising since cNOS enzymes are calcium/calmodulin dependent, while rodent iNOS contains a tightly bound calmodulin which is not calcium sensitive (74). It is likely that some low level of calcium is required for optimal calmodulin binding to human iNOS.

Activity of iNOS can be achieved after activation of cells with LPS or directly with cytokines. Accordingly, lymphokines produced by murine Th1 cells (IL-1β, TNF-α, IFN-γ) are considered to upregulate (108, 112, 167, 293), whereas those secreted by Th2 cells (IL-4, IL-10, IL-13) will downregulate activation of iNOS (293). Thus, suppression of NO production by IL-4 or IL-10 may attenuate the ability of macrophages to kill various NO-sensitive parasites, such as *Leishmania*, *Toxoplasma*, *or Schistosoma* (152, 489). Several significant exceptions from this general rule have been reported. Thus, IL-4, if added after iNOS has already been activated by IFN-γ, can enhance production of NO by murine macrophages (293). Human monocytes were reported to produce NO *in vitro* in response to IFN-γ and IL-4 (249). IL-4 also stimulates production of NO by the low-producer subpopulation of human monocytes (330) and stabilizes IFN-γ-induced iNOS mRNA in human respiratory epithelium (172). Similar controversies were observed with another well recognized NO suppressor, TGF-β (72, 301, 485). Conflicting experimental evidence has been accumulating on the interference of IL-10 in NO biosynthesis. Although considerable experimental evidence favors the view that IL-10 downregulates NO production (489), data are controversial again. Thus, IL-10 did not affect the *in vitro* formation of nitrite and nitrate by LPS-activated mouse mesangial cells (141) nor did it reduce the *ex vivo* NO production by peritoneal macrophages from mice treated systemically with LPS (389). In addition, neither endogenous nor exogenous IL-10 affected mucosal NO production in a model of inflammatory bowel disease in rats (406). In contrast, IL-10 was found to enhance induction of iNOS mRNA in murine RAW 264.7 cells following incubation with IFN-γ or IFN-γ plus LPS (72). Finally, in murine bone marrow-derived macrophages cultured in the presence of IL-10/TNF-α/IFN-γ similar increases in iNOS expression and nitrite release were observed (87). These data support the notion of Th2-type cytokines not being general macrophage deactivating factors. Rather, the combination of Th1- and Th2-type cytokines will often lead to contradictory results. However, it remains to be elucidated whether such combinations are of significance in immune dysfunctions and diseases.

Vice versa, NO will regulate cytokines production. A concentration-dependent increase of LPS-induced TNF-α secretion by the J774 macrophage cell line has been observed after addition of L-NMMA, which was reversed by addition of L-arginine or the NO donor SIN-1. In accordance, the serum levels of TNF-α from LPS-treated rats were increased in animals pretreated with L-NMMA and decreased in those given L-arginine. Similar data were obtained using RAW 264.7 macrophages (126) or human blood mononuclear cells (127).

iNOS activity is mainly transcriptionaly regulated. Analysis of the first 400 bp of the human iNOS promoter for potentially relevant transcription factor consensus sequences revealed the presence of three interferon gamma response elements (γ-IRE), reported to be involved in IFN-γ-induced gene expression (385), and an NFκB site, conserved within the murine promoter, reported to be involved to cytokine and LPS stimulated gene expression (282, 291),

and a TNF-response element site, which confers responsiveness to TNF-α (281). In addition to these elements already mentioned, consensus motifs for NF-IL6 and a shear stress response element could be identified (369). Whether these elements exhibit a functional role in the transcriptional regulation of the human iNOS gene is still unknown. Analysis of the iNOS-promoter sequence reveals many putative cytokine-response elements which are actively being characterized (Figure 4).

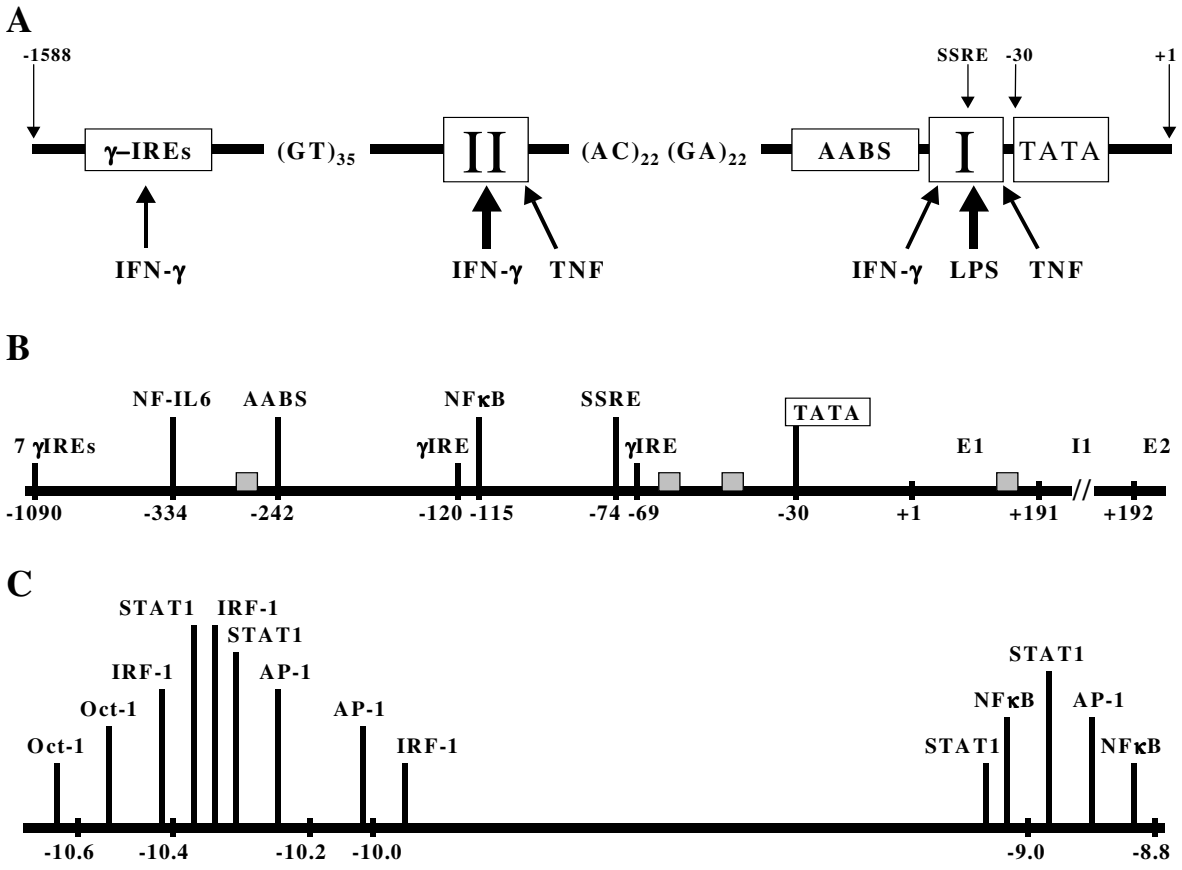


FIGURE 4. Schematic structure of the 5′ flanking region of the inducible nitric oxide synthase and upstream enhancer regions. A and B. *The transcriptional start site is denoted as nucleotide position +1. A likely TATA box begins at –30. TATA-independent iNOS transcripts have alternative splice sites at positions –221, -36, and +191 in the 5′ UTR of the gene. Possible start codons (gray boxes) of open reading frames are located at –256, -65, -45, -40, and +187. Several potential transcription factor binding sites are indicated. The SSRE-box in described only for the human iNOS gene.* ***C.*** *Structure of the distal part of the human iNOS promoter which seems to be a cytokine-responsive enhancer element. This promoter region (-10.9 to –8.7) increases iNOS transcription orientation independently by a factor of 2. It contains multiple binding sites for transcription factors, which are activated in response to either IFN-γ (IRF-1, STAT1) or IL-1β (AP-1, IRF-1).*

γ-IRE, IFN-γ-responsive element; NF, nuclear factor; AABS, activator binding site; SSRE, shear stress responsive element; E, exon; I, intron; IRF, interferon regulatory factor; STAT, signal transducer and activator of transcription. Adapted to Kröncke et al. 1995 (263)

Xie (515) and Lowenstein (302) demonstrated that a 1.7 kb segment of the 5'-flanking region of the murine iNOS gene contains LPS and cytokine responsive promoter elements. Following transfection of deletional iNOS promoter-reporter gene constructs into the macrophage

cell line RAW 264.7, two discrete upstream regions (region I and II) were found to mediate inducibility by LPS and IFN-γ (Figure 4). Region I regulates LPS-induced iNOS expression with specific binding sites for nuclear factor interleukin-6 (NF-IL-6) and transcription factor NFκB/Rel but also contains responsive elements for IFN-γ and an A-activator binding site (AABS) for liver-specific iNOS expression. Region II contains four motifs for binding IFN-regulated transcription factors (327) and an additional NFκB site. Lowenstein *et al.* (302) deleted region I and found that region II by itself has no potential for iNOS induction suggesting that region II has no independent regulatory effect and appears to act primarily as enhancer. Long stretches of GT and AC repeats surrounding region II may form a Z-DNA (515) acting as enhancer. Upstream of region II, LPS inducibility appears to be negatively regulated (302).

Nathan *et al.* (514) identified a functional role for the downstream NFκB element (-86 bp) for LPS activation in murine macrophages. In contrast, the upstream NFκB element was found to contribute to cytokine activation of the iNOS promoter in rat vascular smooth muscle cells (369). Nathan's group also showed that an upstream IRF-E (-913 and -923 bp) bound the nuclear factor IRF-1 and contributed to IFN-γ-dependent transcriptional regulation (327). The requirement for the transcription factor IRF-1 was confirmed in macrophages from mice with a targeted disruption of the IRF-1 gene where iNOS mRNA was barely detectable following stimulation (222). In other studies by Murphy and Russell, the upstream region (region II) functioned in an orientation and position-independent manner consistent with the features of a classical enhancer element (10). An interferon-γ-activated site contained within region II was necessary for full expression of the murine iNOS gene (147). The same group reported a transcriptional basis for hyporesponsiveness of the iNOS gene promoter in human macrophages to LPS and IFN-γ (530). While the controversy over iNOS expression in human macrophages remains (100, 357), it is clear that human macrophages have the ability to produce NO under certain inflammatory conditions (205, 280, 364, 490). There are two explanations for the hyporesponsiveness of the human macrophage iNOS promoter: 1) the presence of multiple inactivating nucleotide substitutions in the human counterpart of the enhancer element which regulates LPS and IFN-γ-induced expression of the murine iNOS gene, and 2) an absence of one or more nuclear factors in human macrophages that are required for maximal expression of iNOS (530). Using *in vivo* footprinting of LPS-stimulated RAW 264.7 macrophages, Goldring (164) reported inducible protein occupation of numerous murine iNOS promoter elements, including NFκB, IRF-1, Oct, and NF-IL6.

Equally important to knowing what upregulates iNOS expression is knowing what turns it off. Glucocorticoids inhibit induced NO synthesis in several cell types (360). Thus, dexamethasone decreases iNOS mRNA levels in hepatocytes (154). This effect is a result of decreased iNOS transcription due to the ability of dexamethasone to upregulate IκB expression

and inhibit cytokine-stimulated NFκB activity (241). TGF-β also partially prevents induced NO synthesis. Suppression of iNOS expression by TGF-β has been demonstrated in macrophages (107), mesangial cells (393), and cardiac myocytes (407). In rat aortic smooth muscle cells, TGF-β decreased IL-1β-stimulated iNOS mRNA by decreasing iNOS transcription (388) and promoter activity (387). In primary murine macrophages, this inhibition occurs at the post-transcriptional level and not by a direct effect on transcription (485). Interestingly, TGF-β actually enhanced induction of iNOS mRNA in Swiss 3T3 fibroblasts (161, 240). These results with dexamethasone and TGF-β underscore the complexity of iNOS regulation and support the notion that species-, tissue-, and cell-specific mechanisms are likely to be important in controlling iNOS expression.

Additional to transcriptional control, post-transcriptional regulation has also been identified for the iNOS gene. TGF-β suppresses macrophage iNOS expression by decreasing iNOS mRNA stability and translational efficiency, and by decreasing stability of the iNOS protein (485). While dexamethasone decreases iNOS transcription, it also exerts post-transcriptional effects by increasing iNOS mRNA stability in IL-1β stimulated rat mesangial cells (272) and vascular smooth muscle cells (388). The precise mechanisms for these post-transcriptional effects, however, have not been elucidated yet.

IMPACT OF iNOS-DERIVED NITRIC OXIDE IN HUMAN DISEASES

INFECTIOUS DISEASES

Soon after the discovery of immunobiological activities of NO it was suggested that this molecule plays a role in the host defense against various infections (89, 215, 225, 361). Indeed, there are good indications for a key role for NO in the human defense against adverse factors from the environment. Inhibition of growth of *Leishmania major* by murine macrophages is solely dependent on NO (17, 292). Similarly, killing of certain intracellular pathogens, such as *Francisella tularensis* (12), *Legionella pneumophila* (455), *Naegleria fowleri* (139), *Toxoplasma gondii* (2), *Trypanosoma cruzi* (351), and many others (96, 215) is largely mediated by NO. For other pathogens, such as *Listeria monocytogenes*, NO is required but not suficient to kill the bacteria (464) and inhibition of *Mycobacterium avium* growth by macrophages is influenced by both NO-dependent and NO-independent mechanisms (14, 28). On the other hand, L-arginine-dependent mechanisms were found ineffective in *Salmonella typhimurium*, *Pseudomonas aeruginosa*, or *Staphylococcus epidermis* (418). Furthermore, chemically generated NO or its derivatives (11) as well as NO derived from macrophages (58), microglial cells (39), or astrocytes (279) had been shown to be fungistatic or fungicidal. Recently, the potent fungicidal activity of Amphotericin B could be linked to an enhancement of the NO-dependent pathway with strong increases in iNOS expression and activity (459).

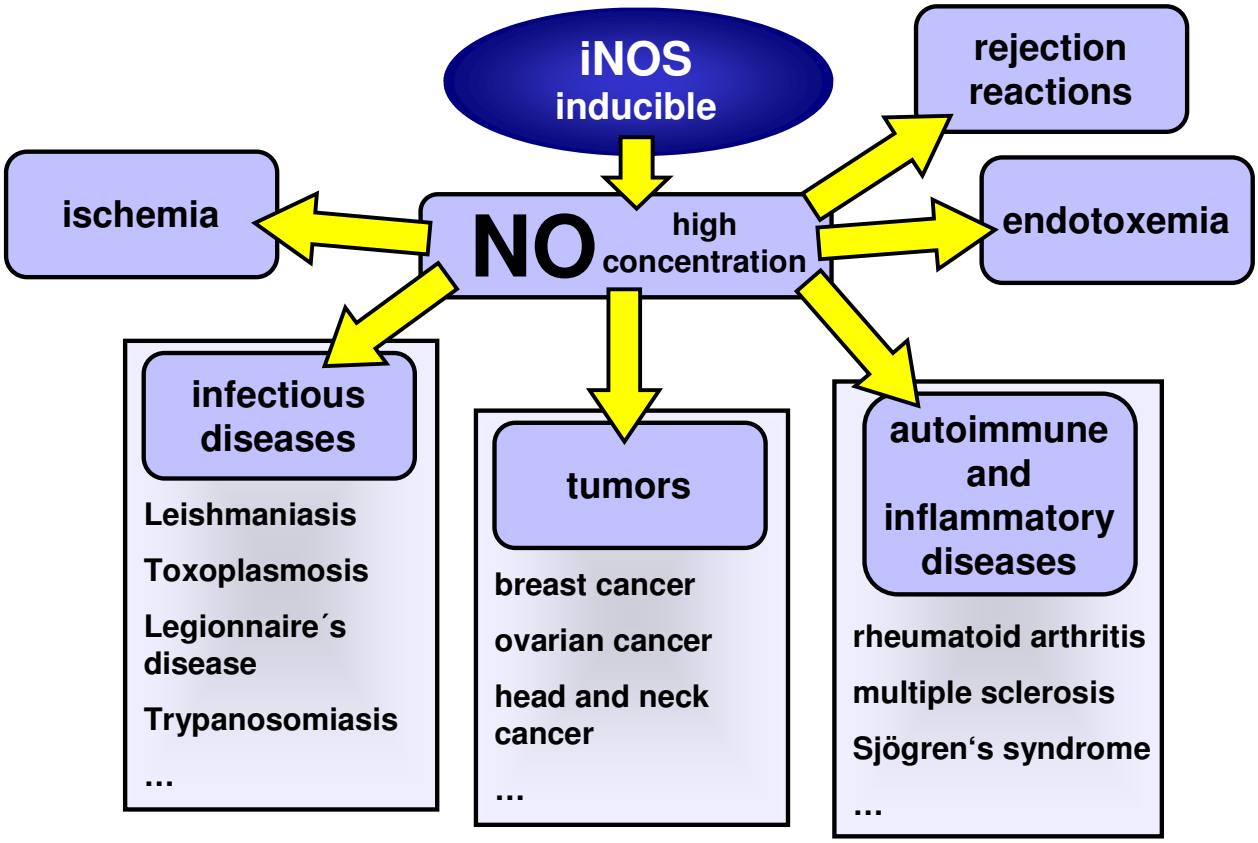


FIGURE 5. Impact of iNOS-derived NO in human diseases. *NO demonstrates its key role in the human defense against adverse factors from the environment by inhibiting bacterial growth and viral replication and by killing certain intracellular pathogens. Further, many chronic inflammatory diseases are associated with sustained iNOS expression, e.g. rheumatoid arthritis, multiple sclerosis, or Sjögren's syndrome. Although numerous in vitro and in vivo studies suggest that endogenous NO synthesis inhibits tumor growth and metastases, recent data support the view that iNOS-derived NO promotes rather than inhibits growth and progression of solid tumors. Tumor cell expression of iNOS has even been found to correlate with the grading of some human tumors, such as breast, ovarian and head and neck cancer. In addition, iNOS-derived NO also plays an important role in ischemia, endotoxemia, and rejection reactions.*

The replication of many viruses can be inhibited by NO and/or its products under *in vitro* conditions (89, 225), but not necessarily *in vivo* (275). For example, although multiplication of vaccinia virus was suppressed *in vitro* (177, 224, 225, 336), inhibitors of NO synthesis did not influence the *in vivo* virus replication or the outcome of infection (408, 409).

Not all viruses appear to be sensitive to the virucidal activity of NO as for instance, induction of NO was ineffective in altering the replication of tick-born encephalitis virus in murine macrophages (254).

Some viruses are able to stimulate production of NO in infected cells, without any suicidal consequences, but with deleterious effects to the host. Many neurotrophic viruses, such as rabies virus or borna disease virus, stimulate NOS activity in the central nervous system and such enhanced NO production may contribute to a variety of neurotoxic and neurodegenerative symptoms, most probably via the disrupting effects of NO on the blood-brain barrier (45). L929 cells spontaneously produce low levels of NO but this activity has no influence on en-

cephalomyocarditis virus replication. However, NO donors inhibit the virus growth in these cells in a dose-dependent manner, albeit via mechanisms other than cytotoxic or direct virucidal effects (170). Direct stimulation of NO production by herpes simplex virus type 1 (HSV-1) infection of human monocyte-like cells U937, widely used in the virus infection studies, did not interfere with their *in vitro* replication; moreover, the replication remained uninfluenced by NO donors or NO inhibitors (300). Other virus infections of these cells can be controlled by NO as documented by the NO-mediated resistance of U937 to parvovirus H-1 infection (300). In contrast, HSV-1 was unable to trigger endogenous NO production in a murine cell line, while its *in vitro* replication could be inhibited by IFN-γ-induced NO synthesis (89). Thus, the spontaneous production of NO, mediated either directly by the host cells or induced by a virus, does not guarantee antiviral protection.

NITRIC OXIDE IN AUTOIMMUNE AND INFLAMMATORY DISEASES

Since the initial notions about deleterious roles for iNOS-derived NO in inflammatory responses, most notably in sepsis, researchers have gathered a wealth of information on iNOS expression in many different human diseases (264, 346). At the beginning of this research the scientific community agreed on the negative contribution to the disease processes, but later this view was modified as more and more evidence for a protective role emerged (497), beginning with the report on NO abrogating the TNF-α-mediated toxicity in endothelia (105). Moreover, it has also become apparent that high-output NO synthesis serves to limit inflammatory reactions (359). On the other hand, however, many chronic inflammatory diseases are associated with sustained iNOS expression representing an apparent contradiction to NO as a factor contributing to limit such reactions. These cases raise the question of whether the iNOS expressed in affected organs and cells is really actively producing NO *in vivo*.

iNOS expression has been described in rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren's syndrome, and many other chronic human inflammatory deseases [for review see (264)]. In active demyelinating lesions of MS patients, macrophages were found to positively stain for iNOS protein (21, 95) and nitrotyrosine (197), at the time thought to be indicative of nitrosative stress. Type-I diabetes, the most prevalent human immune-mediated disease, is the result of > 90% destruction of the pancreatic islet mass. Data concerning iNOS expression during early human prediabetic stages do not exist, but excellent animal models (BB rats and NOD mice) are available which spontaneously develop diabetes closely resembling the human disease. In these animal models, iNOS protein has been detected in islet infiltrating macrophages during early disease stages (239, 416).

iNOS expression has also been found in chronic inflammatory diseases of the airways, the vessels, the bowels, the kidney, the heart, the skin and the apex of teeth. In these various diseases iNOS immunoreactivity has sometimes been localized to macrophages but in most

cases is found associated with epithelial cells around inflammatory foci. In the brain a number of inflammatory mediators, such as cytokines, oxygen free radicals, and NO contribute to the pathogenesis of experimental allergic encephalomyelitis (EAE) and multiple sclerosis (MS) (179). Expression of iNOS mRNA and NO production are enhanced in the central nervous system and correlate with disease severity in animals after induction of EAE (310, 375, 431). In post-mortem biopsies of brains from patients with Alzheimer's or Parkinson's disease, inducible NOS gene expression has been demonstrated in vascular, glial, and neuronal structures (503) and iNOS protein was found (204, 486, 494). Additionally, excessive iNOS expression has been observed in patients with inflammatory bowels diseases (IBD), like ulcerative colitis (UC) and Crohn's disease (CD) (46, 304, 337, 420).

As stated before, iNOS-expression during human inflammatory or autoimmune diseases was afflicted with a negative, disease-promoting role for a long time. However, more recent data apparently demonstrate that the role of the iNOS in human diseases has to be seen more differentiated. For example, rat islet cells are extremely susceptible to NO-induced cell death (259, 267, 450) whereas isolated human islets are also prone to NO-caused cell death, albeit higher concentrations are needed (128). Moreover, the human islet cells can be activated to express iNOS mRNA and to produce NO *in vitro* without any apparent negative effects on their viability (86, 129, 481). Interestingly, a defective iNOS gene expression in the spontaneously diabetic NOD mouse does not significantly alter the course of cytokine-driven β-cell apoptosis (294). In the EAE model, pharmacological manipulation by means of NO inhibitors does not always appear rewarding in this disease. Thus, 7-nitroindazole, a selective inhibitor of nNOS, was found to be only partially effective or ineffective to alleviate disease severity *in vivo* (431). Additionally, application of aminoguanidine, a relatively selective iNOS inhibitor was either ineffective (431) or aggravated disease progression (532) while the non-specific NOS inhibitor N^G-nitro-L-arginine methyl ester either exacerbated disease symptoms or was of no effect (417, 532). Regarding the role of epithelial iNOS activity in chronic inflammatory diseases of the airways as mentioned above, the NO produced by epithelial cells may either serve to limit bacterial invasion or local immune reactions and concomitant tissue destruction during Th1-type immune responses. Further, experiments using animal models indicate that inhibition of inducible NO production decreases gastrointestinal injury induced by LPS (467), peptidoglycans (168), 1,3,5-trinitrobenzene-sulfonic acid (195), or acetic acid (402). These observation, however, do not exclude that at certain stages of disease development NO may excert beneficial effects for instance via reduction of leukocyte infiltration (332). Indeed, NO has been shown to protect mucosa from ischemia reperfusion injury (270), and in accordance with a possible protective action, again inhibitors of NO synthesis remained ineffective or enhanced the development of inflammation (338, 339, 390).

NITRIC OXIDE IN TUMOR GROWTH

Numerous *in vitro* and *in vivo* studies suggest that endogenous NO synthesis inhibits tumor growth (93, 190, 454, 470) and metastases (111, 510-512). High-output NO synthesis has been identified as the intrinsic mediator of BCG-induced host resistance against progression of experimental syngeneic and xenogeneic tumors (134). Although the most important anti-tumor effector cells are macrophages (88, 192, 360), other cell types, such as endothelial cells, also acquire tumoricidal activity (133, 288, 289) after appropriate stimulation for high-output NO production. NO donors, such as the NO-releasing vasodilator isosorbide, may exhibit anticancer and anti-metastatic activity as well (394). However, evidence is now accumulating that NO will promote rather than inhibit growth and progression of solid tumors (81, 295, 471, 472). Thus, tumor cell expression of iNOS has been found to correlate with the grading of some human tumors, such as breast (116, 472), ovarian (471) and head and neck cancer, and with enhanced metastatic activity of breast cancer (116). In addition, malignant central nervous system neoplasms express high levels of NOS activity which may be associated with pathophysiological processes determining their growth (81). The potentiating effect can be contributed to the formation of carcinogenic N-nitroso compounds (296), the risk of which may be enhanced under the conditions of sustained elevation of NO formation, occurring in various persistent inflammatory diseases (374) and in certain viruses (295) or other infections (182). Relationship between endogenous nitrosation and the development of cholangio carcinoma in humans also has been reported (182, 374).

In accordance with these recent data, inhibitors of NOS activity are reported to reduce tumor size and metastatic potential (122, 473). For example, ω3-polyunsaturated fatty acids which were shown to reduce iNOS-mediated NO formation (373) are known to reduce the risk of colon cancer in both man (397) and rat (341). In mice, arginine analogues have been shown to block the promotional phase of neoplastic transformation of mouse fibroblasts (348), but on the other hand, they potentiate pulmonary metastasis of Lewis lung carcinoma and B27 melanoma cells (516).

Angiogenesis is an essential prerequisite for the progression of solid tumors. NO has been shown to exert endothelial growth inhibition (394, 419), however, other experiments demonstrate NO-mediated increases in angiogenesis (531).

The effect of NO on tumor cell growth as well as on normal cell growth regulation is variable depending on the cell type and cell cycle stage, being either inhibitory (150, 151, 165, 342, 362, 401, 520) or stimulatory (181, 251, 352, 531). Furthermore, it has been shown that regulation of growth may depend on the local NO-concentration with enhanced cell proliferation at low NO concentrations, and growth arrest combined with strongly promoted differentiation at higher NO levels (257). One of the participating factors stimulated by NO may be cyclic GMP (cGMP). cGMP is well recognized to promote lymphocytic immune responses. It

has been observed that cGMP stimulates cell growth via the activation of the AP-1 transcription complex, whereas NO predominantly inhibits cell cycling at late steps of growth factor signaling, without dependence on cGMP (430).
Some data suggest that the NOS pathway may be one of the processes that mediate cytotoxicity of natural killer cells (NK). The underlying essential cytokine for the NK activity induced by viable BCG was found to be IFN-γ. Insufficient NO formation activity after inactivation of BCG was due to the inability to induce IFN-γ (518). It has been found that human NK effector mechanisms which cause target cytolysis have a requirement for L-arginine and are closely associated with the accumulation of nitrite and citrulline. Additionally, the NO inhibitor N^G-mono-methyl-L-arginine (L-NMMA) inhibits NK cytolytic activity (509). A prerequisite for NO was also found for lymphokine activated killer cell (LAK) activity in the case of cell stimulation with IL-1. NOS activity gradually increases during LAK generation in parallel with the increasing capability of IL-1-activated NK cells to lyse NK-resistant target cells (78). It was observed that NO inhibitors prevented LAK generation from cultured splenic cells, and generation failed in the absence of L-arginine. In addition, sodium nitroprusside an agent which releases NO, was able to overcome the blocking effects of NMMA. However, NMMA did not affect generation of LAK cells from human peripheral blood cells (220).

This chapter has stressed how complex the impact of NO in various diseases is and that data published so far are still contradictory. Thus, our current knowledge is obviously insufficient to predict whether a disease therapy would benefit from using a selective iNOS inhibitor or might rather profit from exogenously added NO. Various methods to inhibit iNOS have been established to elucidate a protective versus destructive role of NO during various stresses. Hence, the next chapter will focus on different techniques for inhibiting iNOS activity to analyze the effects of NO deprivation on cellular functions.

MECHANISMS TO INHIBIT iNOS ACTIVITY

iNOS INHIBITORS

NO synthases, including iNOS, can be inhibited by a wide variety of drugs that interfere with the catalytic functions of these enzyms (Figure 6). However, many of these drugs are either cytotoxic (33) or do not function as specific iNOS inhibitors blocking the two constitutively expressed members of the NOS family also (9, 62). To some degree, iNOS inhibitors may also interfere with other metabolic pathways (see later chapters).

