iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage

Karsten Hemmrich, Christoph V. Suschek, Guido Lerzynski, and Victoria Kolb-Bachofen
Research Group Immunobiology, Heinrich-Heine-University of Düsseldorf, D-40001 Düsseldorf, Germany
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Hemmrich, Karsten, Christoph V. Suschek, Guido Lerzynski, and Victoria Kolb-Bachofen. iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage. J Appl Physiol 95: 1937–1946, 2003.. First published July 25, 2003; 10.1152/japplphysiol.00419.2003.—In endothelial cells, the expression of the inducible nitric oxide synthase (iNOS) and the resulting high-output nitric oxide synthesis have often been assumed as detrimental to endothelial function, but recent publications have demonstrated a protective role resulting from iNOS expression and activity. To address this question, we used antisense-mediated iNOS knockdown during proinflammatory cytokine challenge in primary endothelial cell cultures and studied endothelial function by monitoring the expression of stress defense genes. Using antisense oligonucleotides, we achieved a block of iNOS protein formation, accompanied by a strong decrease in the expression of the protective stress response genes bcl-2, vascular endothelial growth factor, and heme oxygenase-1 (HO-1). Additionally, cells were also maintained in the presence of limited exogenous substrate concentrations during cytokine challenge, thereby mimicking a situation of low serum arginine level during inflammation. Under these conditions, cytokine addition results in full iNOS protein expression with minimal nitric oxide formation, concomitant with a significant reduction in stress response gene expression and susceptibility to cell death induced by reactive oxygen species. Taken together, our data suggest that cytokine-induced endogenous iNOS expression and activity have key functions in increasing endothelial survival and maintaining function. Thus suppression of iNOS expression or limited substrate supply, as has been reported to occur in atherosclerosis patients, appears to significantly contribute to endothelial dysfunction and death during oxidative stress.

ENDOTHELIAL CELL (EC) production of nitric oxide (NO) plays a pivotal role in the maintenance of vascular function and health (18). Indeed, evidence is accumulating that endothelial dysfunction in hypercholesteremic patients or in animal models of atherosclerosis is linked to impaired NO synthesis (8). Thus inhibition of endothelial NO synthesis leads to increases in adhesion molecule expression, smooth muscle proliferation, and low-density lipoprotein uptake (18).

A number of experimental studies indicate that impaired NO synthesis in vivo may be due to increased systemic levels of asymmetric dimethyl arginine, an endogenous competitive NO synthase (NOS) inhibitor (3, 8). 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 (3, 8). First, measurements of arginine serum levels indicate that low substrate concentrations correlate with disease progression or severity (30). Second, 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 in Ref. 30).

It is generally assumed that the NOS enzyme isoform responsible for the improvement of endothelial function during atherogenesis has to be the constitutively expressed endothelial NOS (eNOS or NOS3) (3, 8). However, under chronic proinflammatory conditions, such as those at work during arteriosclerosis, a local expression of the inducible NOS (iNOS or NOS2) isoform is seen in endothelia and other cell types (42). 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 (34). However, more recent investigations have linked a powerful protective activity toward cellular stress conditions with iNOS-derived NO synthesis (4, 37). Moreover, microarray studies have shown that iNOS-derived NO serves as a modulating activity of the expression of many different genes that also affect protective responses during stress conditions (13, 37, 44).

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 (AS) oligonucleotides (ODN) were used to effectively inhibit iNOS expression, and, alternatively, the cells were maintained under limited ar-
We examined the endothelial stress response by monitoring the expression 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 (HO-1), bcl-2, and vascular endothelial growth factor (VEGF)], expressional increases by NO were already shown at the mRNA as well as at the protein level (12, 14, 32, 37, 44). We here correlate this gene expression control of iNOS-derived NO with endothelial dysfunction and cell death during oxidative stress response.

**METHODS**

*Reagents.* Recombinant human interleukin (IL)-1β was from HBT (Leiden, Netherlands); recombinant murine gamma-interferon (IFN-γ) and recombinant murine tumor necrosis factor (TNF)-α were from Genzyme (Cambridge, MA); butylated hydroxytoluene, EC growth supplement, 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 antiserum, 2-mercaptoethanol, propidium iodide, and anti-tubulin-antibody were from Sigma Chemical (Deisenhofen, Germany); the rat endothelium-specific monoclonal antibody Ox43 was from Serotec (Camon, Wiesbaden, Germany); the monoclonal anti-mouse iNOS antibody was from Transduction Laboratories (Lexington, KT); the iNOS inhibitor 1,N′-(1-iminoethyl)-ornithine (L-NIO) was from Qbiogene (Irvine, CA); the iNOS inhibitor Nω-(1-iminoethyl)-ornithine (L-NIO) was from Qiagen (Hilden, Germany); peroxidase-conjugated porcine anti-rabbit IgG was from DAKO (Hamburg, Germany); peroxidase-conjugated goat anti-mouse IgG was from Zymed Laboratories (San Francisco, CA); trypsin, EDTA, and fetal calf serum (FCS; endotoxin free) were from Boehringer Mannheim (Mannheim, Germany); RPMI 1640 without L-calf serum (FCS; endotoxin free) were from Boehringer Mannheim (Mannheim, Germany); 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 of the manufacturers. After 5.5 h of incubation, cells were activated by cytokine challenge. After 24 h of cytokine incubation, nitrite formation in culture supernatants was detected, and cells were lysed with 2-mercaptoethanol for PCR analysis. Cells that 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 H2O2 for 16 h.