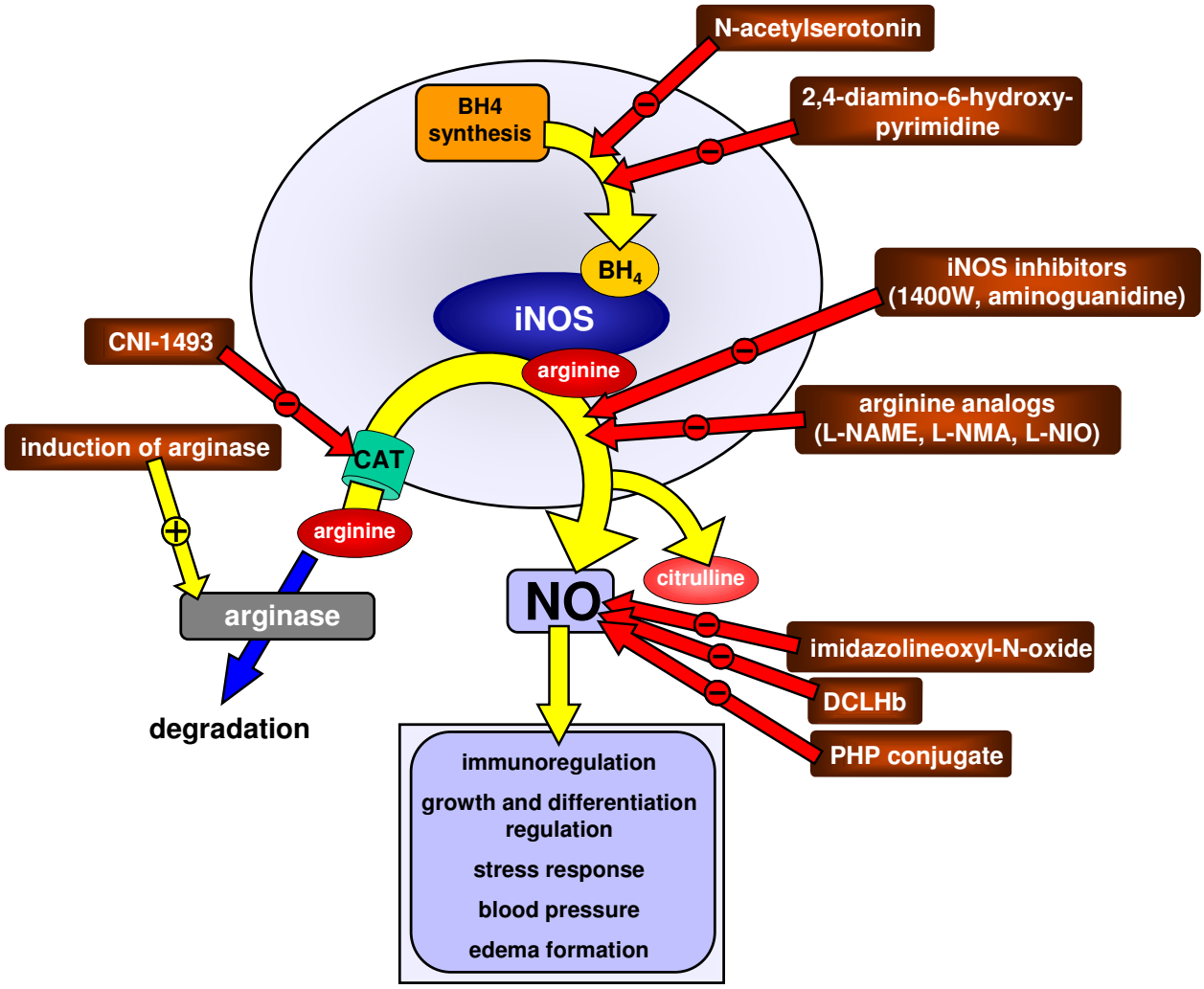


FIGURE 6. Mechanisms to inhibit iNOS activity. *iNOS can be inhibited by a wide variety of drugs that interfere with the catalytic functions of the enzyme. The relatively selective enzyme inhibitors 1400W and aminoguanidine are possible inhibitors of iNOS. Arginine analogs like L-NAME, LNMA, or L-NIO also allow to inhibit iNOS. However, another interesting approach is to limit the availability of L-arginine, either by induction of arginase, or by blocking the cytokine-inducible cationic amino acid transporters (CATs), for instance with CNI-1493, a tetravalent guanylhydrazone and competitive inhibitor of cytokine-inducible L-arginine transport. Since tetrahydrobiopterin (BH_4) is an essential cofactor for all NOS isoforms, BH_4 inhibitors like 2,4-diamino-6-hydroxy-pyrimidine and N-acetylserotonin allow to inhibit NO production. The hydroxy-pyrimidine compound inhibits guanosine triphosphate (GTP) cyclohydrolase I, which is a rate-limiting enzyme involved in BH_4 biosynthesis, and N-acetylserotonin inhibits sepiapterin reductase and thus BH_4 synthesis via this pathway. Another approach is the use of NO scavengers which either rapidly convert NO into less active compounds or bind it so that it cannot exert its widespread actions. Imidazolineoxyl-N-oxide represents such a scavenger molecule selectively reacting with NO. Many investigations concerning NO scavengers have used cell-free hemoglobin molecules and investigations are currently focussing on the two products diaspirin cross-linked hemoglobin (DCLHb) and pyridoxalated hemoglobin poloxyethylene conjugate (PHP). These agents appear to be promising alternatives but current clinical evaluations still need to proof their potency.*

Some authors point out that using iNOS inhibitors in *in vitro* experiments does not serve as a model for *in vivo* studies due to the highly complex *in vivo* system involving several different NO-producing sources. Although the pharmacokinetic properties of these drugs may not be optimal for iNOS inhibition, several excellent reports using the relatively selective enzyme inhibitor 1400W have shown efficacy, for instance in neuroprotection following experimental transient focal ischemia with a therapeutic window of 18 hours (381). High doses of the weak

but relatively iNOS-selective inhibitor aminoguanidine have demonstrated significant potential for reducing infarct size after vessel occlusions (206, 207, 529).

However, the use of iNOS-specific inhibitors revealed a number of disadvantages and side effects: 1) none of these drugs is absolutely specific, evident especially at higher doses, since even most selective inhibitors like 1400W are not completely discriminative at higher concentrations; 2) some inhibitors may affect other cell functions, e.g. arginine transport (43); 3) under certain conditions NO can be produced non-enzymatically from NOS inhibitors (349), thereby producing misleading results; 4) chemical iNOS inhibitors do not allow the study of specific cell types in complex systems.

DEPRIVATION OF ARGININE

Most investigations on iNOS inhibition focus on the development of L-arginine analogs. However, another interesting approach to inhibit iNOS is arginine depletion. Availability of L-arginine as a substrate for NO is critical for the NO production rate (392). *In vitro* studies for example have shown that L-arginine application (or administration) results in an increase of the NO oxidation products nitrate and nitrite, and enhances phagocytosis as well as contractile functions of cardiac myocytes (271, 343).

Two approaches to limit the availability of L-arginine have been investigated so far, induction of arginase or blockade of cytokine-inducible cationic amino acid transporters (CATs) (Figure 6). Arginase is the enzyme responsible for degrading L-arginine, therefore its induction may decrease production of NO by reducing intracellular L-arginine concentrations (65). Theoretically, inhibitors of CATs would act as selective iNOS inhibitors. It has been shown that CNI-1493, a tetravalent guanylhydrazone and competitive inhibitor of cytokine-inducible L-arginine transport, prevents acute inflammation and endotoxin-induced lethality in mice while maintaining vascular NO function (30). In human peripheral blood mononuclear cells, CNI-1493 besides reducing NO production suppresses the release of proinflammatory cytokines (113). However, side-effects of arginine depletion or inhibition of CATs are still unknown but likely to occur.

DEPRIVATION/INHIBITION OF iNOS COFACTORS

Tetrahydrobiopterin (BH_4) is an essential cofactor for all NOS isoforms. Interferon-γ and TNF-α both enhance the *de novo* synthesis of BH_4 in macrophages, fibroblasts, and endothelial cells, thereby promoting NO synthesis. It has also been shown that an increase in the availability of BH_4 is necessary to ensure a sufficient supply for iNOS-generated NO production. Thus, levels of BH_4 may regulate the formation of NO in various tissues (392). Two chemicals have been studied as BH_4 inhibitors, 2,4-diamino-6-hydroxy-pyrimidine and N-acetylserotonin (Figure 6). The hydroxy-pyrimidine compound inhibits guanosine triphos-

phate (GTP) cyclohydrolase I, which is a rate-limiting enzyme involved in BH_4 biosynthesis, and N-acetylserotonin inhibits sepiapterin reductase and thus BH_4 synthesis via this pathway (392). The disadvantages of BH_4 inhibitors are the side effects, since BH_4 is also used by other enzymes, e.g. phenylalanine hydroxylase or tyrosin hydroxylase.

NITRIC OXIDE SCAVENGERS

The main effect of NO scavengers is that they either rapidly convert NO into less active compounds or bind it so that it cannot exert its widespread actions. Imidazolineoxyl-*N*-oxide represents such a scavenger molecule selectively reacting with NO. By converting it to NO_2, the biological effect of NO is abrogated (311), but the resulting NO_2 is probably more toxic than NO.

Many investigations concerning NO scavengers have used cell-free hemoglobin molecules, hemoglobin being a natural NO scavenger (8). To date, investigations are focussing on the search for a safe and reliable hemoglobin substitute. The two products referred to most frequently are diaspirin cross-linked hemoglobin (DCLHb) (131, 491) or pyridoxalated hemoglobin poloxyethylene conjugate (PHP) (355, 400) (Figure 6). Although these agents appear to be promising alternatives, current clinical evaluations will have to proof their potency.

ANTISENSE-MEDIATED GENE KNOCK-DOWN TO INHIBIT iNOS ACTIVITY

A relatively new approach to inhibit iNOS activity involves antisense (AS)-mediated gene knock-down which, at least in theory, provides a highly specific, rapid, and potentially high-throughput method for inhibiting gene expression and thereby also allowing for exploration of gene function (442). This fascinating concept of blocking the expression of a single gene by using AS-oligodeoxynucleotides (ODN) is based on studies in the late 1960s proving that synthetic AS-ODN indeed act in a sequence specific manner. The first attempt to prepare synthetic ODN targeting a defined gene sequence was performed in 1967 by Belikova (24), and Zamecnik and Stephenson (525) were the first to propose a possible therapeutic applications of ODN. In 1978, they demonstrated effective inhibition of Rous Sarcoma Virus replication in infected chicken fibroblasts by adding synthetic ODN directed against a specific viral genome sequence. At that time, such innovative trials were limited by the availability of synthetic ODN and by the low number of genes that had been sequenced so far. Therefore, automation of ODN synthesis was an important step towards a broader availability of AS technology. A relevant topic at the time and still discussed today, was the protection of ODN against their normally rapid degradation. Thus, the development of ODN analogs resistant to cellular breakdown represented an additional step towards the applicability of AS-ODN. Today, the principal fields of application are the investigation of gene function by loss-of-function or decrease-of-function analyses and the development of AS drugs for therapeutic

applications. One possibly promising approach in this direction is the use of iNOS-specific AS-ODN.

TASK OF ANTISENSE OLIGONUCLEOTIDES

In contrast to chemical inhibitors, the AS technique does not primarily affect the protein and its enzymatic activity. AS-ODN are designed to selectively switch off gene expression at the level of or prior to translation. Specific interference with the ribosomal translation of the encoded protein at the mRNA level is thought to be the most frequent effect of AS-ODN. Several studies provide evidence for a selective translational arrest by showing that AS-ODN block the synthesis of the relevant protein without reducing the level of the corresponding mRNA (44, 52, 424). However, two main other mechanisms of AS inhibition have also been proposed: ODN directed to pre-mRNA may interfere with RNA maturation by inhibiting polyadenylation and transport into the cytoplasm (232) (Figure 7, B2), or they may activate RNase H leading to specific cleavage of the mRNA (Figure 7, C4) (90, 193, 443). Indeed, there are several reports stating that the mRNA of the targeted protein is decreased as confirmed by Nothern blot or PCR analysis [for iNOS mRNA decrease after application of AS-ODN see (16, 109, 110)]. Ideally, AS-treated cells develop a deficiency for one specific protein only, while other proteins are not affected and are normally transcribed.

Generally, any gene with a known sequence may be targeted by AS-ODN. The AS technique offers an interesting approach especially in the setting of closely related members of a gene family or for enzymes that cannot be specifically inhibited due to homology problems. Inhibition of the iNOS provides an excellent example to demonstrate the helpfulness and usefulness of the specific inhibition by AS-ODN. Although the AS technique is superior in many ways to other methods of iNOS inhibition, there are also problems, obstacles, and limitations that need to be considered.

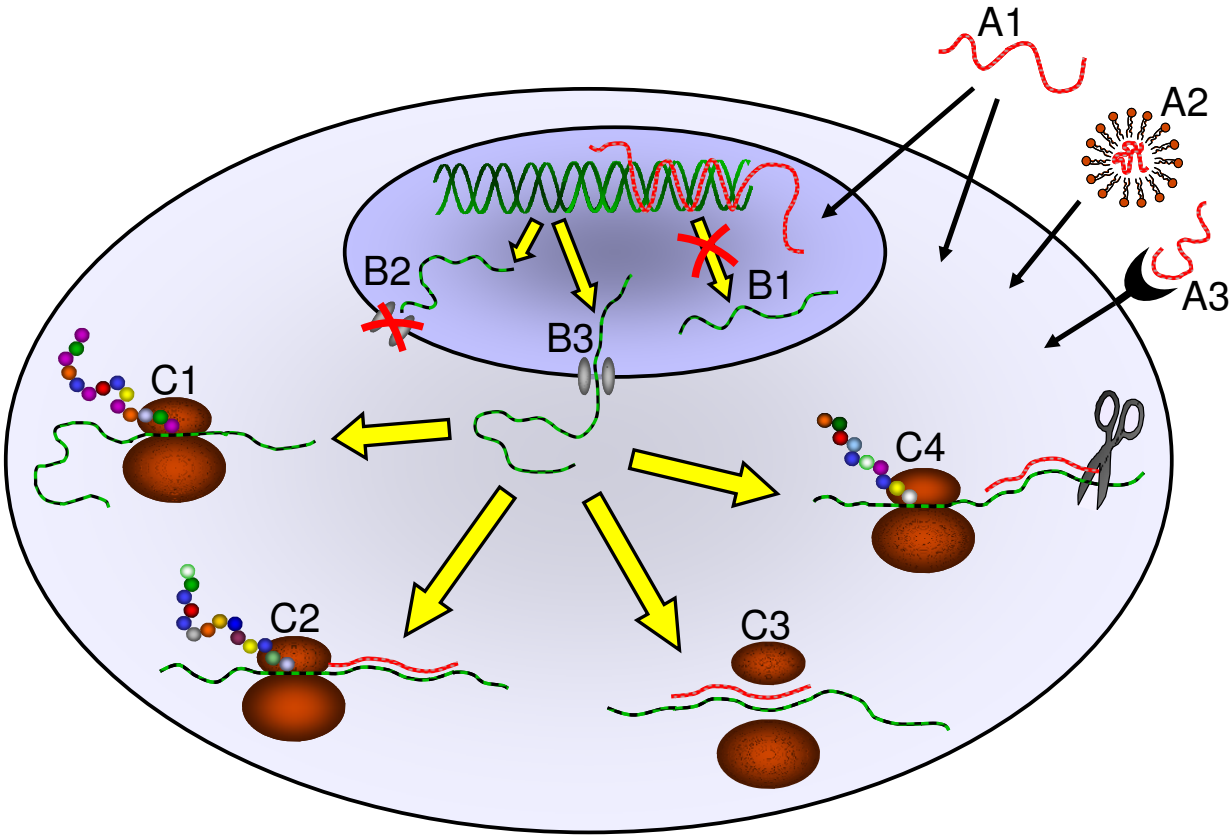


FIGURE 7. Task of antisense oligonucleotides. *The mechanism of how ODN enter cells is not yet completely understood. It is reported by some authors that ODN can enter cells without the application of transmembrane vehicles (A1). However, many other studies state that acceptable results are gained only by using lipids (A2) or similar uptake enhancers. There are also studies on receptor-mediated endocytosis of ODN (A3). ODN either stay in the cytoplasm or they enter the nucleus. In the nucleus, ODN most likely form a triplex with nuclear/cellular DNA thereby inhibiting the generation of mRNA (B1). On the other hand, ODN may prevent polyadenylation and translocation of mRNA from the nucleus into the cytoplasm (B2). If the process of mRNA generation in the nucleus is not affected (B3), the mRNA leaves the nucleus through nuclear membrane pores and enters the cytoplasm. In the absence of ODN, translation would take place at the ribosomes and proteins would be synthesized (C1). In the presence of AS-ODN, several different mechanisms may inhibit translation: ODN directed against the coding region of the mRNA can arrest protein formation by interfering with protein chain elongation (C2) or by inhibiting the initial constitution of the ribosomal complex (C3). It is also reported that the mRNA can become a substrate for the enzyme RNase H, which recognizes the duplex of the ODN and the mRNA resulting in the cleavage of the mRNA strand while leaving the non-cleavable ODN intact (C4).*

OBSTACLES IN ANTISENSE TECHNIQUE

Unfortunately, AS-ODN do not necessarily always function as AS molecules silencing the respective sense sequence. Depending on their sequence, ODN may act as ribozymes with catalytic activity that cleave target mRNA (120). If the ODN sequence resembles a sense sequence of the cellular genome, ODN may also bind transcription factors thereby trapping nucleic acid binding proteins resulting in less specific inhibition of translation (31). Other sequence-specific effects of ODN are: G-quartets have been reported to be antiproliferative and inhibit cell-cell as well as virus-cell interactions. CpG-motifs, in which the CG residues are flanked by two purins on the 5'-end and two pyrimidines on the 3'-end, induce activation

of Toll-like receptor 4-expressing cells (31) and cause immunological reactions (256). Finally, palindromic sequences of 6 or more bases induce type 1 and type 2 interferon production (517). All these examples demonstrate that the search for a proper ODN sequence is a prime task when planning AS experiments. ODN sequences for AS experiments regarding iNOS are listed in Table 7.

In order to prevent degradation and enhance biological activity of synthetic ODN, both their sequence and also their structure are of major importance. Although unmodified phosphodiester oligos offer the advantage of excellent solubility in aqueous solutions and good hybridization characteristics, their use is limited due to rapid degradation by exo- or endonucleases (435). There have been several attempts to modify ODN structure [for review see (6)]. Phosphorothioates in which a non-bridging oxygen is substituted by a sulfur (121) are the most often used type of ODN *in vitro* and *in vivo*. The product of this substitution retains the characteristics of unmodified ODN such as net charge and solubility. Additionally, stability and nuclease resistance are significantly increased *in vitro* and *in vivo* (435, 449) relative to unmodified ODN (492, 493). However, the sulfur substitution converts the ODN into a biologically active molecule different from the unmodified equivalent. Phosphorothioate ODN (PS-ODN) are inhibitors of human DNA polymerases (148) and due to their polyanionic character they can bind to proteins such as heparin binding proteins (136, 173). The affinity of PS-ODN for such proteins is largely sequence-independent (448). As a consequence, PS-ODN of the appropriate length may block the binding of heparin-binding proteins, thereby causing unwanted and biologically relevant effects. Since absolute sequence specificity is not attainable using ODN with phosphorothioate modifications, it is therefore essential to optimize the length of the ODN and, more importantly, to minimize their concentration (442). A too long or too short ODN loses some of its specificity. The minimal length of a particular mRNA sequence specific for one species only out of the entire mRNA population is between 11-14 units in length (160). From this calculation, a relatively short ODN can be used for specific binding to a single mRNA or its gene. Depending on temperature, ionic composition of the system as well as base composition, an ODN of 14 to 20 bases offers optimal specific hybridization properties (160). Longer sequences may cause reduced specificity (188) but increased toxicity due to inhibition of different genes, or they may trigger a cascade of cellular antiviral responses (318). Another factor which can affect binding is the secondary structure of the target or the ODN itself influencing the interaction between the AS-ODN and its target nucleic acid. Since the mRNA secondary structure can be predicted to a certain extend only, the efficiency of AS-ODN has to be determined in practice as shown by Blake et al. (38).

During the last few years, a new group of molecules besides the traditionally used ODN has entered the market: Peptide nucleic acids (PNA) (146, 365, 396) [for iNOS-related experiments see (73, 162)] in which an uncharged pseudo-peptide backbone consisting of N-(2-

aminoethyl)glycine units linked to the purine and pyrimidine bases of natural nucleic acids replaces the complete desoxyribose phosphate moiety (162). This stuctural modification results in a decreased electrostatic repulsion allowing the formation of a stable PNA-DNA hydrogen-bonded double helix (71, 99, 123, 366). Helical structures like that provide specificity of interaction, high thermal stability, and resistance to nucleases and proteases (98). In summary, there are obvious general principles in designing AS-molecules (size, sequence targeted, chemical modifications to increase stability and half-life), but numerous studies indicate that so far there are no definite rules to predict activity in a biological system (340).

UPTAKE AND INTRACELLULAR DISTRIBUTION OF OLIGONUCLEOTIDES

Entering the target cell is a prerequisite for AS action. Polyanions are only poorly taken up by mammalian cells. Investigations using intact cells have demonstrated an active uptake mechanism of polyanionic ODN. This process is mediated by an 80-kDa surface-receptor protein (299) of which the natural function is still unknown. Several authors describe significant ODN incorporation in the absence of transmembrane carriers (16, 166, 367, 405), but many more reports state the necessity of or the improvement by uptake enhancers (157, 276, 286, 329). Table 2 shows a list of the methods most often used to improve ODN uptake. Enhancing delivery to the intracellular target is still an area with comparatively small progress. Preliminary studies suggest that cholesterol conjugation to ODN improves delivery to target RNA (7, 284). *In vitro*, cationic lipid formulations have been shown to improve intracellular delivery of charged ODN by functioning as a nucleotide-transporter, protecting ODN from degradation by nucleases (59) and by increasing the gene-silencing effect (25, 82).
However, in most cases, the combination of chemically modified ODN and synthetic lipid formulations shows toxicity thereby lowering the effectiveness of AS experiments. Furthermore, the *in vivo* applicability of these formulations has yet to be demonstrated. Lipid vehicles will alter the tissue distribution significantly (92) due to preferential uptake by reticuloendothelial cells. An attempt to bypass this has been described by Huang et al. (92, 145) using stealth liposomes. In other approaches, ODN conjugated to a receptor ligand have been applied (507).
Currently, liposomal systems have preferentially been used as uptake enhancers (see Table 2) but some more recent studies show them in the context of stable transfection as well (1, 211). Coincubation of ODN with Lipofectin, one of the widely used lipid formulations, has been reported to increase cellular uptake significantly thereby improving the inhibitory effect of the AS-ODN in cell culture (25, 82) (Figure 17D, 17E), often with the toll of increased non-specific cytotoxicity (521). However, in primary rat aorta endothelial cells, we found Lipofectin to be a suitable and - more important - relatively nontoxic lipid for AS-mediated inhibition of iNOS mRNA expression (185, 186) (see Figure 8)

TABLE 2

METHODS OF ODN DELIVERY	COMMERCIAL SOURCE
Liposomal Systems	
CellFECTIN	Invitrogen
Clonfectin	Clontech
Cytofectene	Biorad
DC-Chol	Sigma
DDAB	Sigma
DMRIE-C	Invitrogen
DOSPER	Roche
DOTAP	Avanti Polar Lipids
Effectene	Qiagen
Escort	Sigma
FuGENE 6	Roche
GeneJammer	Stratagene
GenePorter	GTS
LipofectACE	Invitrogen
LipofectAMINE Plus	Invitrogen
LipofectAMINE 2000	Invitrogen
Lipofectin	Invitrogen
LipoTAXI	Stratagene
Metafectene™	Biontex
MRX-230 and MRX-220	Avanti Polar Lipids
Oligofectamin	Invitrogen
Polyfect	Qiagen
ProFection-CaPO4	Promega
Profection-DEAE-Dextran	Promega
Superfect	Qiagen
Tfx-10, Tfx-20 and Tfx-50	Promega
TransFast	Promega
Transfectam	Promega
Biodegradable pH-Sensitive Surfactants	
Dendrimers	
Transport-enhancing Peptides	
Electroporation	
Nanoparticles	
Microinjection into the nucleus	
Particle delivery by gun shot	

Besides liposomes, there are several other promising delivery systems like the application of transmembrane carriers like dendrimers, transport-enhancing peptides or polymeric nanoparticles and the use of “transfection guns” to shoot ODN into cells, a procedure called biolistics (242, 331, 350), or electroporation (49, 79, 297, 398, 524) (see Table 2). Polymeric nanopar-

ticles theoretically offer advantages for *in vivo* trials. ODN can be delivered after adsorption to macromolecules like lactose- or polyalkylcyanoacrylate-polymers. These formulations are stable, protect ODN from nuclease degradation and therefore allow for a more efficient uptake [for review see (219)]. However, their usefulness for delivery, their effectiveness

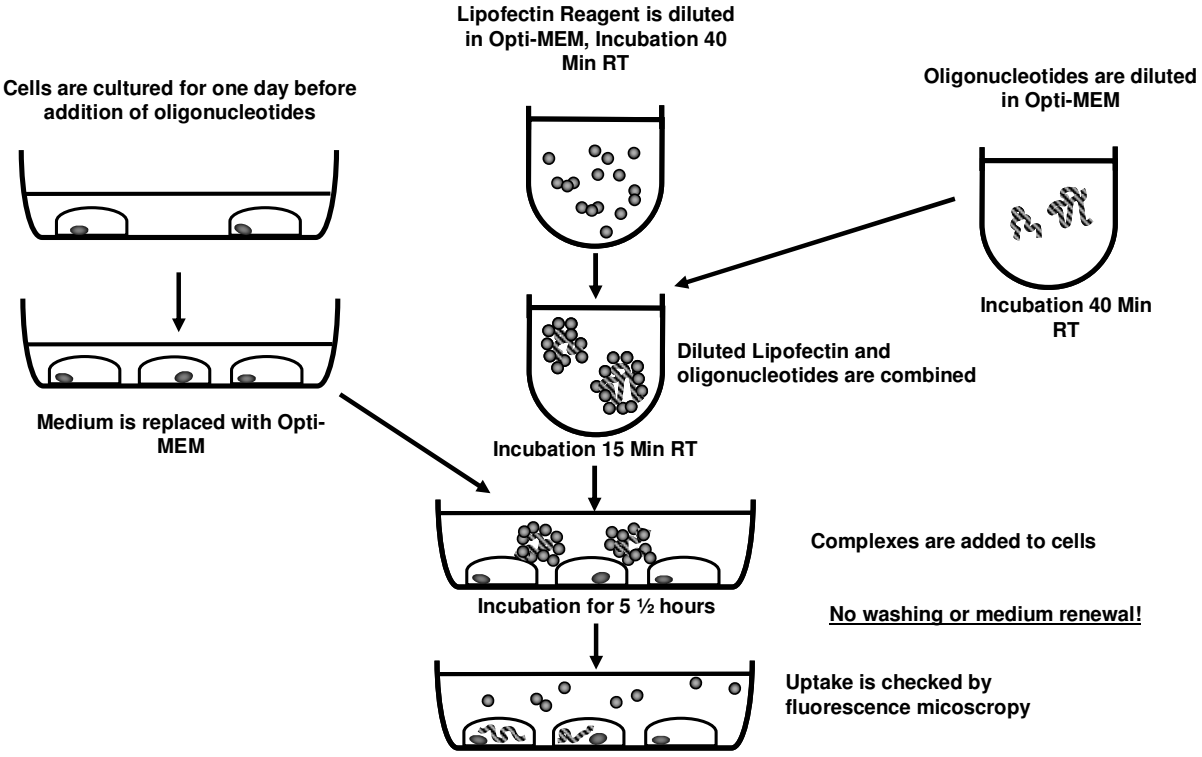


FIGURE 8: Incorporation of oligonucleotides with the uptake enhancer Lipofectin. *ODN are coated with previously diluted Lipofectin to facilitate ODN-incorporation. When complexes have formed, the mixture is added to cell cultures. In contrast to the recommendations of the manufacturer, we do not perform a washing step or medium renewal before adding the prepared complexes since this reduces the extend of inhibition.*

in inhibiting gene expression, and their properties concerning a possible immune response require further examination. Although there are many reports of successful ODN delivery using transmembrane vehicles (19, 25, 59, 276), several problems are connected with the use of these carrier systems. For instance, ODN may not be adequately targeted to relevant intracellular compartments and thus may stay ineffective. We found that AS-ODN to iNOS would only inhibit NO production if they translocate into the nucleus, whereas accumulation in the cytoplasm does not lead to the desired effects (186). On the contrary, other reports state that retention in the *cytosol* determines the activity of an AS-ODN (508). One explanation for this apparent contradiction might be that there are two distinct intracellular locations in which inhibition occurs (444), one of which is nuclear, RNase H-dependent, and can occur at relatively low ODN concentrations applied, whereas the other is cytoplasmic, largely RNase H-independent, requiring relatively high intracellular ODN concentrations (444).

With lipids as uptake enhancers, both uptake and intracellular trafficking of ODN depend on the type of liposome, the charge of the ODN-liposome complex, and the conditions of micelle formation and cell incubation (276). It is often stated that the medium used during ODN incorporation markedly influences the uptake efficacy. Therefore, most authors use fetal calf serum (FCS)-free medium to improve ODN-uptake (16, 32, 34). We found that FCS strongly inhibits ODN transport into the nucleus thereby impeding the inhibition by AS-ODN (186). Besides general problems with lipids as delivery system, these may also have their own impact on the function to be suppressed by AS-ODN. Thus, lipids can induce the gene to be inhibited, which may lead to a less obvious AS success. Indeed, our experiments showed that the cationic lipid Lipofectin enhances the expression of iNOS mRNA and protein as well as increases enzyme activity if added as empty vehicles to cytokine-activated cells (186) (Figure 22, Figure 23). Bilecki et al. (32) found that Lipofectin significantly increases the effectiveness of AS-ODN to decrease iNOS-mediated NO production, but when given to cells without ODN, this vehicle increases the LPS/IFN-γ stimulated iNOS protein synthesis. Interestingly, this enhancing effect is abolished by loading vesicles with ODN, irrespective of their sequence (186). LipofectAMINE, another transfection reagent frequently used, also results in increases of iNOS expression and here, this increment is not abrogated by the addition of ODN (185).

Insufficient uptake, wrong intracellular distribution or weak suppression of iNOS protein expression and NO formation are obstacles which are overcome by generating stably transfected cells containing an iNOS-specific AS-construct (61). However, this method does not allow to study the impact of iNOS activity in primary cells and thus remains of limited use.

NECESSARY CONTROLS IN ANTISENSE EXPERIMENTS

An integral part of every AS experiment are appropriate controls (446). Control ODN are required to differentiate between effects that are caused by a sequence-specific AS mechanism and effects due to sequence- and substance-related side-effects. Negative and positive controls are generally the two types of controls that should be used in AS experiments. Negative controls are normally designed in a way that they do not specifically hybridize to any intracellular target, and serve to ensure that the AS-ODN does not cause remarkable side-effects in the treated cells or organism. A positive control ODN, i.e. an ODN directed to a different sequence of the target mRNA, may provide additional evidence that the observed biological effects are due to a true AS effect.

The most widely used and accepted types of negative controls are the following:

1. Sense controls with a sequence complementary to that of the AS-ODN do not hybridize and thus, no specific effect should derive from this control ODN. However, some sense sequences may represent AS sequences for other genes.

2. Randomized controls, also termed “nonsense” or “scrambled ODN”, are often used, derived by mixing the AS-ODN nucleotides in a randomized fashion. This control has the advantage that a random sequence can be generated that will not interact with other targets.
3. Reverse controls are generated by reversing the AS sequence with respect to its 5’-3’ orientation. These controls bear the same hazard as described in (1.)
4. Mismatch controls are obtained by purposely introducing one or more mismatches into an AS sequence.
5. Empty vehicles or transfection reagent are rarely performed but important controls as they check for unwanted side effects (see previous chapter).

All of the control ODN bear the potential hazard of not being really “negative”. As cell culture does not allow for a restrictive temperature, pairing despite mismatches is likely to occur. Indeed, this obstacle represents a general problem, both in AS-techniques as well as in the siRNA method as has been observed recently (423).

IMPACT OF iNOS-DERIVED NITRIC OXIDE ON CELLULAR STRESS RESPONSE GENES

NO is synthesized by iNOS in the cellular cytosol and transported out of the cell in a way poorly characterized. Since the NO scavenger hemoglobin, which only acts extracellularly, inhibits many NO effects, NO appears to act in a paracrine manner. NO in target cells has been shown to affect transport proteins, ion channels and the plasma-membrane potential, to inhibit the mitochondrial respiration and membrane potential, to destruct metal-sulfur-clusters (Fe-sulfur-clusters, Zn-finger domains) in proteins, to inhibit enzymes with free SH-groups at or near the active centre, and to mediate DNA damage leading to activation of poly(ADP-ribose)polymerase in the nucleus (260). This wide range of actions of the molecule NO is responsible for the high number of genes under NO regulation. Microarray studies which have been performed within the last years further confirm the extensive regulatory role of NO in gene expression (125, 184, 526).

NITRIC OXIDE-MEDIATED GENES: BCL-2, HO-1, VEGF

In the context of endothelial inflammation, examples for stress response genes are bcl-2, HO-1, and VEGF. These genes have been chosen for the studies here due to their affirmed NO-mediated control. For all three proteins, expressional increases by NO have already been shown at the mRNA as well as at the protein level (117, 142, 421, 460, 526). bcl-2 expression is important for inhibiting apoptosis onset (460), up-regulation of HO-1 has been demonstrated to protect during reactive oxygen intermediates-mediated stress (115, 377),

and vascular endothelial growth factor (VEGF) expression is essential for endothelial regeneration after injury (156, 269, 478). Using bcl-2, VEGF, and HO-1 as examples, experiments were performed to correlate the gene expressional control of iNOS-derived NO with endothelial dysfunction and cell death during oxidative stress response (see studies).

BCL-2 FAMILY PROTEINS – GATEKEEPERS OF MITOCHONDRIAL FUNCTION

The bcl-2 family of proteins plays a pivotal role in the regulation of cell death and cell survival. Some proteins of the family such as Bax, Bad, Bid, Bak, bcl-xL, Bik, Bim, Hrk, and *C. elegans* Egl-1 promote cell death while others inhibit cell death including bcl-2, bcl-xL, bcl-w, Mcl-1, A1 (227). bcl-2 family members form homo- and hetero-dimers and the balance between anti- and pro-apoptotic bcl-2 proteins may dictate the response to an apoptotic signal. They can function either independently or in concert (70, 245, 313, 528).

The mechanism by which bcl-2 related proteins exert their function is still unclear. Many bcl-2 family members are anchored by a hydrophobic carboxy-terminal transmembrane domain in mitochondrial and other intracellular membranes. bcl-2 or bcl-xL can prevent the release of cytochrome c from mitochondria, providing a possible mechanistic explanation for their cell survival properties (228, 231, 243, 519). Conversely, Bax, a pro-apoptotic bcl-2 family protein, induces cytochrome c release in intact cells as well as from isolated mitochondria *in vitro* (132, 137, 221, 411). Bax induced cytochrome c release can be blocked by bcl-xL or bcl-2, but not by caspase inhibitors (132, 137, 221, 411). In living cells, Bax resides in the cytosol as an inactive monomer. When a cell receives a pro-apoptotic signal, Bax translocates to mitochondria and integrates into the membrane as a dimer (169, 200, 502). However, the mechanism of Bax translocation is still unclear. It is feasible that Bax undergoes a conformational change which allows for insertion into the mitochondrial membranes (101).

Hints of how bcl-2 family members may exert their function come from three-dimensional structure analysis of bcl-xL which reveal striking similarities with the pore forming domains of bacterial cholera or diphtheria toxins. Furthermore, bcl-2, bcl-xL, and Bax can form ion conducting channels in artificial membranes (13, 20, 425, 426). Other features of bcl-2 with respect to mitochondrial function have been proposed. bcl-2 and bcl-xL enhance proton export from mitochondria and increase mitochondrial Ca^{2+} buffering capacity (20, 438). Moreover, Bax-induced cell death in yeast and in mammalian cells appears to involve a functional Fo/F1-ATPase proton pump (328).

The mechanism by which bcl-2 family proteins control cell survival and death has still to be elucidated but might be related to both, their ability to form ion channels and to form complexes with other proteins.

REGULATION OF BCL-2 BY NITRIC OXIDE

NO is capable of interacting with apoptosis signaling in multiple ways. The variety of interactions of NO include the inhibition of caspase activity by S-nitrosation of the catalytic cysteine residue (105, 287), maintenance of sustained bcl-2 expression in B lymphocytes (155), and a cGMP-independent anti-apoptotic pathway (231). A more recently discovered mechanism is the down-regulation of MAP kinase phosphatase-3 (MKP-3) mRNA levels and, thus, the maintenance of extracellular signal-regulated kinase (ERK)1/2 phosphorylation by NO, which contributes to the protective effects of NO in endothelial cells (412). By stabilizing ERK1/2 phosphorylation via down-regulation of MKP-3 mRNA levels, NO provides constant phosphorylation of the ERK1/2 target bcl-2. Prevention of bcl-2 degradation subsequently prevents the release of cytochrome c from mitochondria and protects cells from apoptosis (Figure 9).

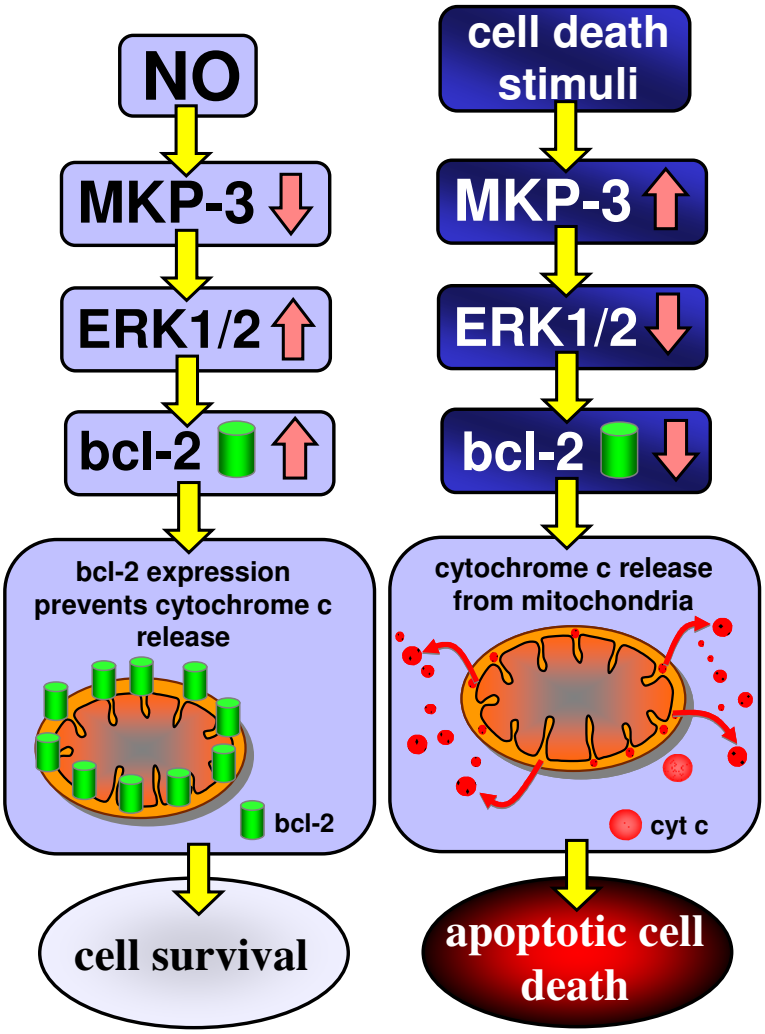


FIGURE 9: Regulation of bcl-2 expression and cell survival by NO. *NO maintains ERK1/2 phosphorylation via down-regulation of MKP-3 mRNA levels, thereby providing constant phosphorylation of the ERK1/2 target bcl-2 which prevents bcl-2 degradation and, subsequently, the release of cytochrome c from mitochondria.*

ERK1/2, extracellular signal-regulated kinase; MKP-3, MAP kinase phosphatase-3; cyt c, cytochrome c

HEME OXYGENASE-1

Heme oxygenases (HOs) are the rate-limiting enzymes in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide (CO), and biliverdin. Three

distinct variants of HOs have been cloned (314). HO-1 is an inducible enzyme, whose synthesis is elicited by inflammatory stimuli (175, 441). By contrast, HO-2 is constitutively expressed and is concentrated mostly in the brain and testes, accounting for most HO activity in the brain (482). HO-3 is also largely localized to the brain, although its enzymatic activity is lower than that of other isoforms (333).

The HO-1 isoform is inducible by stimuli such as cytokines, heavy metals and oxidants that induce inflammatory damage. Induction of HO-1 has been implicated in numerous clinically relevant diseases including lung injury, endotoxic shock, hypertension, atherosclerosis, ischemia/reperfusion after organ transplantation and others.

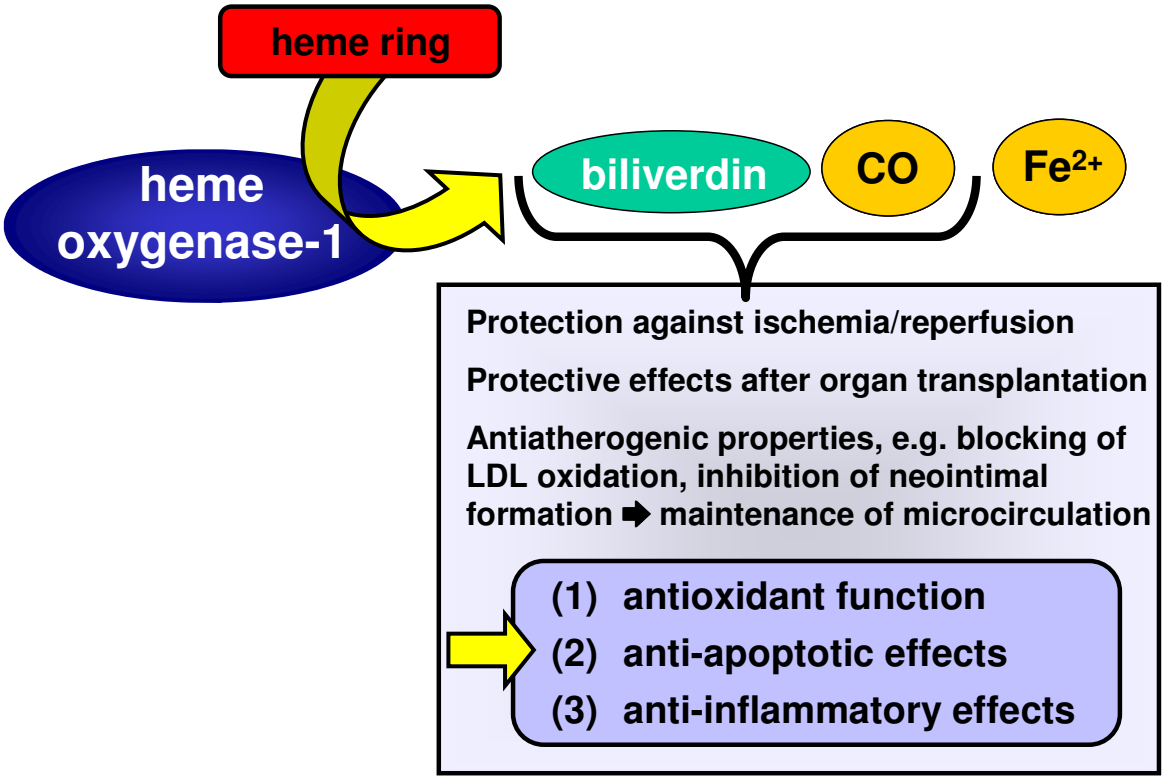


FIGURE 10: Role of heme-oxygenase-1 (HO-1). *Heme oxygenases are the rate-limiting enzymes in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide, and biliverdin. Carbon monoxide and biliverdin function as antioxidants and show anti-apoptotic and anti-inflammatoriy effects. Furthermore, HO-1 and its products reveal antiatherogenic properties by blocking LDL oxidation and by inhibiting neointimal formation.*

HO-1 protects agains atherosclerosis and vascular injury in various ways. It is assumed that HO-1 blocks LDL oxidation thereby minimizing the negative effects of oxidized lipids in plaques, e.g. induction of apoptosis in macrophages and smooth muscle cells, expression of procoagulant and inflammatory genes, and DNA damage (26). Further, HO-1 expression in endothelial cells is protective against vascular stenosis and proliferation (114) since HO-1-induced CO production blocks neointimal formation elicited by arterial injury (475). Antiatherogenic properties of HO-1, however, are not only mediated through CO, but also through the action of bilirubin which inhibits vascular endothelial activation and dysfunction in response to proinflammatory stresses (226). Nevertheless, HO-1-based therapies directed at

the unstable plaques must be closely monitored, as this enzyme could interfere with the normal wound-healing meachnisms involved in plaque repair after sporadic rupture.
The cytoprotective effect of HO-1 is also seen after organ transplantation with enhanced endogenous HO-1 overexpression, and HO-1 downstream mediators (bilirubin, CO), protecting against the ischemia/reperfusion injury (IRI) sequel (3). Indeed, HO-1, which functions to amplify multiple intracellular cytoprotective pathways, may serve as a novel therapeutic concept in transplantation to maximize organ donor pool through their safer use despite prolonged periods of cold ischemia (477).
In summary, HO-1 can be considered a protective player in the vascular response to injury due to a) antioxidant function, b) maintenance of microcirculation, c) anti-apoptotic function, and d) anti-inflammatory effects (210, 429) (Figure 10).

REGULATION OF HEME OXYGENASE-1 BY NITRIC OXIDE

NO induces HO-1 expression in various cell types by a cGMP-independent pathway (37, 119, 176, 178, 233, 237, 323, 461, 462, 501). Both transcriptional and post-transcriptional mechanisms have been implicated in this induction. One study using sodium nitroprusside, an NO donor, concluded that the induction was principally at the transcriptional level, since no change in the stability of HO-1 mRNA was observed (461). A similar conclusion was drawn by Durante et al. (119), who demonstrated that different classes of NO donors increased HO-1 gene transcription 3–6-fold in vascular smooth muscle cells without a significant change in the half-life of HO-1 mRNA. On the other hand, contribution of both transcription and increased mRNA stability were found for vascular smooth muscle cells treated with a NO-donor compound (178) and for human fibroblasts treated with pure NO gas (323). Demple and his colleagues demonstrated that NO-induced stabilization of the HO-1 message occurs immediately in response to NO, with quick disappearance of stabilization after NO exposure ceases (323). Since modulation of HO-1 mRNA stability was found to be rapid and independent of RNA or protein synthesis, they assume that a stable protein already present in the cell may modulate HO-1 mRNA turnover in response to NO (48). One possible candidate is the iron regulatory protein-1 (IRP-1), which regulates iron metabolism at a post-transcriptional level in mammalian cells (47). Once activated by NO, IRP-1 recognizes specific sequences called iron-response elements (IREs) on several mRNAs (e.g. those encoding ferritin, and the transferrin receptor) (35, 64).
In conclusion, it is likely that changes in HO-1 mRNA stability contribute to induction of the enzyme activity of HO-1, but the exact mechanism can vary with the cell type and the NO exposure conditions.

VASCULAR ENDOTHELIAL GROWTH FACTOR

Two sequential mechanisms are mainly responsible for vessel formation (60), vasculogenesis and angiogenesis. *De novo* formation of blood vessels during embryonic development is called vasculogenesis. Mesoderm-derived stem cells (hemangioblasts) form aggregates (blood islands) and develop into primitive hematopoietic and endothelial cells (angioblasts) which then proliferate and differentiate *in situ* to form a primitive network. Angiogenesis, on the other hand, is the formation of new capillaries from preexisting vessels. The principle mechanism of vessel formation in adults is angiogenesis. This process is tightly regulated and required for a number of physiological processes, such as wound healing, ovulation and menstruation as well as embryonic development. Excessive angiogenesis is seen in a wide range of diseases including tumors, diabetic retinopathy, psoriasis, and rheumatoid arthritis. Most of embryonic vessels and proliferating endothelial cells under angiogenesis express receptors for VEGF, suggesting that VEGF plays a key role in vasculogenesis and angiogenesis.

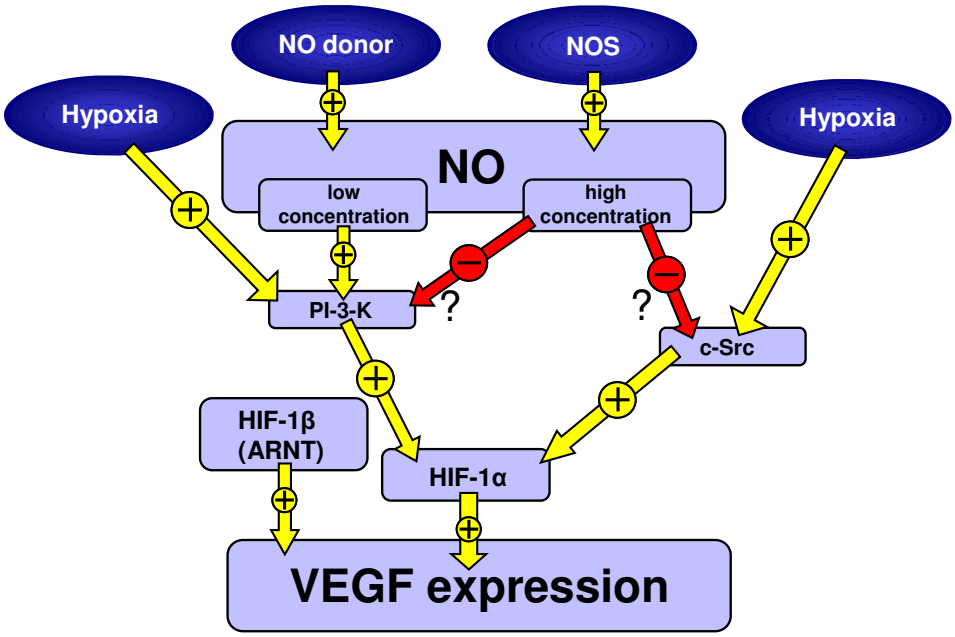


FIGURE 11: Mechanisms of VEGF upregulation by NO and hypoxia. *An optimal amount of NO may upregulate the VEGF gene expression probably through a PI3K-Akt pathway in limited cell lines while an excessive amount of NO inhibits the VEGF expression through an unidentified pathway.*

REGULATION OF VEGF EXPRESSION BY NITRIC OXIDE

A number of angiogenic stimuli have been found to induce VEGF expression, such as cytokines, hormones, phorbol esters, oncogenes, transitional metals, iron chelator and hypoxia (238). Hypoxia is a key inducer of VEGF *in vitro* and *in vivo* (Figure 11), whose mechanisms have been extensively investigated. Inducibility by hypoxia is conferred by the hypoxia response element (HRE), which is located within the 5' promoter of the *VEGF* gene. Compared with HREs of erythropoietin and several glycolytic enzyme genes, these sequences reveal a high homology and similar protein-binding characteristic as hypoxia inducible factor 1 (HIF-

1). HIF-1 is composed of two distinct subunits, both of which belong to the basic helixloop-helix-per-arnt-sim protein family, HIF-1α and HIF-1β. HIF-1α was found as a novel protein, but HIF-1β was identical to aryl hydrocarbon receptor nuclear translocator (ARNT) (495). Transcriptional activation of the VEGF gene is dependent upon HIF-1 binding activity and its protein level (433), although mRNA stabilization under hypoxia is also important for increase in VEGF expression (208, 285). Recently, NO has been reported as an inducer of VEGF synthesis under normoxia. It has been shown that NO donors (except SNP) induce VEGF expression by enhancing the HIF-1 binding activity and accumulation of HIF-1 protein in tumor cell lines, independent of a cGMP pathway (235) (Figure 11). Sandau *et al.* (422) demonstrated that endogenous NO released from overexpressed iNOS as well as NO donors provoked HIF-1 accumulation in tubular LLC-PK cells.

REGULATION OF NITRIC OXIDE PRODUCTION BY VEGF

The angiogenic and inflammatory effects of VEGF can be mediated by NO, which is produced by VEGF-activated eNOS in VEC (356, 379, 380). It has been reported that VEGFR-2 plays a major role in angiogenesis, and its autophosphorylation leads to eNOS activation (135, 183, 258, 474). Immediate NO synthesis by eNOS is mediated through the CaM-Akt pathway whereas delayed NO production results from an activation of the PI3K-Akt pathway or from an upregulaton of the eNOS gene through PKC activation (Figure 12).

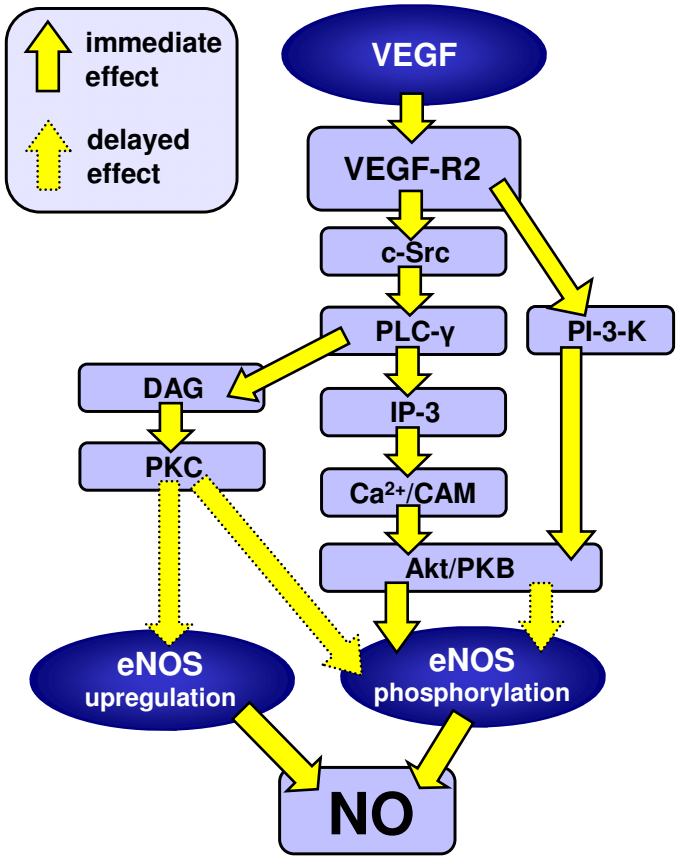


FIGURE 12: Signaling pathways for VEGF-mediated NO synthesis. *VEGF induces immediate NO synthesis through the CaM-Akt pathway. Delayed NO synthesis is mediated by the PI3K-Akt pathway or induced by upregulaton of the eNOS gene through PKC activation.*

RECIPROCAL REGULATION BETWEEN NITRIC OXIDE, NOS, AND VEGF EXPRESSION

As described above, NO at an appropriate concentration induces VEGF synthesis through an HIF-1 mediated pathway, and VEGF enhances NO production by eNOS. These actions may lead to promotion of angiogenesis. However, angiogenesis in normal tissues should be strictly regulated to avoid vascular disaster, and there must be a reciprocal regulation between NO and VEGF. Carbon monoxide (CO) has been reported as a modulator of *VEGF*

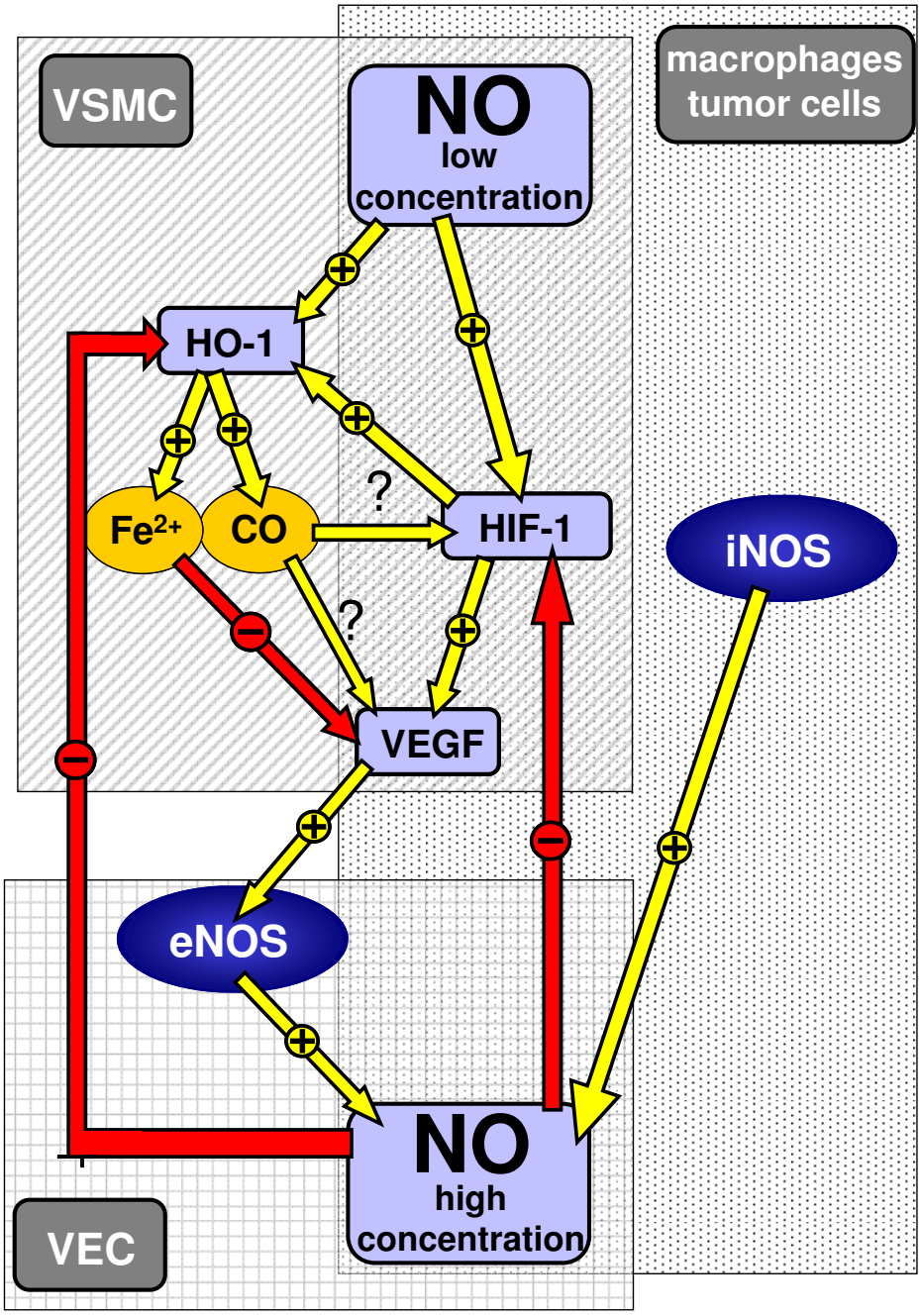


FIGURE 13: Reciprocal relationship between NO and VEGF. *NO is synthesized mainly by eNOS in VSMC and by iNOS in macrophages and tumor cells. NO may modulate positively or negatively VEGF expression through HIF-1 and/or HO-1-mediated pathways.*

expression. Unlike NO, CO is a stable gas, not a free radical, but both gases are endogenously produced. They have similar physiological functions, such as vasodilatation, inhibition of platelet aggregation and neurotransmission, and can act as second messenger molecules

(50, 63). Some authors describe inhibition of HIF-1 and VEGF expression (201, 298) in the presence of high concentration of CO and hypoxia. However, other groups demonstrate that CO at much lower concentrations enhances VEGF expression under normoxia (118, 253, 326), and induces HIF-1α expression in kidneys of rats (410).

As already mentioned above, heme oxygenase is responsible for generating CO and especially HO-1 is inducible after stimulation of cytokines, hypoxia and NO. Dulak et al. (118) demonstrated that cytokine-induced VEGF synthesis in VSMC is dependent upon HO activity, and the HO-derived compounds CO and ferrous ion (Fe2+) (Figure 13). NO itself acts positively or negatively on HIF-1-mediated VEGF gene expression, depending on the concentration. In the vascular wall, a small amount of NO induces activation of the VEGF synthesis in VSMC, and a positive feedback of VEGF leads to more NO production by eNOS in VEC. An excessive amount of NO, in contrast, acts negatively on the VEGF synthesis probably by limiting the HIF-1 activity (Figure 13).
The iNOS is highly expressed in macrophages and tumor cells, and can generate several orders of magnitude more NO than the other constitutive NOS. At first site, it now appears contradictory that tumors have an unregulated angiogenesis since high levels of NO should decrease VEGF synthesis via inhibition of HIF-1 activity. However, it has to be considered that tumor cells are exposed to chronic hypoxia. This effect of hypoxia-mediated VEGF expression via HIF-1 (see Figure 11) is more prominent than the negative feedback of high NO levels inhibiting VEGF gene expression.

THE STUDIES

The topic of this thesis was to establish the antisense method for iNOS gene inhibition and to analyze the relevance of iNOS activity for endothelial stress gene expression.

The first study entitled "**Antisense-mediated knock-down of iNOS expression in the presence of cytokines**" which will soon be published in the journal *Methods in Enzymology* describes in detail methods of how to apply iNOS-specific AS-ODN and also highlights some of the pitfalls of this technique. Especially stressed is the question of how to design AS experiments properly, e.g. how should oligonucleotides be configured, how is ODN incorporation ensured, and how is ODN uptake monitored adequately.

The second study entitled "**Specific iNOS-targeted antisense knock-down in endothelial cells**", published in the *American Journal of Physiology-Cell Physiology*, provides an optimized protocol for AS-mediated knock-down of iNOS. Furthermore, this work presents detailed information on the uptake of fluorescence-labeled ODN, the effects of serum presence during ODN uptake, and the differences between various cationic lipids as uptake enhancers. Although the potency of AS-ODN for blocking iNOS formation is demonstrated, it is also emphasized that application problems of this technique appear when ODN are used in inflammatory settings.

The third work "**iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage**" which has been published in the *Journal of Applied Physiology* has a clinical focus demonstrating that cytokine-induced endogenous iNOS expression and activity have key functions in increasing endothelial survival and maintaining its function. It is shown that AS inhibition of iNOS expression or limited substrate supply as has been reported to occur in atherosclerosis patients, significantly contributes to endothelial dysfunction and death during oxidative stress.

The last study entitled **"What sense lays in antisense inhibition of inducible nitric oxide synthase expression?"** is a review article in revision at the Journal *Nitric oxide - Biology and Chemistry*. In this work, the opportunities, limitations, and experimental problems of iNOS-directed AS-ODN are extensively discussed. Also, all AS experiments since 1993 when first iNOS-directed AS molecules were applied are presented. Furthermore, this review article contains a section about the clinical relevance of AS-ODN and an outlook discussing the question of whether ODN, ds RNA-molecules, PNA, or new structures will be the molecules gaining most attention in the field of iNOS knock-down in the near future.

ANTISENSE-MEDIATED KNOCK-DOWN OF INOS EXPRESSION IN THE PRESENCE OF CYTOKINES

INTRODUCTION

Various methods to inhibit iNOS expression or activity are established that allow for elucidating a protective versus destructive role of high-output nitric oxide synthesis during various stresses. A relatively new method to inhibit iNOS-derived NO production is the antisense (AS) technique which theoretically provides a specific, rapid, and potentially high-throughput method for inhibiting gene expression (442). The concept of blocking the expression of a single gene by using AS-oligodeoxynucleotides (ODN) is based on studies in the late 1960s proving that synthetic AS-ODN indeed act in a sequence specific manner. Today, the principal fields of AS-ODN application are the investigation of gene function by loss-of-function or decrease-of-function analyses and the development of AS drugs for therapeutic applications. In this chapter, we describe in detail methods of how to apply iNOS-specific AS-ODN and we also highlight some of the pitfalls of this technique.

DESIGN OF OLIGONUCLEOTIDES

The AS technique offers an interesting approach especially in the setting of closely related members of a gene family like iNOS and the two other NOS. However, to ensure the specificity of inhibition, the design of the ODN is important. Table 7 gives several examples of AS-ODN and control ODN used for iNOS targeting. However, if you decide to create your own sequence, here are some points that should be considered:

- An ODN sequence resembling a sense sequence of the cellular genome may also bind transcription factors thereby trapping nucleic acid binding proteins resulting in less specific inhibition of translation (31).
- G-quartets have been reported to be antiproliferative and inhibit cell-cell as well as virus-cell interactions.
- CpG-motifs, in which the CG residues are flanked by two purins on the 5'-end and two pyrimidines on the 3'-end, induce activation of Toll-like receptor 9-expressing cells (31).
- Palindromic sequences of 6 or more bases induce α- and γ-interferon production (517).

Concerning length of ODN, it is important to know that the minimal length of a particular mRNA sequence specific for one species only out of the entire mRNA population is between 11-14 units (160). Depending on temperature, ionic composition of the system as well as base composition, an ODN of 14 to 20 bases offers optimal specific hybridization properties (160).

EXPERIMENTAL DESIGN

We here describe the application of AS-ODN for rat aorta endothelial cells (EC). For AS experiments, these cells are best cultured in 6-well tissue culture plates in OPTIMEM I Reduced Serum Medium (GIBCO, Cat. No. 31985) (20% fetal calf serum (FCS)), seeded at a density of 200.000 cells per well and incubated overnight to allow attachment. Next day, culture supernatants are replaced by fresh serum-free OPTIMEM medium and lipid-encapsulated AS-ODN and control-ODN are added. Cytokine activation is then performed and inhibition of NO formation is confirmed on mRNA, protein, and nitrite level 24 hours later.

A) PROCEDURE FOR ODN INCORPORATION

ODN are normally shipped as lyophilized DNA-Na salt (25 or 100 nmol). The pellet may stick to the cap of the tube, therefore start with a short spin in a microcentrifuge prior to opening. To produce a 100 µM ODN solution, add 100 µl of 1xTE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) per 10 nmol of ODN and vortex briefly. ODN are then ready to use or should be stored at -20°C.

To ensure ODN incorporation, lipid vehicles should be used. There are reports on ODN incorporation in the absence of transmembrane carriers (16, 367), however, most authors underline the necessity of or the improvement by uptake enhancers, e.g. (276, 329). In terms of uptake and toxicity, best results were observed with Lipofectin® Reagent (GIBCO, Cat. No. 18292-011). LipofectAMINE® Reagent (GIBCO, Cat. No. 18324-012) is another frequently used lipid which also allows for good and long-lasting nuclear uptake of ODN. However, LipofectAMINE reveals significant, concentration-dependent toxic effects and enhances iNOS expression in the presence of cytokines with the net result of no inhibition of nitrite formation at the end of a respective experiment. We also examined FuGENE^{TM} 6 Transfection Reagent (Roche, Cat. No. 1 815 091) but found no ODN uptake under any condition tested. We therefore recommend the use of Lipofectin for iNOS-targeting AS experiments in primary cells. Here is a step by step procedure:

For preparation of lipid-ODN complexes, prepare the following solutions in sterile tubes: Solution A1: 6 µl of the 100 µM AS- and solution A2: control-ODN preparations are diluted separately with 100 µl OPTIMEM medium.

Solution B: For AS-ODN and control-ODN, 2 tubes are prepared and 6 µl of Lipofectin Reagent are diluted with 100 µl serum-free medium. If you decide to make lipid uptake enhancers yourself, it is of note that a Lipofectin solution of 1 µl ml^{-1} has a final concentration of 0.75 µM DOTMA and 0.68 µM DOPE.

Both solutions A and B are allowed to stand at room temperature for 40 min.

Solutions A and B are then combined, mixed by gentle pipetting and incubated for another 15 min at room temperature to allow for micelle formation. Note: If ODN are fluorescence-

labeled for instance with FITC for tracking and confirmation of successful ODN uptake, light-protected tubes are recommended for preparing solutions A and the mixture of solutions A and B.

In the meantime, supernatant of cells cultured over night (8-12 h) should be replaced by 750 µl of fresh serum-free OPTIMEM medium for each well. Note: In contrast to the recommendations of the manufacturer, cells should not be washed prior to medium replacement since this may reduce the final extend of antisense inhibition.

We find that the omission of FCS during ODN uptake is crucial for effective ODN incorporation.

Next, the 200 µl mixture of solutions A and B is gently pipetted in small droplets onto cells and cultures are further incubated at 37°C in a CO_2 incubator.

Due to the uptake kinetics of Lipofectin-encapsulated ODN (see Figure 14), cell activation by cytokines (IL-1β, TNF-α, and IFN-γ, each at 1000 U ml^{-1}) should be performed 5.5 h after addition of lipid-ODN-complexes. We analyzed mRNA, protein detection, and Griess assays 24 hours after cytokine activation since rat cells show maximum activity at this time point. Other species may have different kinetics. Cytokines should be premixed in a total volume of 50 µl to give a final volume of 1 ml in every well after cytokine addition.

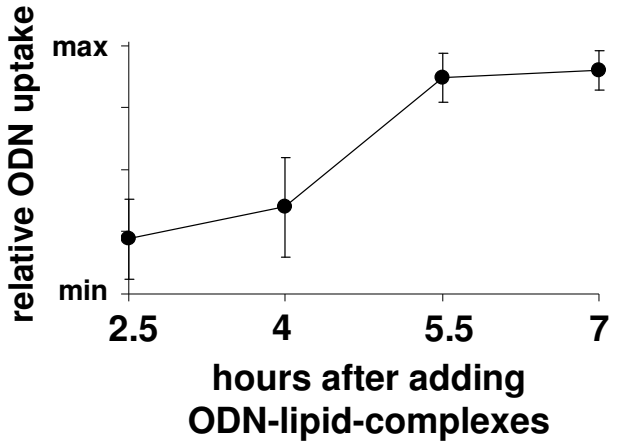


FIGURE 14: Lipofectin-mediated ODN uptake. *The kinetics of Lipofectin-mediated ODN uptake in endothelial cells were analyzed during incubation in Serum Reduced Medium using FITC-labeled ODN. There is a sudden increase in ODN incorporation between 4 and 5.5 hours after addition of ODN-lipid-complexes.*

B) CONTROL FOR ODN UPTAKE

To microscopically ensure successful incorporation of ODN, we recommend to use AS-ODN with FITC-labels (Biognostik, Göttingen, Germany). This allows for confirmation of ODN uptake and accumulation in the nucleus before adding cytokines (see Figure 15).

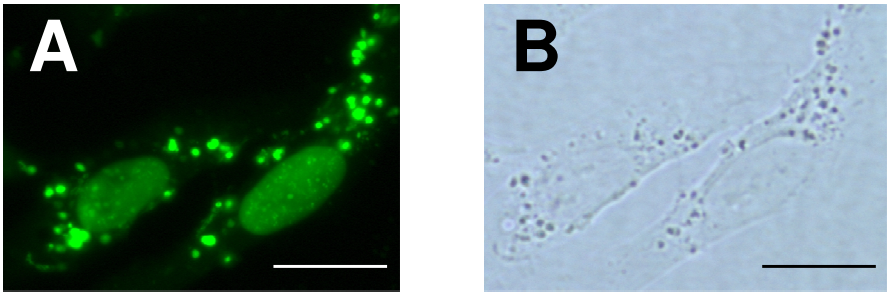


FIGURE 15: Uptake of FITC-labeled oligonucleotides (FITC-ODN) into endothelial cells. *Endothelial cells were cultured in RPMI 1640 without FCS.* ***A****. Incubation for 24 h with ODN (0.75 µM).* ***B****. Bright field micrograph of the identical area. Bar = 50 µm*

It is reasonable to start monitoring of ODN uptake 4-5 hours after their addition since incorporation strongly increases at this time (see Figure 14). Note: in our experimental series, it was crucial to achieve an experimental condition that led to ODN accumulation in the nucleus. Conditions, such as the presence of FCS during uptake, preventing nuclear transport, did not result in any inhibition.

C) ANALYSES OF mRNA FORMATION, PROTEIN EXPRESSION, AND NITRITE PRODUCTION

Real-time PCR has gained increasing importance during the last two years. However, detailed experiments in our laboratory have shown that results are relative identical compared to findings with conventional PCR. Since the newer technique is expensive and does not substantially increase information, we here describe the conventional Reverse Transcription (RT) and Polymerase Chain Reaction (PCR).

In detail, total cellular RNA (with 1 µg RNA/probe) can be prepared using the Omniscript RT Kit and RT carried out at 37°C for 60 minutes with oligo dT (15mer) as primer. The cDNA (500 ng each) is used for PCR with primer ODN and amplification protocols as shown in table 3.

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

For western-blot-analyses of the iNOS protein, cells are washed, scraped from the dishes and lysed, transferred to a microcentrifuge tube, and boiled for 5 minutes in electrophoresis buffer. Proteins (30 µg per lane) are separated by electrophoresis in a 12%-SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Further incubations are: 2 h blocking buffer (2% BSA, 5% non fat milk powder, 0.1% Tween 20 in PBS-buffer), 1 h at 37°C with a 1:2000 dilution of the monoclonal anti-iNOS antibody, and 1 h with a 1:2000 dilution of the secondary horseradish peroxidase conjugated rabbit-anti-mouse-IgG-antibody. Finally, blots are incubated for 5 minutes in ECL reagent (Pierce, Rockford, IL, USA) and

exposed to an autoradiographic film. To control for equal loading of total protein in all lanes, blots are also stained with a mouse anti-α-tubulin antibody at a dilution of 1:2000.
For nitrite determination, cellular NO production is measured by quantifying the nitrite accumulation in culture supernatants of EC using the diazotization reaction as modified by Wood *et al.* and $NaNO_2$ as standard (506).

D) ANTISENSE INHIBITION ON THE mRNA, PROTEIN, AND ACTIVITY LEVEL

mRNA formation will only be partially inhibited (around 20-30% inhibition). On the protein level, expression of iNOS will be strongly decreased by approximately 95% compared to controls. Nitrite levels as determined 24 h after cytokine activation usually showed a reduction of NO formation by around 60 % compared to controls, i.e. cytokines only.
It is of note that Lipofectin, if used as a control empty vehicle, i.e. without the addition of ODN, significantly increases the amounts of mRNA, protein, and nitrite in the presence of cytokines (186). This effect is not due to endotoxin contamination in the reagent and can be prevented by adding any kind of ODN. However, we recommend to check Lipofectin for endotoxin content by the Limulus amebocyte lysate test before starting experiments.

SPECIFIC iNOS-TARGETED ANTISENSE KNOCK-DOWN IN ENDOTHELIAL CELLS

INTRODUCTION

Inhibition of protein formation by adding antisense oligonucleotides (AS-ODN) potentially represents a highly specific technical approach for gene knock-down. Theoretically, the AS method is highly specific since added ODN are supposed to only interact 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 (91).

The power of AS-ODN as specific inhibitors of gene expression is strongly limited by their low spontaneous cellular uptake (278). Furthermore, ODN are rapidly degraded by nucleases if not chemically modified and stabilized (347, 445, 533). To circumvent the obstacle of degradation, a variety of improvements, as for instance phosphate-modified analogs, have been developed (445). The problem of effective cellular ODN uptake will be solved by using uptake enhancers like cationic lipids (59). These substances ensure nucleotide uptake and release from endosomal compartments (203). Here, medium constituents and incubation times have a marked influence on micelle formation as well as incorporation and intracellular localization of ODN (527). Further, it is still controversial whether ODN have to accumulate in the nucleus or whether cytoplasmic accumulation will lead to the desired inhibition (508). Since 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 (29, 57, 445). Since such dysregulated iNOS activity and high concentrations of its product NO have been found to be associated with many diseases (264), the search for a highly specific method for iNOS inhibition is continuing. Substrate deprivation, competitive inhibition by using substrate analogues (143), manipulation of co-factor availability, and the manipulation of the expression or functionality of iNOS-relevant transcription factors like NFκB or IκB (250) 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 (466). 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, since EC represent a prime target for *in vivo* intervention and are inevitably affected by AS-ODN when applied via the blood. Furthermore, the inducible nitric oxide synthase 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 (42, 260, 324, 391). 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, non-transformed endothelial cells (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 fluorescence microscopy we explored how the different conditions and the way 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 IL-1ß, driven by the same cytokines.

MATERIALS AND METHODS

REAGENTS

Recombinant human interleukin-1β (IL-1β) was from HBT (Leiden, Netherlands), recombinant murine gamma-interferon (IFN-γ), and recombinant murine tumor necrosis factor (TNF-α) from Genzyme (Cambridge, MA, USA), endothelial cell growth supplement (ECGS), LPS (from *Salmonella typhimurium*), Neutral Red (3% solution), type I collagen, collagenase (from *Cl. histolyticum*), rabbit anti-human von Willebrand Factor (vWF) antiserum, 2-mercaptoethanol and anti-α-tubulin-antibody from Sigma (Deisenhofen, Germany), the rat endothelium specific monoclonal antibody Ox43 from Serotec (Camon, Wiesbaden, Germany), the monoclonal anti mouse eNOS and anti-mouse iNOS antibodies from Transduction Laboratories (Lexington, KT, USA), peroxidase-conjugated porcine anti-rabbit IgG from DAKO (Hamburg, Germany), peroxidase-conjugated goat anti-mouse IgG from Zymed Laboratories (San Francisco, CA, USA), trypsin, EDTA, and fetal calf serum (FCS, endotoxin free) from Boehringer Mannheim (Mannheim, Germany), Omniscript RT Kit and Taq Core PCR Kit from Qiagen (Hilden, Germany), FuGENE 6 from Roche (Mannheim, Germany), RPMI-1640

(endotoxin free), oligo dT15-primer, Lipofectin, LipofectAMINE, and Opti-MEM Serum Reduced Medium from Life Technologies (Eggenstein, Germany), 3,3'-diaminobenzidine (DAB) from Serva GmbH (Heidelberg, Germany). Antisense oligonucleotides and controls directed to inducible nitric oxide synthase have been designed and manufactured by Biognostik (Göttingen, Germany). Chosen for inhibition of inducible nitric oxide synthase were antisense oligodesoxynucleotides with or without FITC-label (5'-TTTGCCTTATACTGTTCC-3'). As controls, we used two oligodesoxynucleotides with identical purine and pyrimidine content (5'-ACTACTACACTAGACTAC-3' and 5'- ATATCCTTCCAGTACAG-3'), from which the second one was also FITC-labeled.

CELL CULTURES AND CELLULAR CHARACTERIZATION

Rat aorta endothelial cells (EC) were isolated by outgrowth from rat aortic rings exactly as described (457). Briefly, aortic segments were placed on top of a collagen gel (1.8 mg collagen ml^{-1}) in 24-well tissue culture plates and incubated in RPMI 1640 with 20 % FCS and 100 µg ml^{-1} ECGS for 4 to 6 days depending on the degree of cellular outgrowth. Aortic explants were then removed, cells 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 (457). EC showed the antigen phenotype vWF^{high} $Ox43^{high}$ $eNOS^{high}$ exactly as published (457). 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 endothelial cells (EC) were performed with cells from passages 2 to 8. EC or L929 were cultured in 6-well ($2x10^5$ cells) or 12-well ($0.8\text{-}1x10^5$ cells) tissue culture plates in RPMI 1640 (20% FCS for EC, 10% FCS for L929) and incubated overnight to allow attachment before supernatant was replaced by (i) fresh Opti-MEM Serum Reduced Medium, (ii) RPMI 1640/ 10% or 20% FCS or (iii) RPMI 1640 without FCS. Cells were either resident

or activated by cytokines (IL-1β, TNF-α, and IFN-γ, each at 1000 U ml^{-1}) prior to 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 1000 µl (6-well) or 600 µl (12-well) of fresh medium containing the phosphorothioate antisense (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 OLIGONUCLEOTIDE 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 µl to 15 µl in 600 µl Opti-MEM Serum Reduced Medium on 12-well culture dishes. Diluted ODN, used at concentrations from 0.13 µM 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 µl ml^{-1} has a final concentration of 1.03 µM DOSPA and 0.673 µM DOPE. Vehicles and ODN were used at concentrations as indicated in 12-well plates with 600 µl supernatant.

Lipofectin was prepared according to the recommendations of the supplier and used at concentrations as given. A solution of 1 µl ml^{-1} has a final concentration of 0.75 µM DOTMA and 0.68 µM DOPE. Lipofectin was applied in 6-well culture dishes with 1 ml supernatant.

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

CELL TREATMENTS

After overnight attachment of freshly seeded cultures in 6-well 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 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 amebocyte lysate test.

GROWTH RATES OF CELL CULTURES AND VIABILITY

Cell growth was determined by Neutral red staining. Cells were incubated for 90 minutes with Neutral Red (1:100 dilution of a 3% solution), washed twice with PBS, dried, and lysed with isopropanol containing 0.5% 1N 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 using the diazotization reaction as modified by Wood *et al.* and $NaNO_2$ as standard (506).

REVERSE TRANSCRIPTION (RT) AND POLYMERASE CHAIN REACTION (PCR)

Total cellular RNA (with 1 µg RNA/probe) was prepared using the Omniscript RT Kit and RT was carried out at 37°C for 60 minutes with oligo dT (15mer) as primer. The cDNA (500 ng each) was used for PCR with primer ODN and amplification protocols as shown in Table 3.

TABLE 3

LIST OF OLIGONUCLEOTIDES USED FOR iNOS, IL-1β, OR GAPDH cDNA AMPLIFICATION

species/ product		sequence S = sense AS = antisense	product size (bases)	amplification conditions*	
				annealing	cycles
rat iNOS	S	ATGCCCGATGGCACCATCAGA	394	60°C, 30s	26
	AS	TCTCCAGGCCCATCCTCCTGC			
rat IL-1β	S	CCAGGATGAGGACCCAAGCA	519	57°C, 60s	29
	AS	AAGGCTTCCCCTGGAGAC			
rat GAPDH	S	CAACTACATGGTTTACATGTTCC	416	60°C, 30s	26
	AS	GGACTGTGGTCATGAGTCCT			

**, PCR was started with 30 seconds at 94°C and amplication was always followed by a final incubation step at 72°C for 10 minutes.*

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 iNOS PROTEIN

Cells, treated as indicated, were washed, scraped from the dishes and lysed, transferred to a microcentrifuge tube, and boiled for 5 minutes 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% non fat milk powder, 0.1% Tween 20 in PBS-buffer), 1 h at 37°C with a 1:2000 dilution of the monoclonal anti-iNOS antibody, and 1 h with a 1:2000 dilution of the secondary horseradish peroxidase conjugated rabbit-anti-mouse-IgG-antibody. Finally, blots were incubated for 5 minutes in ECL reagent (Pierce, Rockford, IL, USA) 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-α-tubulin antibody at a dilution of 1:2000.

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 endothelial cells (EC) or L929 cells were incubated with various concentrations of oligonucleotides (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, respectively (Figure 16A-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.

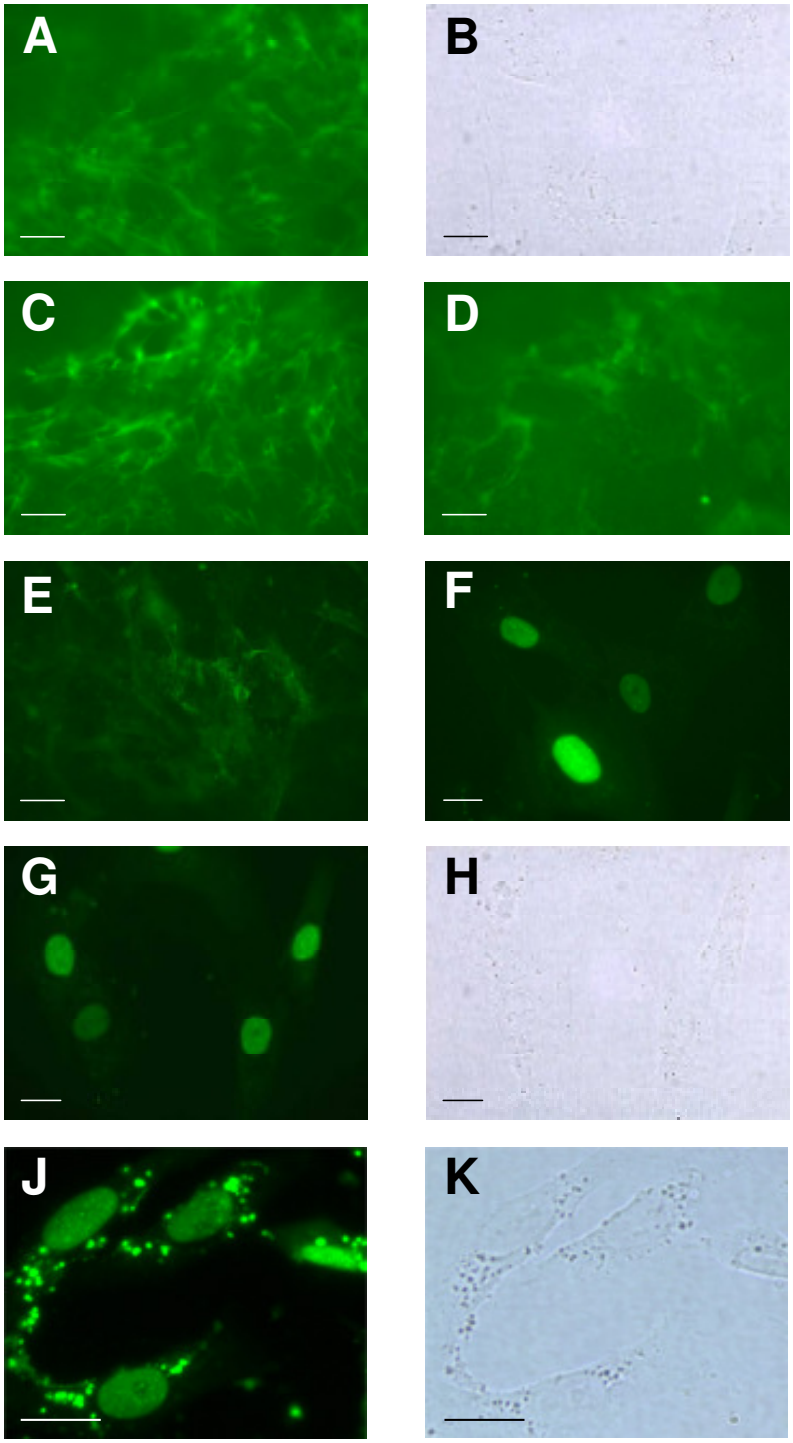


FIGURE 16: Uptake of FITC-labeled oligonucleotides (FITC-ODN) into cultured endothelial cells. *Endothelial cells were cultured in RPMI 1640 or Opti-MEM Serum Reduced Medium in the presence of various concentrations of FITC-ODN with or without fetal calf serum (FCS).* ***A****. Incubation for 24 h with ODN (0.75 µM) in RPMI 1640 without FCS.* ***B****. Bright field micrograph of the identical area.* ***C****. Incubation for 32 h with increased amount of ODN (2 µM) in RPMI 1640 without FCS.* ***D****. Incubation for 24 h with ODN (0.75 µM) and FuGENE 6 (6 µl in 600 µl medium) in RPMI 1640 without FCS representative for the experimental outcome using various concentrations of FITC-ODN with or without FCS and the vehicle FuGENE 6. An overall labeling of the background in A to D indicates unspecific ODN attachment with no accumulation in intracellular compartments.* ***E****. Incubation for 2 hours with LipofectAMINE-encapsulated FITC-labeled ODN (0.7 µM) in Opti-MEM Serum Reduced Medium.* ***F****. As in E but prolonged incubation of 4 hours, showing intranuclear accumulation.* ***G****. As in E with 20 h of incubation.* ***H****. Bright field micrograph of G. Bars in A - H = 50 µm*

J*. Incubation for 5.5 h using 6 µl Lipofectin in 1 ml Opti-MEM and ODN concentrations of 0.6 µM resulting in excellent uptake.* ***K****. Bright field micrograph of the identical area. Bar in J and K = 50 µm*

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 at any combination (Figure 16D).

With LipofectAMINE (Figure 16E-H), good intracellular labeling of cells was observed with 4 µl lipid/ 600 µl 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 prior to cytokine activation. Earlier removal of ODN caused a significantly lower uptake rate and, in addition,

fluorescence microscopy showed ODN being still attached to the outer cell membrane and not yet incorporated (Figure 16E). Incubation of cells for at least 4 h or longer led to accumulation of ODN in the nucleus as evidenced by bright fluorescence signals over the nuclei with hardly any label in the cytoplasm (Figure 16F). ODN were still found in the nucleus after an incubation period of 20 h (Figure 16G, 16H).

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 (Figure 16J, 16K). 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 cytoplasma with the nuclei showing intensive fluorescence signals.

CELL VIABILITY

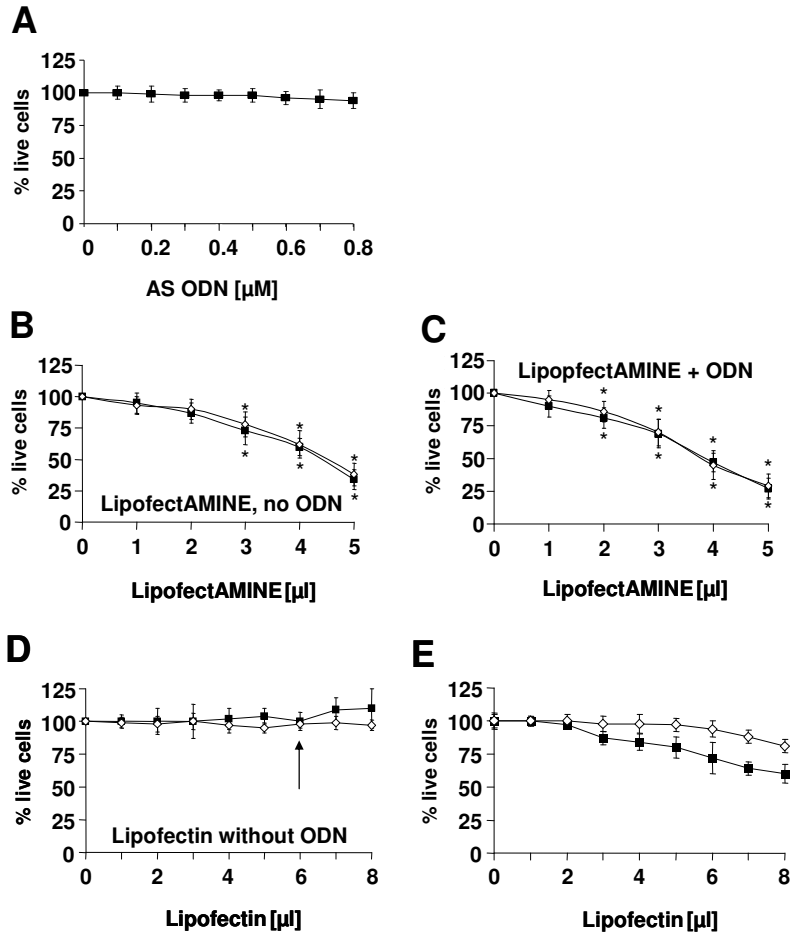
Next, we monitored cell viability under various incubation conditions with or without lipid, with or without ODN (Figure 17). Incubation conditions with negative uptake in the absence of vehicles showed no toxicity irrespective of ODN concentrations (Figure 17A). Unloaded LipofectAMINE vesicles or ODN-loaded micelles showed significant, concentration-dependent toxic effects (Figure 17B, C; Table 4).

With Lipofectin as vehicle, we find no toxicity in the absence of ODN (Figure 17D) and only very low cell loss at Lipofectin concentrations above 6 µl ml^{-1} in the presence of ODN (Figure 17E, Table 4). Interestingly, ODN exerted a significant growth inhibition that was not observed with vehicle alone (Figure 17E).

INCUBATION PROTOCOLS INFLUENCING ODN UPTAKE AND INTRACELLULAR ODN LOCALIZATION

Since LipofectAMINE mediated excellent uptake but caused significant toxicity at the same time, we also examined the impact of the presence of fetal calf serum on viability and uptake modalities in endothelial cells (EC) and L929 fibroblasts incubated and cultured under otherwise identical conditions as EC. We find that using the presence of fetal calf serum during the whole experiment decreases toxicity and does not interfere with uptake but leads to a different intracellular accumulation pattern (Table 4). As shown in Figures 18A-D, in the presence of FCS, label accumulates around the nuclear envelope and not within the nuclear compartment. In contrast, incubation in the absence of serum leads to nuclear accumulation (Figure 18E-H, Table 4). Lipofectin-mediated ODN uptake also reveals cytoplasmic accumulation without any ODN-labels in the nucleus if FCS is present during lipid-ODN-complex formation.

FIGURE 17: Viability of cells in antisense experiments. *Endothelial cells were cytokine-activated and treated with the indicated concentrations of Lipofectin or LipofectAMINE with or without ODN in Opti-MEM Serum Reduced Medium. The percentage of live cells was determined after 24 h by Neutral Red (black squares) and Trypan blue assay (white diamonds). Percentages in cytokine-activated only cultures were set to 100%.*



A*. In the absence of carriers, ODN are not toxic in the tested range (n = 22 individual experiments).* ***B****. Unloaded LipofectAMINE vehicle displayed high toxicity in the presence of cytokines (n = 4 individual experiments). *, p < 0,05 compared to treatment with cytokines only.* ***C****. The same toxicity was seen after uptake of LipofectAMINE-encapsulated ODN (as in Figure 16G). *, p < 0,05 compared to treatment with cytokines only.* ***D****. Lipofectin alone displayed no toxicity (n = 6 individual experiments).* ***E****. Uptake of Lipofectin-encapsulated AS-ODN did not result in increased cell death (black squares) but blocked cell proliferation as seen from decreases in cell number (white diamonds) (n = 22 individual experiments).*

*The arrow in Figure 17D marks the experimental conditions used for the subsequent AS experiments. *, p < 0,05 compared to treatment with cytokines only*

The ratio of lipid to ODN is an important factor determining the formation of intact lipid-ODN complexes and thus, an uptake experiment was performed using fixed ODN concentrations of 0.7 µM and increasing concentrations of LipofectAMINE (Figure 18E-H). If LipofectAMINE amounts were too low compared to ODN concentrations, no adequate lipid-ODN-constructs were formed and FITC-labeled ODN accumulated around cells and in the culture supernatant without any stained nuclei (Figure 18J, 18K). The effect of FCS and the lipid-ODN ratio on ODN uptake was identical in L929 fibroblasts and in endothelial cells.

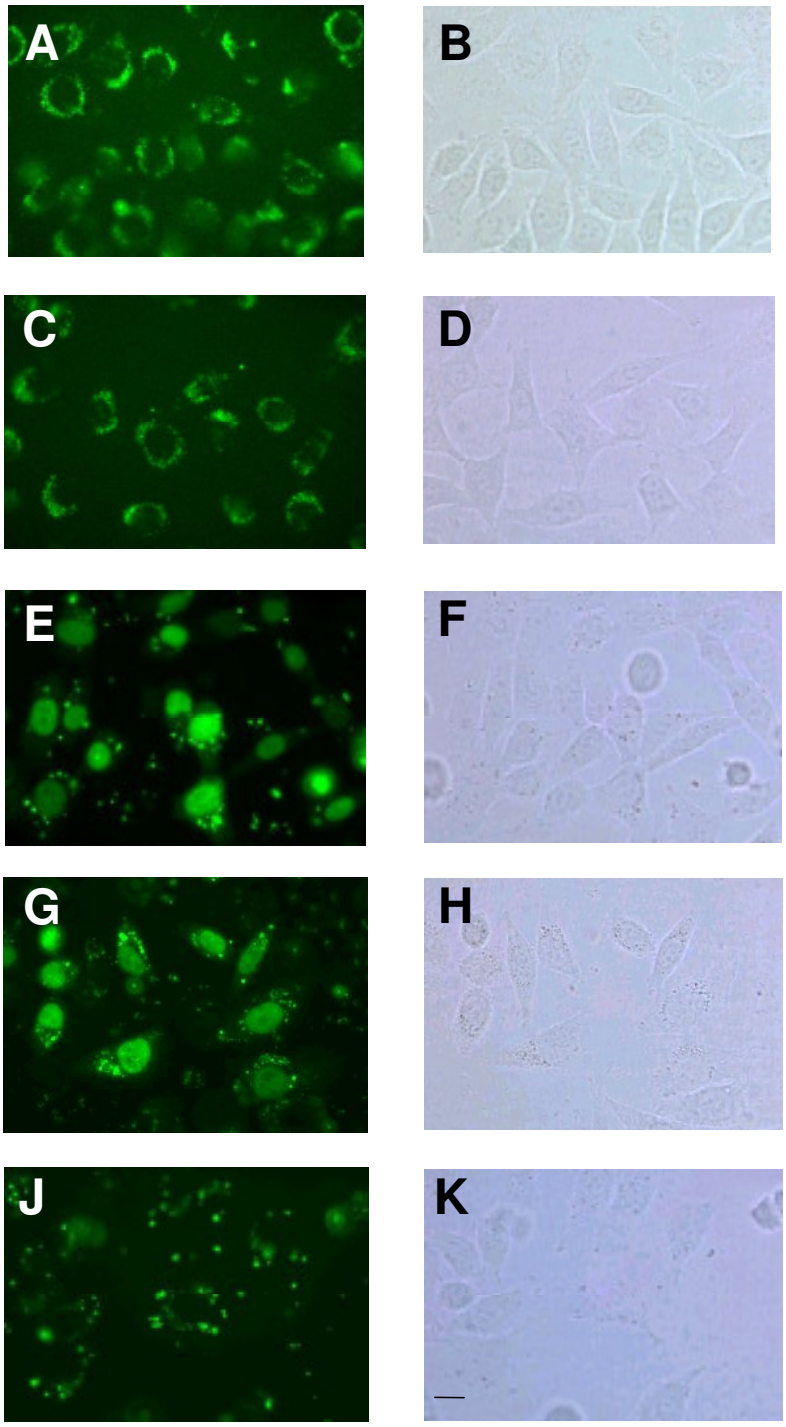


Figure 18: Influence of serum presence and lipid-ODN-ratio on uptake and intracellular localization of ODN in L929 fibroblasts. *The impact of the presence of serum on viability and ODN uptake modalities in L929 fibroblasts incubated and cultured under otherwise identical conditions as endothelial cells were examined by fluorescence microscopy 6-8 h after ODN addition. All micrographs on the right hand of the panel represent the respective bright field images.*

A, B*. Incubation with LipofectAMINE-encapsulated ODN (4µl + 0.7µM) in the permanent presence of FCS (10%) during complex formation and cell incubation.*

C, D*. LipofectAMINE-ODN (4 µl + 0.7 µM) were prepared without FCS and then added to the cells cultured with FCS (10%).*

E, 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, H*. As in E, but using a different compex ratio (4 µl + 0.7 µM ODN)*

J, K*. As in E, but using a different compex ratio (1 µl + 0.7 µM ODN), bar in K = 30 µm.*

Since toxicity was high with LipofectAMINE in both cell types tested (Figure 17B, 17C, Table 4), we now concentrated on the use of Lipofectin and studied the effect of various incubation times. As shown in Figure 19, ODN incorporation into the nuclei of EC and L929 increased significantly between 4 and 5.5 h of incubation from 36.5% ± 21.5% to 88% ± 11% of the cells, while longer incubation periods did not further improve labeling. This strong intracellular accumulation of ODN was achieved in subconfluent cultures only (40-60% of confluency), whereas higher cell densities (confluency of 80% or higher) led to decreases in uptake efficiency (data not shown). Again, data did not differ significantly between L929 and EC.

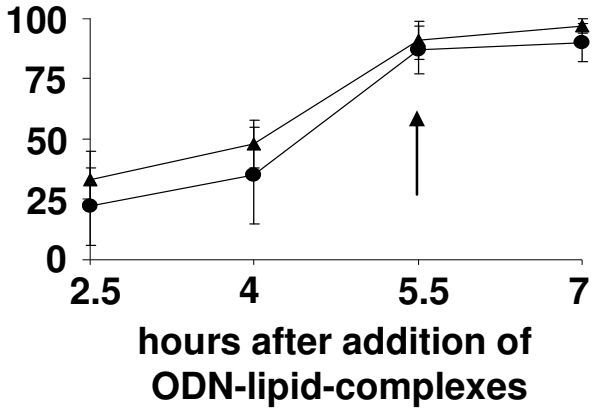


Figure 19: Time-dependent uptake of oligonucleotides.*The kinetics of Lipofectin-mediated ODN uptake in endothelial cells (circles) or L929 fibroblasts (triangles) were investigated during incubation in Serum Reduced Medium and addition of Lipofectin-encapsulated FITC-labeled ODN. The arrow marks the experimental conditions used for all AS experiments in Figure 20. Data are from 18 individual experiments for EC and from 8 separate experiments for L929 fibroblasts.*

In contrast to the recommendations of the manufacturers, ODN-vehicle complexes were not removed during following treatments, since fluorescence microscopy confirmed an improved ODN incorporation if supernatant was not replaced. Furthermore, inhibition of nitrite accumulation in culture media as a read-out for iNOS activity was approximately 10-15% stronger without medium replacement. Addition of cytokines did not affect the process of ODN uptake, neither their intracellular localization nor the kinetics of ODN incorporation.

IMPACT OF ANTISENSE 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, 1000 U ml-1 each) for 24 h. As seen in Figures 20A and 20B, 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 (Figure 20A, lane 1), whereas Lipofectin with either one of two control ODN sequences (s. Reagents) did not alter the cytokine-mediated iNOS expression (Figure 20A, lane 2). Interestingly, the vehicle control, i.e. empty Lipofectin vesicles, led to a significant and dose-dependent increase in iNOS-specific mRNA formation (Figure 20A, lane 4, and 5B). This lipid-mediated increment in iNOS expression was completely abolished by loading the vehicle with control ODN (Figure 20A, lane 2) and was not due to endotoxin contamination of vehicles since no LPS was found with Limulus lysate assay.

TABLE 4

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

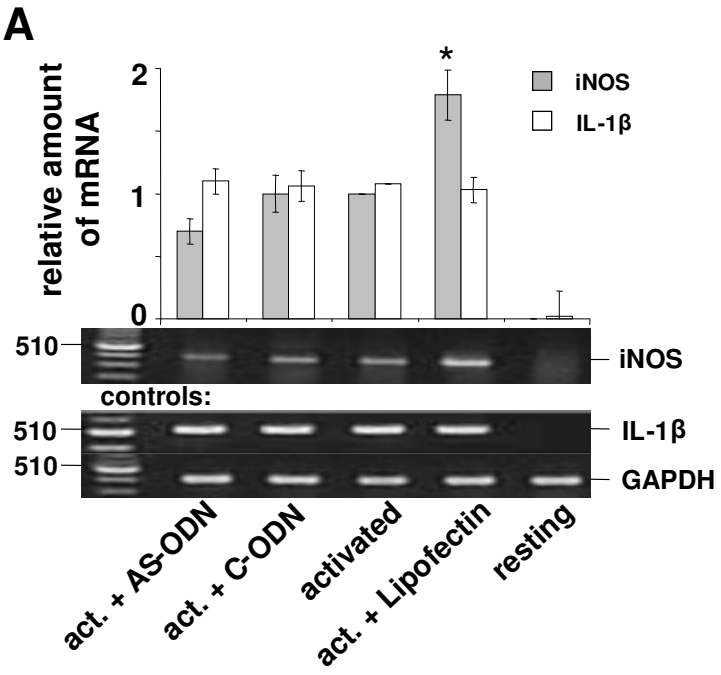
Lipid*	medium during preparation of lipid-ODN-complexes†	medium during incubation of lipid-ODN-complexes on cells	ODN incorporation‡	toxicity**	inhibition of nitrite production
LipofectAMINE	RPMI 1640/ FCS	RPMI 1640/ FCS	cytoplasmic	30 ± 13%	0%
LipofectAMINE	RPMI 1640/ no FCS	RPMI 1640/ FCS	cytoplasmic	28 ± 9%	0%
LipofectAMINE	RPMI 1640/ FCS	RPMI 1640/ no FCS	cytoplasmic/nuclear	46 ± 8%	0%
LipofectAMINE	RPMI 1640/ no FCS	RPMI 1640/ no FCS	nuclear	57 ± 10%	0%
LipofectAMINE	Opti-MEM/ no FCS	Opti-MEM/ no FCS	nuclear	55 ± 11% (s. Fig. 17C)	0%
Lipofectin	RPMI 1640/ FCS	RPMI 1640/ FCS	cytoplasmic	1 ± 2%	0%
Lipofectin	RPMI 1640/ no FCS	RPMI 1640/ no FCS	nuclear	7 ± 2%	0%
Lipofectin	Opti-MEM/ no FCS	Opti-MEM/ no FCS	nuclear	6 ± 6% (s. Fig. 17E)	60 ± 8 % (s. Fig. 21A)

*Inhibition of nitrite formation was only determined in endothelial cells. *, In order 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 1000 µl medium. Both lipids were combined with fixed amounts of 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 (i) during micelle formation and preparation of lipid-ODN-complexes and (ii) during incubation of lipid-ODN-complexes on cells. ‡, ODN incorporation patterns, i.e. aggregation in the nucleus or cytoplasma, 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.*

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 (Figure 20A). Therefore, AS-ODN-mediated inhibition of iNOS must be regarded as specific, not interfering with general cytokine inducibility.

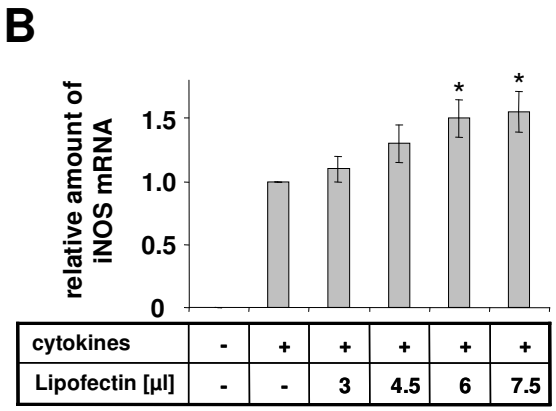
Next, the degree of AS inhibition on protein expression was studied using Western blotting (Figure 20C). We find an almost complete block of iNOS protein formation by AS-ODN (lane 1), while control ODN were of no effect (lane 2). The degree of inhibition for iNOS protein synthesis was better than 95% as compared to cytokine treatment only. Again, a significant increase in iNOS protein formation by empty vehicle addition was seen (lane 4) and complete absence in resident cells (lane 5).

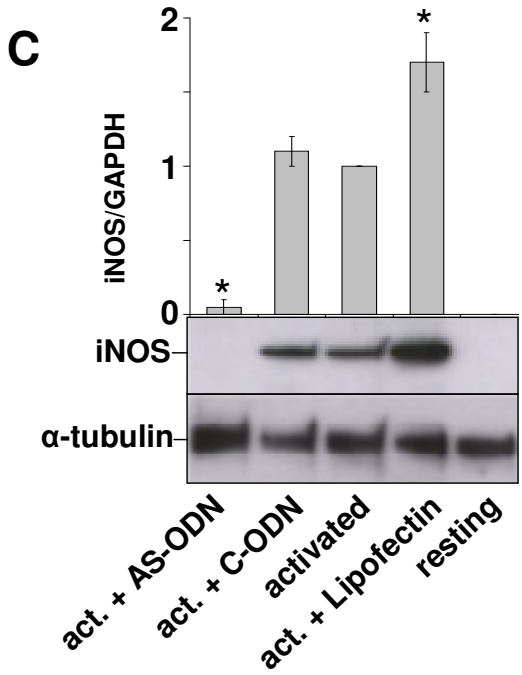
Figure 20: Impact of antisense oligonucleotides and Lipofectin on iNOS mRNA and protein formation. *Endothelial cells were incubated with Lipofectin- control ODN complexes (C-ODN), Lipofectin-encapsulated AS-ODN, or Lipofectin alone for 5.5 h, followed by cytokine activation (IL-1β, IFN-γ, and TNF-α at 1000 U ml^{-1} each) for 24 h. As reference, cells were sham treatment (act). Non-activated, 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.*** *The upper graph gives the relative signal intensities of iNOS-specific amplificatation in relation to the house-keeping gene amplification in each probe and the lower graph shows a representative gel. RT-PCR reveals a small but significant effect of AS-ODN on iNOS mRNA formation and a highly significant upregulation in the presence of empty vehicle. No effects were found on the inducible control gene IL-1ß. Results are based on 6 individual experiments.*



B. *Empty lipid vesicles (Lipofectin) were used as in A (s. 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 as compared to activated (act.) cells*





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 (Figure 21A). Using empty vehicles, again a highly significant and dose-dependent increase in nitrite accumulation was found (Figure 21B). 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.

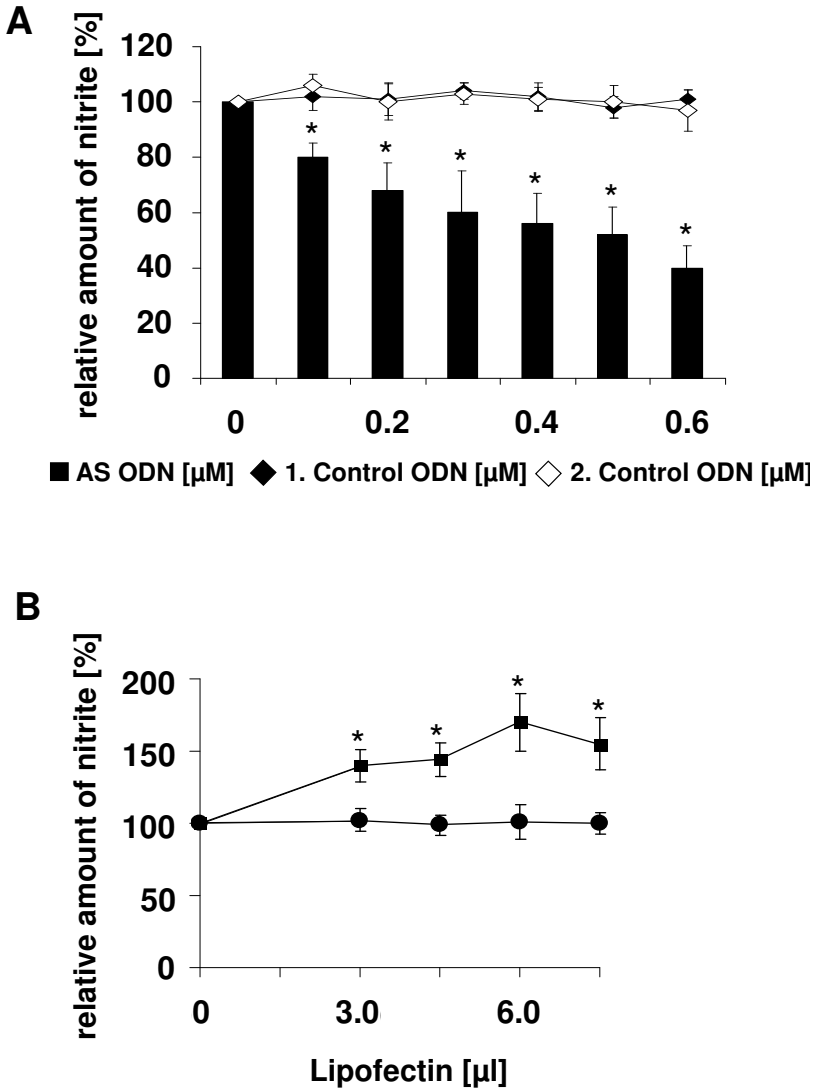


FIGURE 21: Impact of antisense oligonucleotides (AS-ODN) and Lipofectin on nitrite accumulation in culture supernatants

Cells were treated as in Figure 20, and culture supernatants were analyzed for nitrite accumulation as an indirect measurement for NO synthesis.

***A.** Cytokine-activated endothelial cells under antisense treatment are represented by black bars. Black and white diamonds show the effects of the two different control ODN. Data are from 18 individual experiments.*

***B.** The effect of empty Lipofectin vehicles (squares) on endothelial nitrite formation was compared to the effect of vehicles loaded with control ODN (circles). Empty vehicles show a dose-dependent increase in nitrite formation. Data are based on 9 individual experiments.*

**, $p < 0.05$ compared to nitrite formation in cytokine-activated cells*

DISCUSSION

Our experiments demonstrate for the first time that the use of AS-ODN packed into lipid vesicles offers indeed 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 (159, 214) 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 non-transformed 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. Since 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 omit this problem. Nevertheless, it still has to be taken into consideration that in an inflammatory setting, the application of lipid-encapsulated AS-ODN might turn an ODN-mediated iNOS knock-down into an upregulation of NO production, probably due to interactions with charged lipids and cytokine-mediated signaling.

The exact mechanism of AS-mediated inhibition is still under debate but in our experimental setup, we find a distinct correlation between intranuclear accumulation of ODN and successful inhibition of enzyme activities since nitrite production was reduced only when ODN had entered the nucleus. In the continuous presence of FCS, FITC-labeled ODN accumulate 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 to create 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 that ODN get 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 on specific protein synthesis mainly. One explanation for the differences in iNOS mRNA versus protein inhibition might be seen in 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 (110).

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 (110). This may be explained by cytokine-mediated induction of proteins with accessory functions on enzyme activity, as for instance increased arginine transport via cytokine-inducible CAT-2 (80), 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. Taken 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 since no effect was found on concomitant endothelial IL-1β expression, another inflammation marker also synthesized *de novo* in the presence of pro-inflammatory stimuli, following a similar time pattern and in part using identical activation pathways such as NFκB.

In conclusion, we here show 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 to define beneficial versus detrimental effects mediated by this early inflammatory response.

iNOS ACTIVITY IS ESSENTIAL FOR ENDOTHELIAL STRESS GENE EXPRESSION PROTECTING AGAINST OXIDATIVE DAMAGE

INTRODUCTION

Endothelial cell production of nitric oxide (NO) plays a pivotal role in the maintenance of vascular function and health (229). Indeed, evidence is accumulating that endothelial dysfunction in hypercholesteremic patients or in animal models of artherosclerosis is linked to impaired NO-synthesis (85). Thus, inhibition of endothelial NO synthesis leads to increases in adhesion molecule expression, smooth muscle proliferation, and LDL uptake (229).

A number of experimental studies indicate that impaired NO-synthesis *in vivo* may be due to increased systemic levels of asymmetric dimethyl arginine (ADMA), an endogenous competitive NOS inhibitor (41, 85). In addition, insufficient NO production may also be the consequence of limited substrate supply. Two observations support the concept that inadequate endothelial NO synthesis resulting from low arginine availability contributes to atherogenesis (41, 85). Firstly, measurements of arginine serum levels indicate that low substrate concentrations correlate with disease progression or severity (399). Secondly, a number of studies on animal models as well as on human patients find good indications that chronic oral administration of *L*-arginine increases endothelial NO synthesis and inhibits disease progression and arteriosclerosis (as reviewed by Preli et al.)(399).

It is generally assumed that the NOS enzyme isotype responsible for the improvement of endothelial function during atherogenesis has to be the constitutively expressed endothelial NO synthase (eNOS or NOS3) (41, 85). However, under chronic proinflammatory conditions such as those at work during arteriosclerosis, a local expression of the inducible NOS isotype (iNOS or NOS2) is seen in endothelia and other cell types (500). This latter enzyme activity leading to high output NO synthesis was initially perceived to act as a toxic defense mechanism, associated with local tissue destruction in chronic inflammatory conditions (451). However, more recent investigations have linked a powerful protective activity towards cellular stress conditions with iNOS-derived NO synthesis (56, 460). Moreover, microarray studies have shown that iNOS-derived NO serves a modulating activity of the expression of many different genes that also affect protective responses during stress conditions (124, 460, 526).

Thus the aim of the present work was to study the impact of endothelial iNOS activity on oxidative stress during inflammatory conditions, i.e. in the presence of proinflammatory cytokines. We exploited two different ways of interfering with either iNOS expression or enzyme activity despite the continuous presence of cytokines. Antisense oligonucleotides were used to effectively inhibit iNOS expression and alternatively, the cells were maintained under limited arginine supply. We examined the endothelial stress response by monitoring the expressional levels of three examples for stress response genes chosen on the basis of an affirmed NO-mediated control. For all three proteins used – i. e. hemoxygenase-1, bcl-2 and vascular

endothelial growth factor – expressional increases by NO were already shown at the mRNA as well as at the protein level (117, 142, 421, 460, 526). We here correlate this gene expressional control of iNOS-derived NO with endothelial dysfunction and cell death during oxidative stress response.

MATERIALS AND METHODS

REAGENTS

Recombinant human interleukin-1β (IL-1β) was from HBT (Leiden, Netherlands), recombinant murine gamma-interferon (IFN-γ) and recombinant murine tumor necrosis factor (TNF-α) from Genzyme (Cambridge, MA, USA), butylated hydroxytoluene (BHT), endothelial cell growth supplement (ECGS), hydrogen peroxide solution (30%), the Hoechst dye H33342, Neutral Red (3% solution), type I collagen, collagenase (from *Cl. histolyticum*), rabbit anti-human von Willebrand Factor (vWF) antiserum, 2-mercaptoethanol, propidium iodide (PI), and anti-tubulin-antibody from Sigma (Deisenhofen, Germany), the rat endothelium specific monoclonal antibody Ox43 from Serotec (Camon, Wiesbaden, Germany), the monoclonal anti-mouse iNOS antibody from Transduction Laboratories (Lexington, KT, USA), the iNOS inhibitor L-N-(1-iminoethyl)-ornithine (L-NIO) from Qbiogene-Alexis (Grünberg/Germany), peroxidase-conjugated porcine anti-rabbit IgG from DAKO (Hamburg, Germany), peroxidase-conjugated goat anti-mouse IgG from Zymed Laboratories (San Francisco, CA, USA), trypsin, EDTA, and fetal calf serum (FCS, endotoxin free) from Boehringer Mannheim (Mannheim, Germany), RPMI-1640 without L-arginine (custom-made, endotoxin free) from Biochrom (Berlin, Germany), Omniscript RT Kit and Taq Core PCR Kit from Qiagen (Hilden, Germany), oligo dT15-primer, Lipofectin, and Opti-MEM Serum Reduced Medium from Life Technologies (Eggenstein, Germany), and 3,3'-diaminobenzidine (DAB) and L-arginine from Serva GmbH (Heidelberg, Germany). Antisense oligonucleotides and controls directed to inducible nitric oxide synthase have been designed and manufactured by Biognostik (Göttingen, Germany). Chosen for inhibition of inducible nitric oxide synthase were antisense oligodesoxynucleotides with FITC-label (5'-TTTGCCTTATACTGTTCC-3'). As controls, we used two random oligodesoxynucleotides (5'-ACTACTACACTAGACTAC-3' and 5'-ATATCCTTCCAGTACAG-3'), from which the second one was also FITC-labelled.

CELL CULTURES

Rat aorta endothelial cells (EC) were isolated from 5 rats exactly as described (456). In short, aortic segments were placed on top of a collagen gel (1.8 mg collagen ml^{-1}) and incubated in RPMI 1640 with 20% FCS and 100 µg ECGS ml^{-1} for 4 to 6 days. After removing aortic explants, cells were detached and replated onto plastic culture dishes in RPMI 1640/ 20% FCS. Cells were subcultured for up to 8 passages. Each cell batch was routinely characterized by

indirect immunocytochemistry using a crossreacting rabbit-anti-human-vWF antiserum and a rat vascular endothelium specific monoclonal antibody (Ox43) (456). All experiments were performed with different cell batches and data were always comparable with cells obtained from different rats.

EXPERIMENTAL DESIGN

All measurements were performed with cells from passages 2 to 8. EC were cultured in 6-well ($2x10^5$ cells) or 12-well ($1x10^5$ cells) tissue culture plates in 1 ml or 600 µl RPMI 1640/ 20% FCS. Cytokine activation and incubation with oligonucleotides (ODN) was performed in Opti-MEM Serum Reduced Medium. Cytokine-challenge was performed by addition of IL-1β, TNF-α, and IFN-γ, each at 1000 U ml^{-1}. FITC-labelled phosphorothioate antisense (AS)-ODN were used to control ODN-uptake. EC were incubated in RPMI 1640/ 20% FCS on 12-well-plates for 16-24 hours. Supernatant was then replaced by fresh RPMI 1640 (with or without 20% FCS) or Opti-MEM Serum Reduced Medium. Cells were further incubated with the respective ODN at concentrations from 0.08 µM to 4.4 µM for 2 to 72 hours. ODN-uptake was visualized and controlled by fluorescence microscopy.

Lipofectin, a 1:1 liposome formulation (by weight) of the cationic lipid DOTMA and the neutral lipid DOPE, was used as lipid vehicle. A solution of 1 µl/ml has a final concentration of 0.75 µM (DOTMA) and 0.68 µM (DOPE). Experiments were carried out on 6-well-plates. $2x10^5$ cells, incubated in RPMI 1640/ 20% FCS, were allowed to adhere overnight and the supernatant was then replaced by 800 µl of fresh Opti-MEM containing the ODN-carrier complexes which were prepared following the recommendations by the manufacturers. After 5.5 hours of incubation, cells were activated by cytokine-challenge. After 24 hours of cytokine incubation, nitrite formation in culture supernatants was determined and cells were lysed with 2-mercaptoethanol for PCR-analysis. Cells which received cytokine-activation and all medium changes and other treatments but no addition of ODN and/or lipids are referred to as “sham-treated”. Endotoxin concentrations were assayed by the Limulus amebocyte lysate test.

Cell death was induced by incubation with 0.8 mM H_2O_2 for 16 h.

NITRITE DETERMINATION

Nitrite was determined in culture supernatants using sulfanilamide and naphthylethylene-diamine in the Griess diazotization reaction as modified by Wood *et al.* using $NaNO_2$ as standard (505).

DETERMINATION OF GROWTH RATES AND VIABILITY AND DETECTION OF CELL DEATH

Cell growth was determined at different times by Neutral Red staining (217). Additionally, viability of EC was controlled routinely at the beginning and end of every experiment using the trypan blue exclusion assay.

Apoptosis (nuclear chromatin condensation, nuclear fragmentation) was detected by using the Hoechst dye H33342 (8 µg/ml, excitation: 355 nm, emission: 465 nm) or by detecting DNA strand breaks with the *in situ* nick-translation method, both exactly as described (460). In each of the samples a minimum of 500 cells was counted and cells positive for apoptosis were expressed as a percentage of the total cell number.

DETERMINATION OF LIPID PEROXIDATION

Resting endothelial cells ($2x10^7$) were incubated with H_2O_2 at concentrations indicated in the absence or presence of the respective additives for 18 hours. Then lipid peroxidation was stopped by addition of butylated hydroxytoluene (BHT, 10 µM). Cells were lysed by repeated freezing and thawing. Lipid peroxidation was measured by determination of thiobarbituric acid reactive substances (TBARS) with HPLC and expressed as malondialdehyde (MDA)-equivalents exactly as described previously (199).

REVERSE TRANSCRIPTION (RT) AND POLYMERASE CHAIN REACTION (PCR)

Total cellular RNA (1µg each) was prepared from resting or cytokine activated cells using the Omniscript RT Kit and RT was carried out at 37°C for 60 minutes with oligo dT (15mer) as primer. The cDNA (500 ng each) was used as template for PCR, primed by using oligonucleotides and conditions as shown in Table 5. Extensive testing for each gene product was performed, to ascertain that amplifications were always in the linear amplification range.

After amplification, 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 using the KODAK 1D software (KODAK, Stuttgart, Germany). To further ensure the correctness of the PCR-procedure, the amplification products of IL-1β and bcl-2 have been sequenced.

WESTERN-BLOT-ANALYSIS OF iNOS PROTEIN

Cells treated as indicated were washed, scraped from the dishes, lysed, transferred to a microcentrifuge tube, and boiled for 5 minutes in an 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 hours blocking buffer (2% BSA, 5% non fat milk powder, 0.1% Tween 20 in PBS-buffer), 1 hour at 37°C with a 1:2000 dilution of

the monoclonal anti-iNOS antibody, and 1 hour with a 1:2000 dilution of the secondary horseradish peroxidase-conjugated rabbit-anti-mouse-IgG-antibody. Finally, blots were incubated for 5 minutes in ECL reagent (Pierce, Rockford, IL, USA) and exposed to an autoradiographic film. To control equal loading of total protein in all lanes, blots were also stained with a mouse anti-α-tubulin antibody at a dilution of 1:2000.

TABLE 5

LIST OF OLIGONUCLEOTIDES USED FOR iNOS, IL-1β, VEGF, HO-1, BCL-2 OR GAPDH cDNA AMPLIFICATION

species/ product	acc. no.		sequence S = sense AS = antisense	product size (bases)	amplification conditions*	
					annealing	cycles
rat iNOS	D14051	F R	ATGCCCGATGGCACCATCAGA TCTCCAGGCCCATCCTCCTGC	394	60°C, 30s	36
rat IL-1β	E05490	F R	CCAGGATGAGGACCCAAGCA AAGGCTTCCCCTGGAGAC	519	57°C, 60s	29
rat VEGF	NM_031836	F R	CTGCTCTCTTGGGTGCACTG CACCGCCTTGGCTTGTCACAT	431 563 635	57°C, 45s	26
rat HO-1	J02722	F R	CACCAGCCACACAGCACTAC CACCCACCCCTCAAAAGACA	1043	57°C, 60s	22
rat bcl-2	NM_016993	F R	TATGATAACCGGGAGATCGTG CAGATGCCGGTTCAGGTACTC	521	60°C, 60s	27
rat GAPDH	M17851	F R	CAACTACATGGTTTACATGTTCC GGACTGTGGTCATGAGTCCT	416	60°C, 30s	26

**, PCR was started with 30 seconds at 94°C and amplication was always followed by a final incubation step at 72°C for 10 minutes.*

STATISTICAL ANALYSIS

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

RESULTS

IMPACT OF ANTISENSE OLIGONUCLEOTIDES ON iNOS mRNA SYNTHESIS, PROTEIN EXPRESSION, AND ENZYME ACTIVITY

Rat aorta endothelial cells (EC) were cultured in RPMI 1640 (with or without 20% FCS) or Opti-MEM Serum Reduced Medium and ODN were added at the concentrations indicated.

When administered at various concentrations, different medium conditions, and incubation times of up to 72 hours, the fluorochrome-labelled ODN were always attached to the outer cell membrane and uptake with subsequent accumulation in intracellular compartments was never observed in the absence of transport vehicles (data not shown).

Various transmembrane vehicles were examined for positive ODN uptake, and good intracellular accumulation of labelled ODN was achieved with Lipofectin, a 1:1 liposome formulation of DOTMA and DOPE. Examination by fluorescence microscopy after an incubation period of 5-6 hours demonstrates bright nuclear fluorescent signals with additional labels in cytoplasmic vesicles (labelling efficiency of >90% of the cells). Viability of cells as assayed by trypan blue exclusion and Neutral red proved that neither ODN alone nor lipid carriers alone, nor their combination exhibit toxic or growth-inhibiting effects at the tested concentration range (data not shown).

With the optimal conditions as defined above, we next determined the effect of AS-ODN on iNOS mRNA and protein expression as well as enzyme activity. Complexes of lipids with either one out of two control-ODN sequences or AS-ODN or empty lipid vesicles were added to the endothelial cell cultures, followed by a 5.5 hour-incubation period and subsequent cytokine activation (IFN-γ, IL-1β, TNF-α, 1000 U ml^{-1} each) for 24 hours. Analysis of iNOS mRNA expression showed that the cytokine-mediated activation induced the *de novo* formation of iNOS mRNA as expected (Figure 22A, lane 5). Interestingly, the vehicle control, i.e. empty lipid vesicles, led to a significant increase in iNOS-specific mRNA formation (Figure 22A, lane 3), which was not due to endotoxin contamination as ascertained by the Limulus lysate assay. This lipid-mediated augmentation of iNOS expression was completely abolished when lipid vesicles were loaded with control-ODN (Figure 22A, lane 2). Incubation with AS-ODN plus lipid carriers resulted in a moderate, but significant decrease in iNOS mRNA expression (Figure 22A, lane 4). Thus, we find a sequence-specific inhibition of iNOS mRNA formation of about 20% relative to sham-treated or control-ODN-treated, cytokine-activated cells.

Next, we examined the effect of AS inhibition on iNOS protein expression. Again, we observed that iNOS protein expression was significantly enhanced in the presence of unloaded lipid vesicles (Figure 22B, lane 3). We find that AS-ODN almost completely inhibit iNOS protein formation (inhibition = 95% in Figure 1B, lane 4) while control-ODN (Figure 22B, lane 2) were identical to sham-treated controls (Figure 22B, lane 5).

To also assess the effect of AS-ODN on NO formation, nitrite accumulation in the culture supernatants was measured as described in Materials and Methods. Again, we observed a dose-dependent decrease of nitrite accumulation with AS-ODN plus lipid vehicles and no effect whatsoever with any of the two control-ODN (Figure 23A). A remarkable increase in nitrite accumulation was seen when lipid carriers, i.e. Lipofectin, were incubated with cytokines in the absence of ODN (Figure 23B).

In conclusion, we find a significant AS-ODN-mediated inhibition of iNOS at all levels of enzyme synthesis and activity.

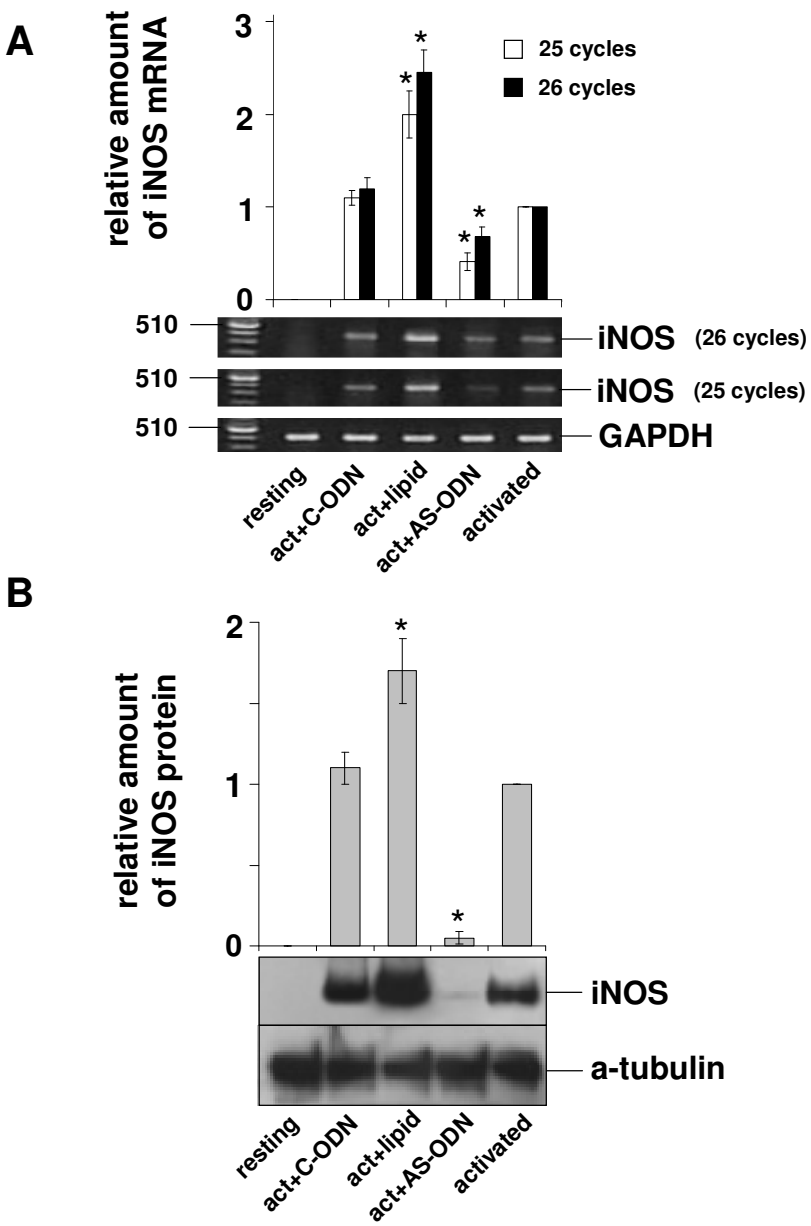


FIGURE 22: Effect of antisense oligonucleotides (AS-ODN) on iNOS-specific mRNA and protein formation. *Complexes of different control (C)-ODN with Lipofectin, AS-ODN with Lipofectin or lipid alone were incubated with cultured endothelial cells for 5.5 hours, followed by cytokine activation (IL-1β, IFN-γ, and TNF-α at 1000 U ml^{-1} each) for 24 hours. The presence of iNOS-specific mRNA was analysed by semiquantitative RT-PCR and iNOS protein formation was investigated by Western blotting.*

Band 1: resting cells, band 2: cytokine-activated (act, activated) cells with C-ODN, band 3: cytokine-activated cells with empty lipid vesicles only, band 4: cytokine-activated cells in the presence of AS-ODN, band 5: cytokine-activated, sham-treated cells

A. *Results shown for 25 and 26 cycles of PCR amplification are from the same cycle sample. Findings are based on 6 individual experiments.*

B. *Shown is a representative blot from three individual experiments.*

**, $p < 0.05$ as compared to cytokine-activated cells (band 5)*

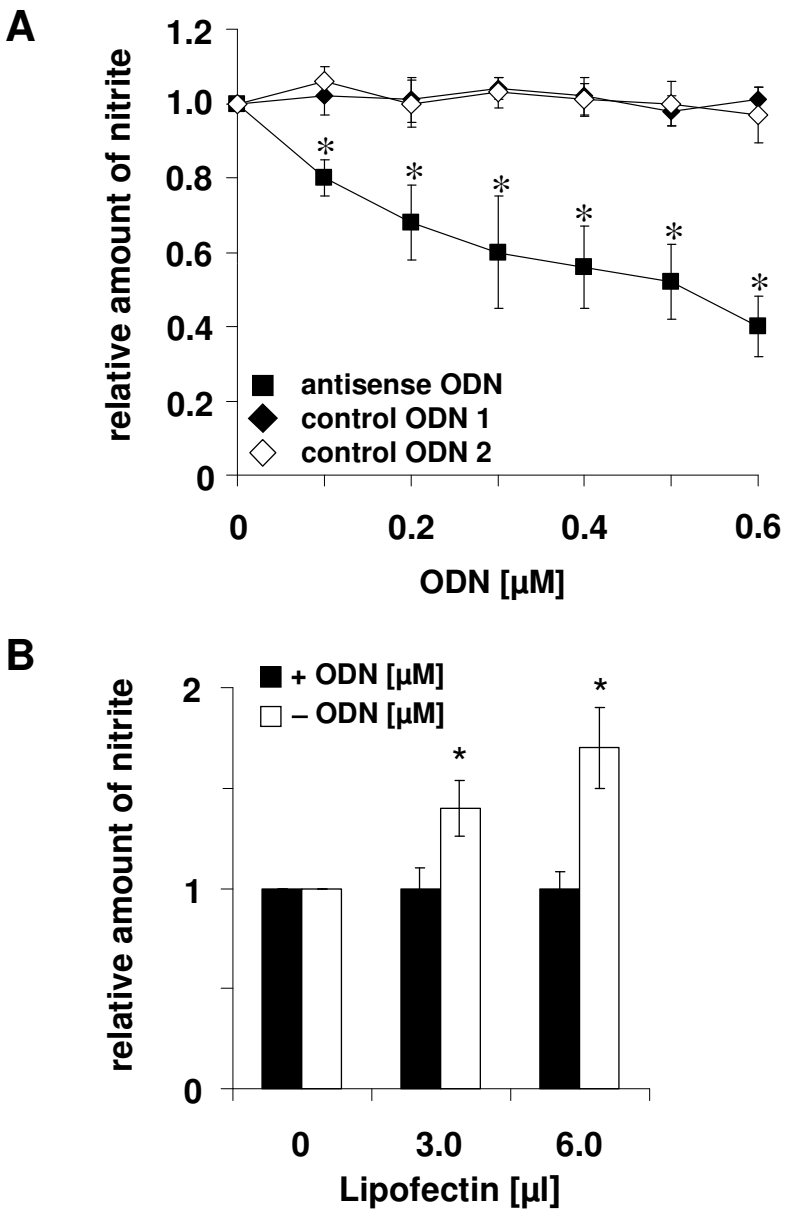


FIGURE 23: Impact of AS-ODN and lipid carriers on nitrite accumulation. *Cells were treated as in Figure 22 and culture supernatants were analyzed for nitrite accumulation as an indirect measurement for NO synthesis.*

A. *Cytokine-activated endothelial cells under AS treatment are represented by black squares. Black and white diamonds show the effects of two different C-ODN. Data are from 18 individual experiments.*

B. *The effect of lipid carriers on nitrite formation was examined in the presence or absence of C-ODN. Data are based on 9 individual experiments.*

**, $p < 0.05$ compared to nitrite formation in cytokine-activated cells*

IMPACT OF iNOS ANTISENSE INHIBITION ON THE EXPRESSION OF STRESS RESPONSE GENES

To ascertain the specificity of the AS-mediated effect, we monitored the impact of iNOS AS-ODN or control-ODN on a gene which is also expressed *de novo* in the presence of proinflammatory cytokines. In this respect, 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 (Figure 24, E). Thus, AS-ODN-mediated inhibition of iNOS must be regarded as specific, not generally interfering with the expression of cytokine-inducible genes.

FIGURE 24: Specific effects of AS-ODN on gene expression of stress-related genes. *Cells were treated as in Figure 22 and the impact on the endothelial mRNA expression of bcl-2, vascular endothelial growth factor (VEGF) and heme oxygenase 1 (HO-1) was examined by RT-PCR. Also determined was the effect on IL-1β expression as an example of cytokine-inducible genes serving as a specificity control for the AS-mediated inhibition.*

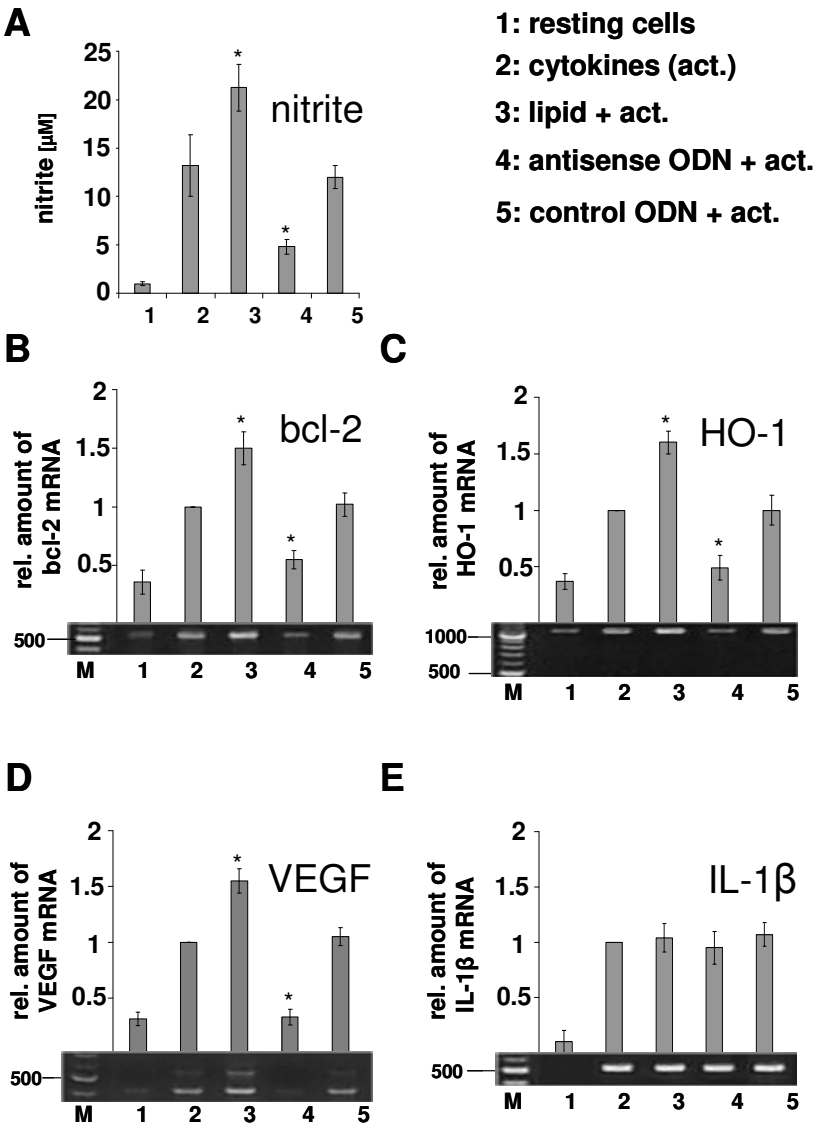


Figure 24B to 24D present the correlation between iNOS-derived NO production and the expression of the endothelial proteins bcl-2, VEGF, and HO-1. Lane 4: effect of iNOS-targeting AS-ODN on the mRNA expression of bcl-2, HO-1, and the three splice variants of VEGF, lane 3: effect of C-ODN, lane 2: cytokine-activated cells (IFN-γ, IL-1β, TNF-α, 1000 U ml^{-1} each), lane 1: resting cells. Levels of nitrite production are given to show the correlation between nitrite formation and the expression of the three stress-response genes (Figure 24A). In Figure 24E, the de novo mRNA expression of IL-1β during cytokine activation was examined in the presence of lipid vesicles alone or the combination of lipid with either AS- or C-ODN. Data on nitrite formation were gained from 18 individual experiments while data on bcl-2, HO-1, VEGF, and IL-1β are from 4 individual experiments.

**, p <0.05 as compared to the controls*

We then examined the impact of AS-mediated iNOS inhibition on the expression of genes with protective activity during oxidative stress and endothelial injury. As examples for such genes we chose the stress response gene heme oxygenase-1 (HO-1), the anti-apoptotic protein bcl-2, and the injury response protein vascular endothelial growth factor (VEGF). Indeed, we find a direct correlation between iNOS expression and activity and the endothelial expression of the three gene products (Figure 24, B-D). Of note is that with VEGF, inhibition affects all three splice variants that can be detected with the primer pair used (117). This finding also demonstrates the correct identity of the VEGF-product. Further support for a NO-mediated modulation of bcl-2, VEGF, and HO-1 expression came from the control experi-

ments with empty lipid vesicles, as these increase the mRNA-expression of all three investigated genes to the same degree as the iNOS mRNA expression.

IMPACT OF EXOGENOUS ARGININE CONCENTRATIONS ON ENDOTHELIAL iNOS ACTIVITY AND EXPRESSION OF STRESS RESPONSE GENES

Next, we examined the impact of low versus high substrate concentrations on iNOS-mediated NO production and on stress gene expression. Resting or cytokine-activated cells were incubated at high physiological (200 µM) or depleted (5 µM) arginine concentrations to determine the effect on endothelial iNOS activity (Figure 25). As expected, cytokine-challenge of EC leads to induction of identical iNOS mRNA expression at both arginine concentrations. Nitrite concentrations were determined in culture supernatants and demonstrated high-output NO synthesis at 200 µM of arginine (Figure 25, lane 2), which was inhibited by addition of the NOS inhibitor L-NIO (0.5 mM) (lane 3). In contrast, cytokine-induced nitrite formation was below the detection limit in cultures grown under arginine-depleted conditions (lane 5).

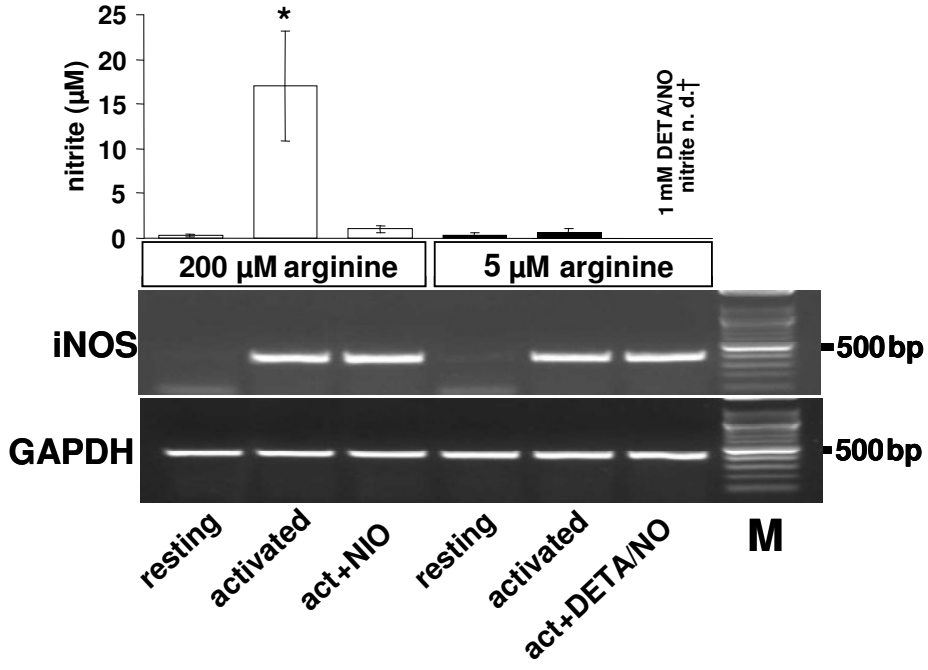


FIGURE 25: Effects of arginine-concentrations on endothelial nitrite production. *Resting or cytokine-activated (IFN-γ, IL-1β, TNF-α, 1000 U ml^{-1} each) cells were incubated for 24 hours at physiological (200 µM) or depleted arginine concentrations (5 µM). Nitrite concentrations in culture supernatants were determined as an indirect parameter for endothelial NOS activity. Additionally, for iNOS-mRNA detection iNOS-specific PCRs were performed. Values represent mean ± SD of 4 individual experiments. Micrographs show a representative gel from three individual experiments with similar results. *, p <0.001. †, nitrite concentrations for NO donor experiments were not determined.*

When analyzing the effect of arginine on the expression of endothelial stress reponse genes (Figure 26), we find that endogenous iNOS-derived NO-synthesis completely parallels the

expression of the genes bcl-2, HO-1, and VEGF. When compared to non-activated cells, cytokine challenge in the presence of 200 µM arginine with resulting high-output NO synthesis increases bcl-2-mRNA expression by the factor of 3.5±0.4 and HO-1- or VEGF-mRNA expression by 2.0±0.2- or 2.3±0.6-fold, respectively (Figure 26, lane 2). Both competitive inhibition by the NOS inhibitor L-N(5)-(1-iminoethyl)ornithine (L-NIO) (lane 3) or limitation of arginine supply (lane 5) significantly decrease the mRNA expression of these three genes to control levels. Addition of the NO-donor molecule diethylenetriamine nitric oxide (DETA/NO, 1 mM) fully restores the increases in bcl-2-, HO-1-, and VEGF-mRNA expression in the absence of arginine (Figure 26, lane 6). Thus, the effect of AS-mediated knock-down of iNOS expression is comparable with the effect of limited substrate supply as regards the expression of the stress response genes tested.

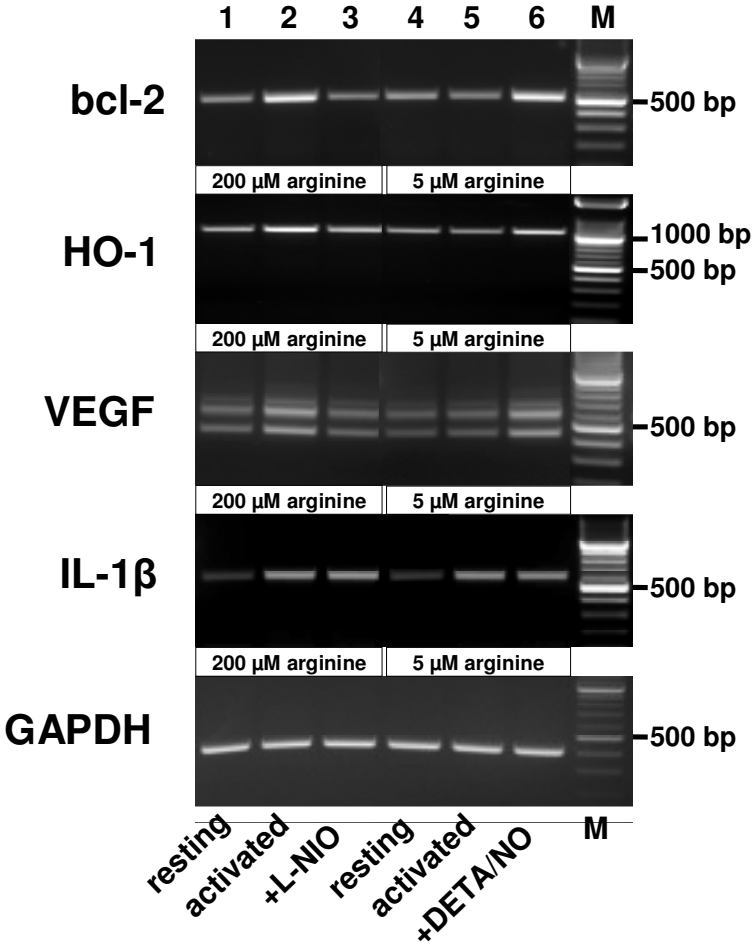


FIGURE 26: Impact of arginine concentration on endothelial bcl-2, heme oxygenase-1, and VEGF expression. *The mRNA expression levels of bcl-2, HO-1, VEGF, and IL-1β were compared in resting and cytokine-activated EC cultures under the influence of arginine (lane 1-3: 200 µM arginine) or arginine depletion (lane 4-6: 5 µM arginine). Lane 3 shows the effect of the competitive NOS inhibitor L-NIO (0.5 mM). The influence of exogenous NO addition by DETA/NO (1 mM) is seen in lane 6.*

The gel micrograph is representative for 3 to 4 individual experiments with similar results.

THE ROLE OF iNOS ACTIVITY IN INCREASING ENDOTHELIAL RESISTANCE TOWARDS H_2O_2-INDUCED APOPTOSIS

We next examined whether this altered gene expression profile correlated with endothelial survival during oxidative stress, experimentally induced by the addition of H_2O_2. To study this, we chose the model of restricted arginine supply, because relative sensitivity in cells

treated with cationic lipids alone was markedly influenced, pointing to problems in using knock-down experiments for such a study.

Resting EC cultured at the high-physiological arginine concentration of 200 µM in the presence of increasing hydrogen peroxide concentrations showed a concentration-dependent increase in cell death as determined after 18 hours. Cytotoxicity reached a half-maximal level at 0.6±0.05 mM H_2O_2 and maximal death of >80% of the cells at and above 0.8 mM H_2O_2 (Figure 27A). Cytokine activation with concomitant iNOS-mediated high output NO-synthesis resulted in full protection from peroxide-induced death. This effect was abrogated by the NOS-inhibitor NIO (0.5 mM) (Figure 27A).

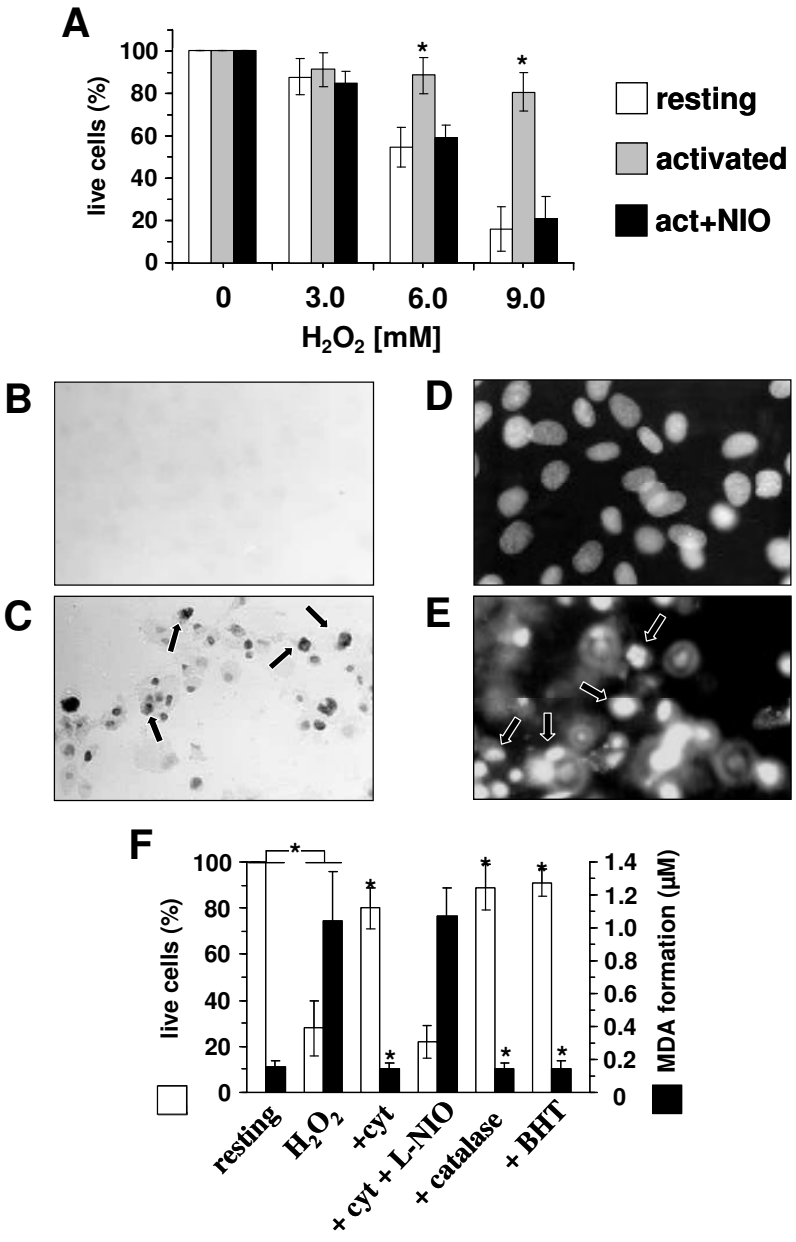


FIGURE 27: Role of iNOS activity on apoptosis and lipid peroxidation during oxidative stress. A. *With resting cells (white bar), H_2O_2 (0.8 mM for 18 hours) leads to endothelial cell death in a concentration-dependent manner. Cytokine activated cells (black bars) were fully protected. Incubation in the presence of the NOS inhibitor L-NIO (grey bars, 0.5 mM). completely reversed this protection. Values represent the mean ± S.D. of 6 individual experiments. *, p<0.001 as compared to resting or NIO treated cultures.* ***B-E.*** *Apoptosis was monitored by in situ nick translation (Figure 27C) detecting nuclei with DNA strand-breaks or by detecting pyknotic and shrunken cells with condensed and/or fragmented chromatin/nuclei using the Hoechst-stain (Figure 27E). B and D are representative micrographs of 3 individual experiments with identical outcome. Magnification: x 650.* ***F.*** *H_2O_2 challenge of resting cells leads to a strong increase in lipid peroxidation as ascertained by monitoring MDA-formation. However, the presence of catalase (2000 U/ml) or the addition of BHT (10 µM) inhibited this increase. After cytokine challenge (**+cyt**) lipid peroxidation was completely suppressed, but inhibition of iNOS activity with L-NIO (0.5 mM) abrogated this suppression. Bars represent the mean ± S.D. of 3 individual experiments. *, p<0.001 as compared to only H_2O_2 challenged cells*

Cell death occurs via apoptosis as confirmed by detecting DNA strand breaks using *in-situ*-nick-translation (Figure 27B and 27C) or by staining with the Hoechst dye (Figure 27D and 27E). Addition of catalase (2000 U/ml) or butylated hydroxytoluene (BHT, 10 µM), the latter an inhibitor of lipid peroxidation, blocks this apoptosis (Figure 27F) indicating that lipid peroxidation represents an initiating event. Indeed, H_2O_2 challenge leads to a marked increase in lipid peroxidation in resting cells as analyzed by detection of malondialdehyde formation (Figure 27F, black bars). In contrast, hydrogen peroxide-induced lipid peroxidation was completely suppressed after cytokine challenge with concomitant iNOS expression and high output NO-synthesis, Inhibition of iNOS activity by adding NIO (0.5 mM) again abrogated this protective effect The impact of arginine on H_2O_2-induced cell death is demonstrated by the close correlation between the exogenous arginine concentration and the degree of cell survival (Table 6). Thus, cytokine-activated EC grown in the absence of arginine are highly sensitive towards H_2O_2-induced death, comparable to non-activated cells at any arginine concentration. Activated cells grown in the presence of 200 µM arginine are fully protected from H_2O_2-induced cell death (Table 6). Again, in the absence of exogenous arginine, protection can be fully restored by addition of the NO donor DETA/NO (1 mM), demonstrating that the arginine-dependent cell resistance is indeed due to high-output NO synthesis.

TABLE 6

INPUT OF iNOS ACTIVITY ON H_2O_2-INDUCED CELL DEATH OF ENDOTHELIAL CELLS

	Live cells (%)	
	200 µM arginine	5 µM arginine
resting	16 ± 4	21 ± 9
activated	86 ± 11*	20 ± 3
activated + L-NIO	21 ± 3	nt
activated + DETA/NO	nt	77 ± 8

Resting or cytokine-activated (IL-1β + TNF-α + IFN-γ, 500 U/ml each) rat aortic endothelial cells grown at physiological (200 µM) or at depleted (5 µM) arginine concentrations were incubated for 16 hours with 0.8 mM H_2O_2. The number of live cells was then determined using the neutral red dye assay. Irrespective of the arginine concentration used, incubation of resting cells with H_2O_2 leads to cell death of app. 80% of the cells. In contrast, high-output NO formation in cytokine-challenged cells grown at 200 µM arginine fully protects endothelial cells from reactive oxygen species-induced cell death. Protection can be diminished by reducing iNOS activity as a result of incubation with the NOS inhibitor L-NIO (0.25 mM) or arginine depletion. On the other hand, protection can be restored again by exogenously applied NO in the form of a diethylenetriamine nitric oxide adduct (1 mM DETA/NO). Values represent mean ± SD of 4 individual experiments. ***nt****, not tested. *, p<0.001*

DISCUSSION

The expression of iNOS has to be considered as an early marker for inflammatory processes (261). Moreover, in a number of chronic inflammatory diseases, high-output NO synthesis is thought to contribute to local tissue destruction (262). When expressed in infiltrating macrophages (266) and in endothelial cells (EC) (451), iNOS may trigger apoptotic and necrotic cell death in the neighbouring tissue via high-output NO synthesis. In atherosclerosis, such a negative role for endothelial or macrophage iNOS-expression and activity was repeatedly postulated, especially as an increase in iNOS mRNA and protein in EC was indeed observed, for instance in transplant coronary artery disease (404). Thus, specific inhibition of iNOS enzyme activity or gene expression was often considered a suitable target for therapeutic intervention in chronic inflammatory diseases such as atherosclerosis. However, attempts to inhibit high-output NO synthesis have underscored the dichotomous role of iNOS-derived NO as a molecule which also displays protective and thus beneficial activity (483). Indeed, inhibition of iNOS activity severely aggravated transplant artherosclerosis (436). Further, in one of the earliest studies on NO-mediated protection, it was seen that NO protects murine endothelia from TNF-α (104). A second study has shown that either an NO-donor or transfection of iNOS into sheep endothelia will protect from LPS-induced death (66). Interestingly, in this latter study, a NO-mediated effect on gene expressional levels of bcl-2 and HO-1 was not observed, a controversy that remains to be explained. However, later investigations on this system by the same group have demonstrated that this protective effect correlates with the long known zinc-mediated anti-apoptotic effect (465) and this metal has been repeatedly proven to alter stress response gene expression (247).

The experiments presented here were performed to achieve a successful AS-mediated iNOS-inhibition in non-transformed endothelial cells in the presence of proinflammatory stimuli and then to confirm the impact on the expression of genes with known protective functions. Using cationic lipids as delivery vehicles we achieve a specific and efficient inhibition of iNOS expression which was better than 90% at protein level. This AS-mediated decrease of protein formation must be regarded as highly specific for several reasons:

Nucleotide-matched control ODN showed no effect and cytokine-driven IL-1ß expression was not affected, although this *de novo* induction uses partly identical pathways and follows a similar time pattern. Further, it is impossible that iNOS-targeted ODN interacted with eNOS or nNOS expression since there were no sequence matches between the iNOS-specific AS-ODN chosen here and eNOS or nNOS.

Surprisingly, we always observed that the addition of unloaded lipid vesicles significantly enhanced iNOS expression as well as NO formation in the presence of cytokines, an effect that served as an additional control for NO-mediated effects. A similar finding has also been reported in RAW 264.7 murine macrophages (32).

When investigating the impact of AS-ODN 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. A similar observation has been made with using iNOS-specific AS-inhibition in mouse mixed glial cell cultures (110). The reason for this finding may be the cytokine-mediated induction of proteins with accessory functions on enzyme activity, as for instance increased arginine transport via cytokine-inducible CAT-2 (80), but lack of feedback inhibition might also be responsible for the few enzyme molecules formed to work at maximal activity. A role for increased endothelial NOS3 activity can be ruled out as the source of NO production since NOS3 is down-regulated in these cells during cytokine-challenge as shown previously (458).

When looking into the impact of AS-mediated inhibition on stress-relevant gene expression, our data demonstrate that the blockade of iNOS expression leads to diminished gene expression of proteins representing protective endothelial responses to various stresses. Thus up-regulation of bcl-2 expression prevents apoptosis onset (460), up-regulation of HO-1 has been demonstrated to protect during reactive oxygen intermediates-mediated stress (115). The NO-driven expressional increase of HO-1 might well be the most important effect when considering the impact in atherosclerosis, as it was recently shown that carbon monoxide, an enzyme product of HO-1, can suppress lesion formation (377). Further, the increased VEGF expression promotes endothelial regeneration after injury (156, 269, 478). Indeed, our results are in accord with earlier reports on the impact of iNOS enzyme activity or exogenously added NO on the expressional modulation of stress response genes (142, 421, 460). Our findings are also in complete agreement with the numerous recent reports demonstrating a protective activity of iNOS-mediated NO synthesis (234, 268, 335, 460, 476). However, the modulating effect of NO on the expression of stress response genes has not been directly linked to endothelial survival. We here show for the first time that this tight regulatory control of NO on these genes mediated by the endogeneously expressed iNOS is of prime importance in endothelial function.

By limiting exogenous substrate supply for iNOS enzyme activity, and thus again interfering with endogenous iNOS-derived NO synthesis despite the presence of proinflammatory cytokines, we fully corroborate the results obtained with AS-mediated knock-down. Again, the outcome of these experiments demonstrates the highly significant impact of high-output NO synthesis on the endothelial stress response.

In addition, we can also prove the protective impact on oxidative stress as mimicked by incubating the cell cultures with H_2O_2 and monitoring cell survival. This set of experiments was not performed in knock-down cells, as the multiple treatments necessary for successive knock-down alters the cellular functions considerably and renders results difficult to control without affecting the experimental outcome, as highlighted also by the impact of empty vehi-

cles on cytokine responses. However, the impact on stress gene expression is comparable to cells grown under arginine-restricted conditions. These experiments also show that iNOS activity in EC completely depends on exogenous arginine availability and that under limiting concentrations of exogenous arginine, iNOS activity can drop to control levels with a concomitant loss of cytokine-induced protection. Indeed, a number of studies (as recently reviewed by Preli et al. (399)) have found that relatively low arginine serum levels appear to correlate with disease progression in artherosclerosis and that oral arginine supplementation may be beneficial in animal models for hypercholesterolemia as well as in patients (85). Investigators have usually speculated but not shown that the beneficial effects of arginine supplementation on progression of artheroma are due to increases in constitutive endothelial NOS activity (84, 85). In contrast, we here give evidence that disease progression might be associated with inappropriately low endothelial iNOS activity and thus failure of the endothelial lining cells to mount a protective response in the inflammatory environment. Indeed, in the absence of exogenous arginine, we can fully restore this stress response by addition of an NO donor. Although the concentration used here (1mM of DETA-NO) appears high at a first glance, calculation of the amount of NO generated shows a concentration that corresponds to reasonable levels: We had determined a half-life of of 7.7±0.8 h for this compound in culture medium at 37°C, pH 7.4. According to the first-order kinetics with which DETA-NO will spontaneously release its NO, 1mM will thus generate 3.3 µM of NO per minute (27) and a calculation of the steady-state concentration of NO above an actively producing cell monolayer resulted in a value of about 4-5 µM of NO in the immediate vicinity (277). This demonstrates that indeed the level of the NO donor used is comparable to iNOS activity. With these findings the additional question of why local iNOS expression is closely associated with chronic inflammatory conditions, yet its inhibition seems to exacerbate disease progression, may also be answered. It appears reasonable to speculate that under oxidative stress such as occurs during inflammatory reactions, iNOS-derived NO serves two tasks, (i) it helps to down-regulate the production of inflammatory mediators (248), and (ii) it helps to mount protective stress responses. If, however, such a reaction occurs under limited arginine concentrations, these regulatory tasks will be incomplete and the inflammatory reaction may become a chronic condition.

WHAT SENSE LAYS IN ANTISENSE INHIBITION OF iNOS EXPRESSION?

INTRODUCTION

The signal molecule nitric oxide (NO) is synthesized on demand in a tightly regulated fashion for short periods of time by two constitutively expressed NO synthases (endothelial and neuronal NOS, cNOS). Additionally, after activation by proinflammatory cytokines (e.g. interleukin-1ß, tumor necrosis factor-α, interferon-γ, etc.) and/or bacterial products (e.g. lipopolysaccharides (LPS)), an inducible NO synthase (iNOS) is expressed, which produces NO for prolonged periods of time (hours to days) in an apparently unregulated fashion. The impact of NO on cell function or cell death is complex and data published are contradictory. Thus, our current knowledge is obviously insufficient to predict whether a disease therapy would benefit from using a selective iNOS inhibitor or might rather profit from exogenously added NO. Therefore, it is important to elucidate the role of iNOS activity during different diseases as well as to establish new therapeutic concepts. A relatively new method involves antisense (AS)-mediated gene knock-down which, at least in theory, provides a highly specific, rapid, and potentially high-throughput method for inhibiting gene expression and thereby also allowing for exploration of gene function (442). This fascinating concept of blocking the expression of a single gene by using AS-oligodeoxynucleotides (ODN) is based on studies in the late 1960s proving that synthetic AS-ODN indeed act in a sequence specific manner. The first attempt of preparing synthetic ODN targeting a defined gene sequence was performed in 1967 by Belikova (24), and Zamecnik and Stephenson (525) were the first to propose a possible therapeutic applications of ODN. In 1978, they demonstrated effective inhibition of Rous Sarcoma Virus replication in infected chicken fibroblasts by adding synthetic ODN directed against a specific viral genome sequence. At that time, such innovative trials were limited by the availability of synthetic ODN and by the low number of genes that had been sequenced so far. Therefore, automation of ODN synthesis was an important step towards a broader availability of AS technology. A relevant topic at the time and today still discussed, was the protection of ODN against their normally rapid degradation. Thus, the development of ODN analogs resistant to cellular breakdown represented an additional step towards the applicability of AS-ODN. Today, the principal fields of application are the investigation of gene function by loss-of-function or decrease-of-function analyses and the development of AS drugs for therapeutic applications. One possibly promising approach in this direction is the use of iNOS-specific AS-ODN, and we here review the current knowledge in this special field.

The chapters „Mechanisms to inhibit iNOS activity", "Task of Antisense Oligonucleotides", "Obstacles in antisense technique", "Uptake and intracellular distribution of oligonucleotides", and "Necessary controls in antisense experiments" can be found in the Introduction.

TABLE 7

ANTISENSE AND CONTROL SEQUENCES USED IN EXPERIMENTS AIMED AT iNOS KNOCK-DOWN

cell type / animal	in vivo/ in vitro	antisense sequences (ODN or PNA)	controls: scrambled, nonsense, sense, missense sequences (ODN or PNA)	reference
rat pulmonary artery smooth muscle cells	in vitro	5-AAACTTCCAGGGGCAAGC-3	5-GCTTGCCCCTGGAAGTTT-3	Thomae-KR. et al., 1993 (469)
macrophages (bone marrow derived)	in vitro	5-CTTCCAGGGGCAAGCCATGTCTGAG-3 5-GGACTTGCAAGTGAAATCC	5-TCAGACATGGCTTGCCCCTGGAAG-3 5-CATCGGATTTCACTTGCAAGTCC-3	Flesch-IE. et al., 1994 (140)
liver cells from male Wistar rats	in vitro	5-GTGCTAATGCGGAAGGTCATG-3	5-CATGACCTTCCGCATTAGCAC-3 sense	Kurose-I. et al., 1996 (273)
BSC-1 African green monkey kidney cells	in vitro	5-ACAGGCCATCTCTATGGATTTACA-3 (bp 85-62 on human iNOS cDNA)	5-TGTAAAGCCATACAGATGGCCTGT-3 (sense, bp 62-85) 5-TGTCCAATTAGCTCCGAGTCATAC-3	Peresleni-T. et al., 1996 (386)
J774.1A mouse macrophage cell line	in vitro			Rothe-H. et al., 1996 (415)
mouse mixed glial cell cultures from cerebral cortex of SJL/J-mice	in vitro	5-CTAAGTTCAAAAGCTGGGCAT-3	5-ATGCCCAGCTTTTGAACTTAG-3 5-AGCTAGTTACAGTGCAAGTCA-3	Ding-M. et al., 1996 (110)
mouse peritoneal macrophages C3H/HeN	in vitro	5-TCCAGGGGCAAGCCATGTCT-3	5-AGGTCCCCGTTCGGTACAGA-3 5-CTGCGAGTCGCACATTGAGC-3 5-TCTGTACCGAACGGGGACCT-3 5-TCTGGACCCAATGGGGACCT-3 5-TCCTGGGGCAAACCAGGTCT-3	Arima-H. et al., 1997 (16)
macrophage- and T-cell depleted bone marrow cells	in vitro	5-GGTGCTGCTTGTTAGGAGGTCAAGTAA-AGGGC-3	5-TGGCCCAGAAGGGGGTGCTGCATGCG-GTGCAC-3	Selleri-C. et al., 1997 (432)
RAW 264.7 murine macrophages	in vitro	5-CCAGGGGCAAGCCATGTCTG-3 (bp 251-70) 5-CAAGCCATGTCTGAGACTTT-3 (bp 244-63 5-GGGCAAGCCATG-3 (bp 254-70) 5-AAGCCATGTCTG-3 (bp 259-70) 5-AAGGGCA-3 (bp 253-259)	5-GACGTGCGAGTCAGCACTGC-3 random 5-CAGACATGGCTTGCCCCTGG-3 sense	Bilecki-W. et al., 1997 (32)
mouse macrophage cell line J774.2	in vitro	bp 2476-2969		Cartwright-JE. et al., 1997 (62)

female SJL mice	in vivo	5-CAAGCCATGTCTGAGACTTTG-3	5-CAAAGTCTCAGACATGGCTTG-3	Ding-M. et al., 1998 (109)
mouse macrophage cell line RAW 264.7	in vitro	5-AATTAAGCTTGCAGCTAAGTATTAGAG-CGGCG-3	5-AATTAGATCTCACCTTGGTGAAGG-GACTGAGC-3	Giovine-M. et al., 1998 (162)
rat peripheral blood natural killer (NK)-cells and spleen-NK cells	in vitro	5-CTTCAGAGTCTGCCCATTGCT-3	5-TCTCAGTGAGCCCTCATTCTG-3	Cifone-MG. et al., 1999 (77)
human breast cancer cell line MCF-7	in vitro	5-AAATTTCCAAGGACAGGC-3	5-GCCTGTCCTTGGAAATTT-3	Binder-C. et. al., 1999 (34)
murine C3H 10T1/2 fibroblasts	in vitro	5-GAACGGGGACCTTCA-3 (bp 260-74) 5-ACCGAGGGGCGTCGA-3 (bp 402-16) 5-GGTCGGCGTGGTGGG-3 (bp 2480-94) 5-TTCTCCGACGGGGGG-3 (bp 2686-700)	5-CACTGTTGACTGGGG-3 (nonsense) 5-ATCGGACGCAGGCTA-3 (missense)	Lesoon-Wood-LA. et al., 1999 (283)
murine endothelial cell line s-End-1	in vitro			Cartwright-JU. et al, 2000 (61)
A7r5 vascular smooth muscle cells	in vitro	5-CAGGGGCAAGCCATGTC-3	5-CACCGCCATGGCATCTG-3	Ishigami-M. et al., 2000 (209)
cultured human vascular endothelial cells	in vitro			Tanjoh-K. et al., 2000 (466)
C57BL/J6 mice	in vivo	5-CACCTCCAACACAAGATC-3	5-CCTTCGTACCCTTTTTCC-3	Dick-JM. et al., 2001 (103)
C6 glioma cells	in vitro			Yin-JH. et al., 2001(522)
male Sprague-Dawley rats	in vivo	5-GGCAAGCCATGTCTG-3	5-ACCGACCGACGTGT-3	Parmentier-Batteur-S. et al., 2001 (382)
human colon carcinoma cell line HT-29	in vitro	5-CAGAAATTTCCAAGGACAGGCCAT-3		Chun-YJ. et al., 2002 (75)
murine macrophages	in vitro	5-Lys-CCTTTTCCTCTTTC-Gly-3 (PNA)	5-Lys-CTTCTCCCTTTTTC-Gly-3 (PNA)	Chiarantini-L. et al., 2002 (73)
Lewis male rats	in vivo	5-CTAAGCTCAAACGCTGGGCGT-3-NH_2	5-TGCGGGTCGCAAACTTGAATC-3-NH_2	Voigt-M. et al., 2002 (487)

rat aorta endothelial cells	in vitro	5-TTTGCCTTATACTGTTCC-3	5-ACTACTACACTAGACTAC-3 5-ATATCCTTCCAGTACAG-3	Hemmrich-K. et al., 2003 (186)
murine osteoblastic MC3T3-E1 cells	in vitro	bp 52-264, GenBank M 84373 (305)		Abe-T. et al., 2003 (1)
human fibroblasts	in vitro	5-ACAGCTCAGTCCCTTCACCAA-3	5-TTGGTGAAGGGACTGAGAGCTGT-3	Grasso-S. et al., 2003 (166) Renis-M. et al., 2003 (405)
adult femal Sprague-Dawley rats	in vivo	5-CTTCAGAGTCTGCCCATTGCT-3	5-TCTCAGTGAGCCCTCATTCTG-3	Pearse-DD. et al., 2003 (384)
male Sprague Dawley rats	in vivo	5-GGCAAGCCATGTCTG-3	5-CGTCCCTATACGACC-3	Steiner-J. et al., 2004 (449)
mouse cholangiocytes 603 B cells (cell line)	in vitro	bp 207-641		Ishimura-N. et al., 2004 (211)

EXPERIMENTS TARGETING THE iNOS GENE

First experiments on AS-mediated inhibition were performed in 1978. However, it took another 15 years until the first trial targeting iNOS with AS-ODN was published, designed to determine whether AS-mediated blocking of iNOS expression in cytokine-stimulated rat pulmonary artery smooth muscle cells inhibited NO production. In this study, a concentration-dependent inhibition by AS-ODN was observed reaching a 36% reduction of nitrite formation in the absence of uptake enhancers. A similar effect on basal NO production in the absence of cytokines was also found, indicating a lack of iNOS specificity (469).

The number of experiments focussing on AS-mediated inhibition of iNOS-dependent NO formation increased during the following years (109, 110, 140, 273, 386, 415), some reaching full abrogation of NO formation (16) and full inhibition of iNOS protein expression (432). Arima et al. (16) established a protocol for specific inhibition of NO production in murine macrophages by using PS-AS-ODN. They found that activation of cells and inhibition of NO formation require a proper time management. Furthermore, they noticed a 50% reduction in nitrite production with control nonsense and mismatch ODN. Selleri et al. (432) analyzed whether Fas-receptor (Fas-R) triggering causes an induction of iNOS in haematopoietic cells. Using AS-ODN homologous to iNOS mRNA and the competitive iNOS inhibitor N^G-monomethyl-L-arginine (*L*-NMA), they studied the effects of iNOS inhibition in haematopoietic cells treated with a Fas-R agonist to induce apoptosis. They found that specific iNOS enzyme inhibitors failed to block iNOS expression whereas inhibition via AS technology was very successful showing complete abrogation. Uptake enhancers in iNOS-related AS experiments were first used in murine macrophages resulting in a time- and dose-dependent decrease in iNOS mRNA and protein formation (32). Stable or transient transfections of cells for production of iNOS AS molecules started in 1996 by Rothe et al. (415). Specificity and selectivity are often put forward to be the major advantages of the AS technique. To prove the selectivity of the AS molecules, Peresleni et al. (386) used ionomycin to show that constitutive NO synthases remain unaffected by AS-ODN application, retaining functional competence. They investigated the effect of iNOS AS-ODN on epithelial cell viability under oxidative stress, generated by addition of H_2O_2 or LPS. They found that selective inhibition of iNOS as well as a decrease in NO production by AS treatment improved endothelial cell viability after oxidative stress. Cells pretreated with AS-ODN failed to release NO in response to H_2O_2 whereas ionomycin-induced NO generation was unaffected (386).

As stated in the introduction, there is a plethora of observations that relate NO to cytotoxicity (106, 317, 383, 523), but there is also an increasing number of studies stating the contrary [for review see (290)]. Analyzing the possible reasons for such diverse findings, one might consider the insufficient selectivity and specificity of the available NOS inhibitors (244). For

instance, it has been demonstrated in mice that the lack of one out of three NOS isoforms may affect cell viability in a different manner (202).

Analyzing iNOS-targeting AS experiments in cell culture, there appears to be no absolute requirement for uptake enhancers to allow ODN uptake (see Table 8). There are several reports of complete or near complete inhibition of iNOS protein formation and nitrite production in the absence of uptake enhancers with murine or bone marrow-derived macrophages (16, 77, 432). However, ODN incorporation without uptake enhancers only seems to work well in macrophages, maybe due to the phagocytotic activity of the cells. For cell types other than macrophages, uptake enhancers are required or improve the extend of iNOS inhibition. Surprisingly, when using lipid vehicles, so far only Lipofectin has been applied as uptake enhancer for external ODN application with iNOS as a target (Table 8). For transfections a variety of methods have been used, such as electroporation (415), poly-L-ornithine (61, 62), LipofectAMINE (1, 211), and SuperFect (522) (Table 8). Chiarantini et al. (73) used AS-PNA to inhibit iNOS in murine macrophages by selective targeting. Besides using this relatively new technique, they also present an innovative method to improve incorporation of molecules by using red blood cells for delivery. PNA were encapsulated in erythrocytes following a procedure of hypotonic dialysis and isotonic resealing.

In vivo experiments in which iNOS was inhibited by AS-ODN were first carried out by Noiri et al. (367) to examine the potential of iNOS AS-ODN for protection of rat kidneys against ischemia after acute renal failure. They reported that the selective AS knock-down of iNOS resulted in functional disparity of NOS's in the kidney and attenuated ischemia-induced dysfunction, while the non-selective NOS inhibitor L-NAME resulted in increased deterioration of renal functions as compared to untreated animals. Earlier experiments had shown that NOS inhibitors in *in vitro* experiments protected renal tubular epithelium against hypoxic injury (523), whereas they invariably aggravated renal dysfunction in different *in vivo* models of acute renal failure (5, 40, 428). The results of Noiri et al. (367) demonstrate that this discrepancy between *in vitro* and *in vivo* effects of NOS inhibitors appears due to the poor selectivity of the available inhibitors (244, 322, 492).

None of the *in vivo* experiments performed resulted in complete block of NO formation, emphasizing the differences between cell culture experiments and *in vivo* studies (Table 9). In animal experiments, AS-ODN targeted to iNOS were injected intravenously (103, 198), intracardially (367), intracerebroventricularly (109, 382, 449) or intraspinally (384) but always without uptake enhancers. Most studies with mice or rats reveal iNOS inhibition or reduction in iNOS activity being stronger than 50% (109, 198, 367, 384, 449, 487). However, Parmentier-Batteur et al. (382) demonstrate that even a 39% inhibition of iNOS protein expression and activity bears the high potency of AS-ODN in treating transient focal cerebral ischemia in rats, where the partial iNOS inhibition significantly reduced the infarct volume and improved

recovery of sensorimotor functions. They speculate that iNOS AS-ODN application may be a novel therapy in cerebral ischemia. Steiner et al. (449) also focused on brain damage demonstrating that attenuation of iNOS mRNA exacerbates hypoperfusion and upregulates endothelin-1 expression after brain trauma. They show that intracerebroventricular injection of iNOS AS-ODN resulted in a significant but strongly time-dependent inhibition of iNOS synthesis (55% at 4h, 40% at 24h, and 35% at 48h post traumatic brain injury) (449). Pearse-DD. et al. (384) compared iNOS inhibition by AS-ODN versus pharmacological inhibitors after spinal cord injury in rats. Their findings demonstrate that AS inhibition of iNOS is more efficacious than the pharmacological agents amonoguanidine and 1400W to inhibit iNOS-mediated pathophysiological reactions. They hypothesize that the benefit of AS-ODN is due to the fact that pharmacological agents act extracellularly on the iNOS protein whereas AS-ODN can enter cells and interfere with the translation of iNOS mRNA and subsequent protein synthesis (384).

Voigt et al. (487) published that the innovative method of transcorneoscleral iontophoresis can deliver ODN to a body region difficult to access, i.e. retrocorneal parts of the eye. Voigt et al. used iNOS-targeting AS-ODN in a rat model of endotoxin-induced uveitis (EIU). They found that iontophoresis facilitated the penetration of intact ODN into the intraocular tissue of the rat´s eye. Only the eyes receiving ODN via electrical current demonstrated intact ODN within the ocular tissue, and resulted in significantly down-regulated iNOS expression and nitrite production in the iris/ciliar body. Therefore, a high potential for successful gene therapy in human eye diseases is envisaged (487). Most experiments emphasize the potency of ODN to selectively reduce NO overproduction, however, researchers also report problems, e.g. a carcinogenic potential in AS-ODN treatment (283). Lesoon-Wood et al. (283) have shown that the treatment with both iNOS AS- and missense ODN significantly increases the number of neoplastic foci in 3-methylcholanthrene treated murine fibroblasts and this effect corresponds with the inhibition of NO production. It remains an unsolved question whether the observed carcinogenic effect is a consequence of inhibiting a protective NO formation, or whether it is a direct ODN-induced effect. It must be taken into consideration that negative effects after AS-induced iNOS inhibition might be a direct result of impaired NO-mediated gene expression. High-output NO formation is now known to affect the cellular gene usage, among others of apoptosis-regulating factors like bcl-2 (460), growth factors like VEGF (117, 142), inflammation-regulating enzymes like heme oxygenase (421) or superoxide dismutase and many other proteins. It is therefore important to bear in mind that AS-ODN to iNOS are highly specific but may display additional effects by altering gene expression. Establishing the AS technique targeted to iNOS in endothelial cells (EC), we found that the manipulation of NO production highly significantly modulates the expression of genes that are under NO-mediated expressional control (185). Thus, cells that had been treated with iNOS AS-ODN

and subsequently challenged with cytokines showed a significant inhibition in mRNA-formation of bcl-2, VEGF, and HO-1.

Five AS experiments have been carried out with human cells so far and all studies show a weak to medium inhibition of nitrite formation (34, 75, 166, 405, 466). Tanjoh et al. (466) found that AS-ODN designed against the human iNOS successfully blocked the translation from iNOS mRNA to protein formation in human vascular endothelial cells (466). To completely prevent iNOS caused multiple organ failure in patients with severe sepsis (230, 344, 378, 466), they recommend suppressing iNOS activity prior to transcription by two pathways: by using anti-TNF-α monoclonal antibodies, which block iNOS expression prior to transcription, and additionally at the translational stage by AS-ODN application (466).

As stated in the introduction, enzyme inhibitors of iNOS do not always specifically and sucessfully inhibit NO production (367, 432) and toxic effects may occur at high concentrations (33). Some authors therefore recommend a combination of AS-ODN and iNOS inhibitors to block new iNOS protein expression and to inhibit iNOS activity simultaneously (34). This procedure may allow for lower concentrations of both, enzyme inhibitor and ODN. For instance, when investigating the role of NO in TNF-α-induced apoptotic cell death in the human breast cancer cell line MCF-7, it was found that when NO synthesis was inhibited by *L*-NAME, the rate of apoptosis was reduced (34). Simultaneous inhibition of NO generation and iNOS expression by combining *L*-NAME and iNOS AS-ODN completely prevented apoptosis.

Besides analysing NO synthesis, some authors recommend to monitor mRNA formation also. Indeed, with endothelial cells, we found that iNOS mRNA formation was inhibited by AS-ODN by about 20% and iNOS protein formation was decreased by about 95%; nitrite formation, however, was decreased by about 60% only as compared to cytokine-activated sham-treated endothelial cells (186). Although this AS effect on mRNA formation is rather weak, it supports the theory that AS-ODN induce RNase H activity thereby reducing the newly formed mRNA as put forward by Ding et al. (110) who also observed decreases in both, protein and iNOS mRNA formation.

The question of whether a complete AS-mediated inhibition of iNOS expression and subsequent NO production is warranted remains an important issue. In many experimental settings, a significant but not complete inhibition of iNOS is enough to improve disease symptoms by diminishing dysregulated harmful NO-overproduction, e.g. in experimental autoimmune encephalomyelitis in SJL/J mice (109), multiple sclerosis (110), or endotoxin-induced shock (198). Whenever total inhibition of NO production is desired, the experimental time management plays a pivotal role (16, 32, 110). Thus, it was found that the inhibitory effect of the AS-ODN gradually diminishes with time, for instance during an LPS/IFN-γ stimulation. While blockade of NO formation of 82% was observed at day 1, an inhibition of 31% only was measured on day 3 (110). Steiner et al. (449) also demonstrate a time-dependency

when inhibiting iNOS with AS-ODN *in vivo*. Furthermore, the time of AS-ODN addition relative to the time of stimulation is also important (16). Most authors add ODN to the cell culture first and subsequently stimulate iNOS expression by cytokines or IFN-γ plus LPS.

STABLE TRANSFECTION WITH iNOS-DIRECTED AS-ODN VERSUS EXTERNAL APPLICATION

As mentioned before, in some cases of inadequate intracellular ODN distribution and failure of AS inhibition, it might help to use stably transfected cells which express an AS sequence endogenously. However, these endogenously produced AS-ODN have a length of around 600 to 1000 bps and are therefore much longer than the externally applied ODN (62, 415). Since this approach offers the opportunity to specifically determine the cell of interest, it is possible to investigate the involvement of endogenously produced NO in various processes (61) or to analyze the contribution of iNOS activity to regulatory and immune defence functions (415). In addition, transfection with an AS DNA vector has the advantage that the AS molecules are synthesized within the target cell, eliminating the need for continuous addition of exogenous ODN (1, 439, 440, 447). Cartwright et al. (62) created transfected macrophages and endothelial cells and investigated the role of NO in adhesion modulation of macrophages to endothelial cells. In addition, they studied the involvement of endogenously produced NO in endothelial cell proliferation using stably transfected endothelial cells (61). Rothe et al. (415) produced stably transfected macrophages to determine the contribution of iNOS activity to macrophage immune defence functions. By using stably transfected C6 glioma cells, Yin et al. (522) confirmed the ability of iNOS-derived NO to confer resistance against chemotherapy drugs in brain tumor cells. Abe et al. (1) demonstrated that transfection of murine osteoblasts with iNOS-directed AS DNA potently reduces the cytokine-induced inhibition of osteoblastic activity, thereby having potential impact on inflammatory diseases like rheumatoid arthritis and osteoporosis.

TABLE 8

INHIBITION OF iNOS – CELL CULTURE EXPERIMENTS					
cell culture	iNOS mRNA expression	inhibition of iNOS protein expression	inhibition nitrite formation	administration, uptake enhancers	reference
rat pulmonary artery smooth muscle cells			36%	no uptake enhancers	Thomae-KR. et al., 1993 (469)
macrophages (bone marrow derived)	significant inhibition		50%	no uptake enhancers	Flesch-IE. et al., 1994 (140)
mouse mixed glial cell cultures from cerebral cortex of SJL/J-mice	significant inhibition	significant inhibition	31-82%	no uptake enhancers	Ding-M. et al., 1996 (110)
liver cells from male Wistar rats			strong inhibition	no uptake enhancers	Kurose-I. et al., 1996 (273)
BSC-1 African green monkey kidney cells			83%	no uptake enhancers	Peresleni-T. et al., 1996 (386)
J774.1A mouse macrophage cell line			66-84%	stable transfection with electroporation	Rothe-H. et al., 1996 (415)
macrophage- and T-cell depleted bone marrow cells		completely inhibited		no uptake enhancers	Selleri-C. et al., 1997 (432)
mouse peritoneal macrophages C3H/HeN	substantial reduction	strong inhibition	100%	no uptake enhancers	Arima-H. et al., 1997 (16)
mouse macrophage cell line J774.2		strong inhibition	22-97%	stable transfection with poly-L-ornithine	Cartwright-JE. et al., 1997 (62)
RAW 264.7 murine macrophages	strong inhibition	inhibition	90%	Lipofectin	Bilecki-W. et al., 1997 (32)
mouse macrophage cell line RAW 264.7		100%		use of peptide nucleic acid as AS molecule, no uptake enhancers	Giovine-M. et al., 1998 (162)

human breast cancer cell line MCF-7			50%	no uptake enhancers, FCS-free medium	Binder-C. et. al., 1999 (34)
murine C3H 10T1/2 fibroblasts	strong inhibition	strong inhibition	83%	Lipofectin	Lesoon-Wood-LA. et al., 1999 (283)
rat peripheral blood natural killer (NK)- cells and spleen-NK cells		almost complete inhibition		no uptake enhancers	Cifone-MG. et al., 1999 (77)
murine endothelial cell line s-End-1		strong inhibition	82%	stable transfection with poly-L-ornithine	Cartwright-JU. et al, 2000 (61)
A7r5 vascular smooth muscle cells			60%	no uptake enhancers	Ishigami-M. et al., 2000 (209)
human vascular endothelial cells			50%	no uptake enhancers	Tanjoh-K. et al., 2000 (466)
C6 rat glioma cells		42-56%	40%	transient transfection using SuperFect	Yin-JH. et al., 2001 (522)
mouse macrophages		40%	33%	use of peptide nucleic acid as AS molecule, incorporated in red blood cells	Chiarantini-L. et al., 2002 (73)
human colon carcinoma cell line HT-29		inhibition		no uptake enhancers	Chun-YJ. et al., 2002 (75)
rat aorta endothelial cells	20%	90-95%	65%	Lipofectin	Hemmrich-K. et al., 2003 (186)
human fibroblasts		30%		repeated addition of ODN, no uptake enhancers	Grasso-S. et al., 2003 (166) Renis-M. et al., 2003 (405)
murine osteoblastic MC3T3-E1 cells		strong inhibition	75%	stable transfection with LipofectAMINE reagent	Abe-T. et al., 2003 (1)
mouse cholangiocytes 603 B cells (cell line)		100%	strong inhibition	stable transfection with LipofectAMINE and Plus reagent	Ishimura-N. et al., 2004 (211)

TABLE 9

INHIBITION OF iNOS - ANIMAL EXPERIMENTS					
animal	iNOS mRNA expression	inhibition of iNOS protein expression	inhibition in nitrite formation	uptake enhancers, route of administration	reference
male Sprague-Dawley rats (kidney cells)		strong inhibition	73%	no uptake enhancers, intracardially	Noiri-E. et al., 1996 (367)
female SJL mice	complete inhibition	strong inhibition	60-70%	no uptake enhancers, intraventricular	Ding-M. et al., 1998 (109)
male Wistar rats		67%	50%	no uptake enhancers, i.v. (femoral vein)	Hoque-AM. et al., 1998 (198)
C57BL/J6 mice			36%	no uptake enhancers, intravenously	Dick-JM. et al., 2001 (103)
male Sprague-Dawley rats		39%	39%	no uptake enhancers, intracerebroventricular injection	Parmentier-Batteur-S. et al., 2001 (382)
male Lewis rats	21%		81%	no uptake enhancers, transcorneoscleral iontophoresis	Voigt-M. et al., 2002 (487)
adult femal Sprague-Dawley rats			82%	no uptake enhancers, intraspinally	Pearse-DD. et al., 2003 (384)
male Sprague-Dawley rats		35%-55% (time-dependent)		no uptake enhancers, intracerebroventricular injection	Steiner-J. et al., 2004 (449)

CLINICAL ASPECTS

The expression of iNOS is an early marker for inflammatory processes (261) and in a number of chronic inflammatory diseases, high-output NO synthesis is considered to contribute to local tissue destruction (262). When expressed in endothelial cells (EC) (451) and in infiltrating macrophages (266), iNOS may trigger apoptotic and necrotic cell death in the neighbouring tissue via high-output NO synthesis. Therefore, specific inhibition of iNOS enzyme activity or gene expression was often considered a suitable target for therapeutic intervention in chronic inflammatory diseases as for instance in atherosclerosis. However, attempts to inhibit high-output NO synthesis have underlined the dichotomous role of iNOS-derived NO as a molecule also displaying protective and thus beneficial activity (483). Thus, it comes as no surprise that inhibition of iNOS activity severely aggravates transplant artherosclerosis (436). Furthermore, in one of the earliest studies on NO-mediated protection, it was shown that NO protects murine endothelia from TNF-α induced cell death (104). A second study described that either a NO-donor or transfection of iNOS into sheep endothelia will prevent LPS-induced cell death in pulmonary artery endothelial cells (66). However, there are also settings in which an inhibition of NO production might be reasonable, for instance in tumor genesis. The exact role of NO in the context of tumor growth and progression is still under debate, but iNOS-specific knock-down may represent a promising target for tumor treatment since neoangiogenesis in malignomas is known to be driven by iNOS-derived NO among other factors. Since NO is a key regulator of anti- and pro-apoptotic proteins as well as pro-angiogenic genes like VEGF, it is certainly a promising approach to inhibit NO production by AS-molecules thereby affecting tumor angiogenesis and thus general malignant growth. Furthermore, the absence or decline of NO levels would upregulate proapoptotic and downregulate antiapoptotic proteins (76, 265) thereby contributing to inhibition of tumor cell proliferation.

In the last few years, a number of first generation synthetic ODN have entered into human clinical trials. Currently AS-ODN are tested in the treatment of CMV retinitis (97, 484), Crohns Disease (158, 191), Psoriasis (499), Rheumatoid Arthritis (158, 315), various types of tumors (4, 67, 94, 325, 363, 376, 463), HIV (309), coronary restenosis (144, 236), various lymphoma (15, 488), and other diseases. Targets in these clinical trials are for instance VEGF (180), ICAM-1 (158), C-raf (163, 496), Ha-ras (496), c-myc (36, 496), and bcl-2 (67). Although ODN for the local treatment of cytomegalovirus retinitis have been accepted already, this new class of compounds still faces many obstacles.

Cellular ODN internalization is a prerequisite for any AS effect and lipid formulations are often the easiest tool to reach this goal. It is quite surprising that most researchers used Lipofectin or LipofectAMINE since there is a large variety of similar transfection reagents and uptake enhancers with only slight changes in the molecular structure. A promising approach

to an efficient uptake is the use of ODN-encapsulating substances that only attach to cells with specific receptors on the plasma membrane. Encapsulating AS molecules into erythrocyte membranes for instance targets macrophages only (73). Cholesterol-ODN associate with low density lipoproteins (LDL) and are taken up by LDL receptor-carrying cells only (255). Furthermore, immuno-liposomes carrying anti-CD32 or anti-CD2 show high potency for cell specific incorporation. Compared with non-encapsulated ODN (307), an increased ODN uptake into human leukaemic cells by 3 - 5 fold results from the use of an antibody-linked immunoliposome (308).

OUTLOOK

Over the last 25 years, the AS method has constantly received changes, especially concerning the structure of the ODN, e.g. backbone modification. At the same time, alternative molecules like double-stranded (ds) RNA (also called siRNA technique) have been examined for their inhibitory potential in comparison to the established AS method. RNA interference is a process of sequence-specific, post-transcriptional gene silencing, initiated by ds RNA with complete sequence homology to the silenced gene (53, 138, 174, 218, 434, 479). Undoubtedly, the concept of RNA-interference might represent an interesting alternative (130, 403). However, to date, no trial using ds RNA for iNOS knock-down has been reported. The effectiveness between the two PNA-applying studies so far performed varies widely, although both researchers used the same cell type (inhibitory success 40% compared to 100% inhibition) (73, 162). Therefore, it is not yet clear whether ODN, ds RNA-molecules, PNA, or new structures will be the molecules gaining most attention in the field of iNOS knock-down over the next years.

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INPUT OF AMPHOTERICIN B ON ENDOTHELIAL EXPRESSION AND ACTIVITY OF THE INDUCIBLE NITRIC OXIDE SYNTHASE

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Nitric oxide (NO), released by the inducible nitric oxide synthase (iNOS), contributes to anti-microbial defence mechanisms by the innate immunity. Recent data examining the role of the endothelium as a defence barrier against microorganisms implicate a considerable role for endothelial cells expressing iNOS as an effective tool of the innate immune system.
Using reporter gene assays, RT-PCR, Western blot analysis, and by the quantification of nitrite in culture supernatants we have tested the effects of the anti-fungal agent amphotericin B (AmB) on the expression and activity of the iNOS in endothelial cells. AmB alone does not induce iNOS but cytokine-challenge in the presence of amphotericin B, used at a concentration optimal for anti-fungal action, led to significantly enhanced iNOS mRNA and protein expression, and subsequent increases in nitrite formation, as compared to cytokine activated cells only. These effects are due to an amphotericin B induced rise of endothelial expression of the iNOS-inducing cytokine IL-1\{b}, increase in iNOS mRNA stability, and improvement of iNOS gene transcription efficiency. In contrast, incubation of cytokine activated endothelial cells with a higher albeit subtoxic concentration of amphotericin B resulted in reverse effects, abolishing iNOS mRNA and protein expression accompanied by a highly significant reduction of endothelial NO production as a result of a strong decrease in iNOS promoter transcription rate.
The experiments demonstrate a concentration dependent biphasic effect of amphotericin B on the expression and activity of the iNOS in endothelial cells by affecting endogenous cytokine expression, iNOS mRNA stability and gene transcription rates. These effects may contribute to the anti-fungal activity of amphotericin B pointing to endothelial iNOS as an effector tool of the innate immune system.

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EXPRESSION OF STRESS DEFENCE GENES IS IMPAIRED BY ANTISENSE OLIGO-DESOXYNUCLEOTIDE-MEDIATED INHIBITION OF ENDOTHELIAL iNOS FORMATION

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High output nitric oxide (NO) synthesis by the inducible nitric oxide synthase (iNOS) occurs in many cell types after pro-inflammatory challenge. Cellular iNOS expression and high-output NO formation may contribute to cell death in chronic inflammation, but have recently also been acknowledged as regulators of gene expression and of the immune response. The aim of the present study was to define a suitable protocol for inhibiting iNOS protein expression using antisense oligodesoxynucleotides (AS-ODN) in non-transformed endothelial cells as this cell type plays a major role during inflammatory reactions and represents a possible target for *in vivo* intervention. We achieved specific antisense inhibition in cells pre-incubated with complexes of ODN and lipids in serum-free medium and subsequent activation by cytokines (IL-1β, IFN-γ, and TNF-α, each at 1000 U ml^{-1}). Lipid vesicles had to be used since ODN uptake was absolutely negative in the absence of vehicles. Expression of iNOS mRNA, protein, and NO production were monitored under antisense and control treatments. Additionally, the expression of the injury response protein vascular endothelial growth factor, the stress response gene heme oxygenase-1, the anti-apoptotic protein Bcl-2, and the inflammation marker IL-1β were examined. We find a significant downregulation of iNOS-mRNA, protein and NO-formation, which is specific since cytokine-induced IL-1β formation is not affected and a scrambled sequence control ODN as well as a non-related control ODN gave no inhibition. We observe a simultaneous inhibition of heme oxygenase-1, and Bcl-2 as well as a strong downregulation of vascular endothelial growth factor, all three gene products being part of the cellular stress defense. These results confirm the role of NO in modulating gene expression and demonstrate that lipid-encapsulated antisense oligodesoxynucleotides represent a powerful tool for inhibition of iNOS expression and NO formation. Due to the downregulation of important stress responses, iNOS antisense oligonucleotides might serve as therapeutic agents in tumor treatment.

OPTIMIZED ANTISENSE OLIGONUCLEOTIDE-MEDIATED INHIBITION OF THE INDUCIBLE NITRIC OXIDE SYNTHASE CO-SUPPRESSES NO-MEDIATED STRESS RESPONSE IN ENDOTHELIUM

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The input of the inducible nitric oxide synthase (iNOS) and high output nitric oxide (NO) formation during inflammation are controversially discussed in endothelial biology and pathobiology. In the sequence of established methods for iNOS inhibition, the antisense (AS) technique represents the most specific one. Unfortunately and in contrast to the expected results, AS-experiments with endothelial cells (EC) have not been very satisfactory so far. The aim of the present study was therefore to optimize the procedure of AS-iNOS-blockade for EC to reach the effectiveness which is expected in theory. EC were incubated with iNOS-specific antisense and control-oligonucleotides (ODN) in Opti-MEM Serum Reduced Medium for 5.5 hours and then activated by cytokine addition (IL-1β, IFN-γ, and TNF-α, each at 1000 U ml^{-1}). Lipofectin was used as vehicle because ODN uptake was negative without uptake enhancers. Intracellular ODN accumulation was analyzed by fluorescence microscopy and expression of iNOS mRNA, protein, and NO production were monitored. We here show for the first time that in EC, an optimized AS-technique represents a more specific and less toxic, and therefore a more promising instrument for the inhibition of iNOS than other methods, leading to a nearly complete inhibition of iNOS protein formation. This effect is accompanied by a significant reduction in the expression of the stress response genes Bcl-2, VEGF, and HO-1, giving further evidence for the pivotal role of the iNOS in regulating the expression of these vascular factors.

iNOS-INHIBITION BY ANTISENSE OLIGONUCLEOTIDES IS HIGHLY EFFECTIVE FOR REGULATING NITRIC OXIDE-MEDIATED STRESS RESPONSE IN ENDOTHELIUM

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Introduction: The effects of inducible nitric oxide synthase (iNOS) expression and high output nitric oxide formation during inflammation are still discussed controversially. The antisense (AS) technique represents a very suitable method for specific inhibition of iNOS protein expression and concomitant investigation of the dichotomous functions of the signal molecule nitric oxide. However, regarding the inhibition of endothelial iNOS, AS-experiments have not been very satisfactory so far.

Materials & methods: Rat aorta endothelial cells were isolated, grown following a routine protocol, incubated with iNOS-specific AS- or control-ODN in serum-free medium and finally activated by cytokine addition. Expression of iNOS mRNA and protein as well as nitric oxide production were monitored.

Results: We here show for the first time that in endothelial cells an optimized AS-technique represents a powerful instrument for an almost complete inhibition of iNOS activity. This effect is accompanied by a significantly reduced expression of the stress-response genes Bcl-2, VEGF, and heme oxygenase-1.

Conclusions & discussion: Our results verify that the antisense technique is more promising for *in vivo* trials than other methods of iNOS inhibition and may open up new perspectives for the treatment of endothelial dysfunctions and inflammatory disorders like systemic inflammatory response syndrom or shock.

PROTECTIVE STRESS RESPONSE AND REGULATION OF INFLAMMATION BY iNOS: DOES LIMITED SUBSTRATE AVAILABILITY EXPLAIN CHRONIC INFLAMMATION?

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Nitric oxide (NO) locally released during inflammatory immune-mediated diseases was initially perceived as a contributor to tissue destruction, but recent studies demonstrate that iNOS-derived NO modulates cellular gene expression and exerts important immunoregulatory functions contributing to the downregulation of inflammatory responses. Paradoxically, a large number of chronic proinflammatory diseases are closely associated with iNOS expression, posing an unsolved question on the role of iNOS activity in these disorders.
We now have searched for an explanation for this paradoxon under the hypothesis that in these situations the iNOS enzyme activity might be inappropriately inactive.
First we find that maintenance of various human and rat cell types (HepG2, AKN, HaCat, human keratinocytes, rat hepatocytes and endothelial cells) at physiological L-arginine levels of 60 to 100 µM does not allow for maximal iNOS activity due to coexpressed arginase-1 enzyme activity. Blocking the latter, leads to increases of iNOS activity by 40 to 60% in the various cells types, with the exceptions of HaCAT and AKN cell lines, that completely lack arginase-1 expression.
We also examined the impact of iNOS activity on the increased expression of cellular stress response genes. With the use of iNOS-specific antisense oligonucleotides we find a highly significant impairment of stress response gene expression in HepG2 and primary rat endothelial cells (EC). An analogous disfunction in stress response is also seen in EC, when cultured under restricted arginine supply, a condition that renders these endothelial cells highly sensitive to oxidative stress.
We then re-examined the situation in human psoriasis, a chronic proinflammatory skin disease where iNOS expression is always observed. We find a highly significant overexpression of arginase-1 mRNA and protein in psoriatic lesions and can link arginase overexpression and impaired iNOS activity to keratinocyte hyperproliferation, a hallmark of this skin disease. These findings raise the possibility, that inadequately low iNOS-derived NO-synthesis due to restricted L-arginine availability or due to arginase-1 overexpression might contribute to the chronicity of immune-mediated proinflammatory disease conditions.

IMPAIRED INTRACELLULAR ARGININE SUPPLY ENHANCED REACTIVE OXYGEN SPECIES-INDUCED CELL DEATH OF CYTOKINE-ACTIVATED ENDOTHELIAL CELLS

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Endothelial cells, like macrophages, display a capacity and represent a source for nitric oxide production by cytokine-induced expression of inducible NO-synthase (iNOS). Although being toxic under some conditions, NO can exert significant protection from reactive oxygen species (ROS)-induced cell death. Since iNOS activity may be modified by availability of its substrate, the objectives of the present study were to elucidate the influence of endothelial L-arginine supply on iNOS-mediated protection from ROS-induced cytotoxicity. Cytokine activated (IL-1β+TNF-α+IFN-γ, 500 U/ml each) rat aortic endothelial cells (RAEC) were cultured for 24 hours in the presence of L-arginine at various concentrations (0 to 1000 µM) or in addition were incubated with hydrogen peroxide (H_2O_2;0 to 0.9 mM). After H_2O_2-challenge a good correlation between iNOS activity and protection from ROS-induced cell death was observed: as compared to cytokine-activated RAEC grown in the absence of arginine (0.5 µM nitrite and 83% toxicity), activated cells grown in the presence of 200 µM arginine were fully protected from H_2O_2-induced cell death (15 µM nitrite and 10% toxicity). Inhibition of iNOS (0.5 mM L-NIO) completely abrogated iNOS-mediated protection. Increased cell death was also observed with restriction of L-arginine transport in the presence of L-lysine+L-ornithine (20 mM each). Addition of exogenously applied NO (DETA/NO, 1mM) led to full protection in these conditioned cells. In conclusion, we here describe that in cytokine-activated endothelial cells impaired intracellular L-arginine supply (or utilization) as may be found locally during wound healing or inflammatory disorders will enhance ROS-induced cell death via impairment of iNOS activity.

ENDOTHELIAL STRESS RESPONSE IS IMPAIRED BY LIMITED ARGININE AVAILABILITY OR ANTISENSE MEDIATED KNOCK-DOWN OF INDUCIBLE NITRIC OXIDE SYNTHASE

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In endothelial biology, the input of inducible nitric oxide synthase (iNOS) and high output nitric oxide (NO) formation are generally assumed to be detrimental to endothelial function. Thus, a temporary cessation of iNOS expression or activity should prevent endothelial cell dysfunction. This study aimed at using an antisense (AS)-mediated iNOS knock-down during proinflammatory cytokine challenge to study endothelial expression of stress defense genes. Rat aorta endothelial cells were incubated with iNOS-specific AS- or control-oligonucleotides (ODN), loaded in Lipofectin, in serum-free medium for 5.5 h and then activated by cytokine addition. Moreover, cells were maintained in the presence of limited exogenous substrate concentrations during cytokine challenge to mimic a situation of low serum arginine level and inflammation. Expression of iNOS mRNA, protein, and NO production were monitored. By using AS-ODN, we achieved a block of iNOS protein formation and a strong decrease in the expression of the protective stress response genes (BCL-2, VEGF, HO-1). Limited exogenous substrate concentrations revealed similar results. Our data show that cytokine-induced iNOS expression and activity have key functions in increasing endothelial survival and function. Suppression of iNOS or limited substrate supply contribute to endothelial dysfunction and cell death during oxidative stress.

ANTISENSE OLIGONUCLEOTIDES TO INDUCIBLE NITRIC OXIDE SYNTHASE AS INTERVENTION STRATEGY IN NITRIC-OXIDE MEDIATED SKIN DISEASES?

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Nitric oxide (NO) is an important molecular mediator with physiological and pathophysiological functions in all organ systems, including the skin. NO is generated by the enzyme family of NO synthases that metabolize arginine and molecular oxygen to citrulline and NO. Expression of the cytokine-inducible NO synthase (iNOS) and subsequent high-output NO synthesis represent an early consequence and marker of proinflammatory reactions also in a number of acute and chronic skin diseases. Several studies point to abnormal iNOS expression or activity or defects in NO signalling as factors contributing to disease pathogenesis especially in chronic skin diseases. The use of antisense oligonucleotides (AS-ODN) potentially allows for effective and specific inhibition of iNOS expression and activity, further, for a successful *in vivo* application endothelial cells represent prime target cells. Therefore, we here present data on the effect and specificity of this technique in primary endothelial cells in an inflammatory surrounding.

Primary rat aorta endothelial cells (EC) were incubated with iNOS-specific AS- and control-ODN, loaded in carrier vehicles, in Opti-MEM Serum Reduced Medium for 5.5 hours and then activated by addition of proinflammatory cytokines. Intracellular ODN accumulation was analyzed by fluorescence microscopy and expression of iNOS mRNA, protein, and NO production were monitored.

In EC, an optimized AS-technique leads to a nearly complete inhibition of iNOS protein formation and represents a specific and promising instrument for inhibition of iNOS activity with little toxicity. Lipid vehicles are necessary and pose a problem under inflammatory conditions, as they can potentially increase instead of decrease iNOS expression in the presence of cytokines. This inhibition is specific not interfering with other cytokine-inducible gene products. One additional result of our study is, that iNOS-derived NO is essential for the expression of the protective stress response genes Bcl-2, VEGF, and HO-1, giving further evidence for the pivotal role of iNOS activity in regulating gene expression.

NITRIC OXIDE INDUCES PREADIPOCYTE DIFFERENTIATION – A PROMISING REAGENT FOR ADIPOSE TISSUE ENGINEERING

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In this study, we analyzed the effects of nitric oxide (NO) on preadipocytes, stem-cell like progenitor cells in the adipose tissue, and endothelial cells (EC) in a setting as found after transplantation of a 3D-biohybrid composed of viable EC and adipose precursor cells. NO is an important molecular mediator with physiological and pathophysiological functions in all organ systems, including the endothelium and adipose tissue. Expression of the cytokine-inducible NO synthase (iNOS) and subsequent high-output NO production represent early consequences of proinflammatory reactions as found in wounds after transplantations.

Preadipocytes were isolated from human subcutaneous adipose tissue samples, cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with 10% fetal calf serum (FCS), and differentiated after culture expansion by adding insulin, isobutylmethylxanthine, pioglitazone, dexamethasone, and transferrin without FCS. The influence of NO on proliferation and differentiation was evaluated after adding DETA/NO at various concentrations for varying periods of time. Parallely, primary rat aorta endothelial cells (EC) were isolated, cultured in RPMI 1640/ 20% FCS, and treated with DETA/NO to analyze the effect of NO on the stress response genes Bcl-2, vascular endothelial growth factor (VEGF), and heme oxygenase (HO)-1.
We find that NO in combination with the conventionally used differentiation-inducing factors significantly enhances maturation of adipogenic precursors to adipocytes. Proliferation, in contrast, is inhibited by NO. Treatment of endothelial cells with DETA/NO causes the upregulation of Bcl-2, VEGF, and HO-1. Our results emphasize the pivotal role of iNOS activity in regulating gene expression. Further, they are encouraging for applying NO during transplantation of preadipocytes and EC in a three-dimensional construct since NO helps to optimize differentiation of adipogenic precursor cells, to inhibit apoptosis by upregulating Bcl-2, and to stimulate angiogenesis by inducing production of the proangiogenic VEGF.

OPTIMIZED ADIPOSE TISSUE ENGINEERING WITH THE USE OF NITRIC OXIDE-DONATING AGENTS

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Introduction: This study aims at analyzing the effects of nitric oxide (NO) on preadipocytes and endothelial cells in a setting as found after transplantation of a biohybrid composed of viable endothelial cells and adipose precursor cells in a three-dimensional matrix. Such an implant may represent an adequate solution for correcting soft tissue defects, e. g. extensive deep burns, tumor resections, hereditary and congenital defects. NO is an important signalling molecule regulating physiological and pathophysiological functions throughout the body, including endothelial and adipose tissue activities. NO is generated by the enzyme family of NO synthases that metabolize arginine and molecular oxygen to citrulline and NO. Expression of the cytokine-inducible NO synthase (iNOS) and subsequent high-output NO synthesis represent an early consequence and marker of proinflammatory reactions as found in wound situations after transplantation.

Materials & Methods: Preadipocytes were isolated from human subcutaneous adipose tissue samples and cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) with 10% fetal calf serum (FCS). After 7-14 days of culture expansion, differentiation was induced by insulin, isobutylmethylxanthine, pioglitazone, dexamethasone, and transferrin in the absence of FCS. To evaluate the influence of NO on proliferation and differentiation, the NO donor molecule DETA/NO was added at various concentrations. Proliferation was evaluated by microscopy and the extent of differentiation was assayed after 15 days by cell counting and analysis of the enzyme glycerophosphate dehydrogenase highly expressed in mature adipocytes.

In parallel, primary rat aorta endothelial cells (EC) were isolated and cultured in RPMI 1640/ 20% FCS for up to 8 passages. DETA/NO was added at a concentration of 1 mM to analyze the effect of NO on the stress response genes Bcl-2, vascular endothelial growth factor (VEGF), and heme oxygenase (HO)-1.

Results: We find that NO in combination with the conventionally used differentiation-inducing factors significantly enhances maturation of precursor cells to adipocytes. NO also increases the number of differentiated cells if adipogenic conversion is only promoted by dexamethasone, insuline, and transferrin. Proliferation of preadipocytes, in contrast, is inhibited in

the presence of NO. Treatment of EC with DETA/NO causes the upregulation of the antiapoptotic protein Bcl-2, the pro-angiogenic VEGF, and the antioxidative HO-1.

Conclusion: Our results emphasize the pivotal role of iNOS activity in regulating gene expression. Further, they are encouraging for applying NO-donors during transplantation of preadipocytes and EC in a three-dimensional setting since it helps optimizing differentiation of adipogenic precursor cells and stimulating angiogenesis by induction of the proangiogenic VEGF. Therefore, NO-supplemented implants with optimized preadipocyte maturation and improved vascularisation open new perspectives for adipose tissue engineering to treat extended soft tissue defects.

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ANTISENSEHEMMUNG DER INDUZIERBAREN STICKSTOFFMONOXIDSYNTHASE – IST DIES SINNVOLL?

ABSTRACT

Die Bedeutung von Stickstoffmonoxid (NO), welches durch eine induzierbare Stickstoffmonoxidsynthase (iNOS) aus Arginin nach Aktivierung durch proinflammatorische Zytokine oder durch Bakterienbestandteile gebildet wird, ist immer noch widersprüchlich. Die Expression der iNOS im Rahmen von Entzündungen ist vorwiegend zu finden in Zellen epithelialen Ursprungs. In diesem Kontext wirkt NO protektiv durch Begrenzung von Gewebsschäden und durch Hemmung der Ausbreitung von Pathogenen. Die lokale Entzündungsreaktion wird so limitiert durch Anstoßung einer Th2-Antwort im Sinne eines klassischen Feedback-Mechanismus. Allerdings weist die Literatur der iNOS bzgl. chronischer Krankheiten mit vorwiegend Th1-gesteuerten Reaktionen eine destruktive Rolle zu. Für diese Konstellationen erscheint eine spezifische Enzymhemmung überaus wertvoll. Diverse Methoden zur iNOS-Hemmung sind in der Vergangenheit schon etabliert worden, um die Kontroverse von protektiver versus destruktiver Wirkung von NO klären zu können. Die Antisense-mediierte Genhemmung stellt eine relativ neue Methode zur Hemmung der NO-Produktion dar. Da Endothelzellen ein besonders geeignetes Ziel für eine potentielle in vivo Anwendung der Antisensetechnik darstellen, beschäftigt sich diese Dissertation mit der Etablierung und Optimierung der Antisensemethodik an primären Rattenendothelzellen. Vorgestellt wird ein optimiertes Protokoll für die iNOS-Hemmung in Endothelzellen mit Ergebnissen über die Aufnahme fluoreszenzmarkierter Oligonukleotide (ODN), die Einflüsse von Serum im Medium sowie die Unterschiede zwischen verschiedenen Lipiden, die zur Verbesserung der ODN-Aufnahme getestet wurden. Die Ergebnisse werden eingebettet in die Diskussion über bereits etablierte Techniken sowie deren Erfolge. Dabei geht es insbesondere um das Design von Antisense-Experimenten: Wie sollen ODN aussehen, wie kann die Aufnahme der ODN nach intrazellulär sichergestellt werden und wie kann diese zelluläre Inkorporation adäquat kontrolliert und dargestellt werden. Zwar konnte eine 95%ige iNOS-Hemmung erreicht werden, jedoch zeigte sich dabei auch eine Hemmung der protektiven, NO-gesteuerten Gene bcl-2, VEGF und Hämoxygenase-1. Diese Expressionhemmung protektiver Gene ließ sich auch bei Kultivierung der Endothelzellen in Arginine-armem Medium demonstrieren, da hier aufgrund von Substratmangel trotz Stimulation der iNOS durch Zytokine kaum NO produziert wurde. Unsere Ergebnisse zeigen somit, daß eine iNOS-Hemmung durch Antisense-ODN zwar möglich ist, jedoch aufgrund der Nebenwirkungen höchst kritisch zu betrachten ist. Zum Schutz und zur Funktionserhaltung des Endothels erweist sich die iNOS als extrem wichtig. Eine Hemmung des Enzyms genauso wie niedrige Argininspiegel, wie sie z.B. im Rahmen der Atherosklerose auftreten, fördern endotheliale Schädigung und Dysfunktion.