**Nitrite determination.** Nitrite was determined in culture supernatants by using sulfanilamide and naphthalene-diamine in the Griess diazotization reaction, as modified by Wood et al. (43) using NaN3O2 as standard.

**Determination of growth rates and viability and detection of cell death.** Cell growth was determined at different times by neutral red staining (17). Additionally, viability of EC was controlled routinely at the beginning and end of every experiment by 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 detection of DNA strand breaks with the in situ nick-translation method, both exactly as described previously (37). 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 ECs (2 × 105) were incubated with H2O2 at concentrations indicated in the absence or presence of the respective additives for 18 h. Then lipid peroxidation was stopped by addition of butylated hydroxytoluene (10 μM). Cells were lysed by repeated freezing and thawing. Lipid peroxidation was measured by determination of thiobarbituric acid reactive substances with high-performance liquid chromatography and expressed as malondialdehyde equivalents exactly as described previously (16).

**RT and PCR.** Total cellular RNA (1 μg each) was prepared from resting or cytokine-activated cells by using the Omniscript RT kit, and RT was carried out at 37°C for 60 min with oligo(dT) (15-mer) as primer. cDNA (500 ng each) was used as template for PCR, primed by using ODNs and conditions as shown in Table 1. Extensive testing for each gene product

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was performed to ascertain that amplifications were always in the linear amplification range.

After amplification, PCR products were subjected to ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed by 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 the iNOS protein. Cells treated as indicated were washed, scraped from the dishes, lysed, transferred to a microcentrifuge tube, and boiled for 5 min in 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 h in blocking buffer (2% BSA, 5% nonfat milk powder, 0.1% Tween 20 in PBS buffer), 1 h with a 1:2,000 dilution of the secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody. Finally, blots were incubated for 5 min in enhanced chemiluminescence reagent (Pierce, Rockford, IL) 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 1:2,000.

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

RESULTS

Impact of AS ODN on iNOS mRNA synthesis, protein expression, and enzyme activity. Rat aorta 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 h, the fluorochrome-labeled ODNs 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 labeled of 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 h demonstrates bright nuclear fluorescent signals with additional labels in cytoplasmic vesicles (labeling 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 of two control ODN sequences, AS ODN, or empty lipid vesicles were added to the EC cultures, followed by a 5.5-h incubation period and subsequent cytokine activation (IFN-γ, IL-1β, TNF-α; 1,000 U/ml each) for 24 h. Analysis of iNOS mRNA expression showed that the cytokine-mediated activation induced the de novo formation of iNOS mRNA as expected (Fig. 1A, lane 5). Interestingly, the vehicle control, i.e., empty lipid vesicles, led to a significant increase in iNOS-specific mRNA formation (Fig. 1A, 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 (Fig. 1A, lane 2). Incubation with AS ODN plus lipid carriers resulted in a moderate, but significant, decrease in iNOS mRNA expression (Fig. 1A, lane 4). Thus we find a sequence-specific inhibition of iNOS mRNA formation of ~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 (Fig. 1B, lane 3). We find that AS ODN almost completely inhibited iNOS protein formation (inhibition = 95% in Fig. 1B, lane 2).
lane 4), whereas control ODN (Fig. 1B, lane 2) was identical to sham-treated controls (Fig. 1B, lane 5).

To also assess the effect of AS ODN on NO formation, nitrite accumulation in the culture supernatants was measured as described in 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 ODNs (Fig. 2A). A remarkable increase in nitrite accumulation was seen when lipid carriers, i.e., Lipofectin, were incubated with cytokines in the absence of ODN (Fig. 2B).

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

Impact of iNOS AS 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 that 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 (Fig. 3E). Thus AS ODN-mediated inhibition of iNOS must be regarded as specific, not generally interfering with the expression of cytokine-inducible genes.

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 HO-1, the anti-apoptotic protein bcl-2, and the injury response protein VEGF. Indeed, we find...
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 vs. 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 (Fig. 4). 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 (Fig. 4, lane 2), which was inhibited by addition of the NOS inhibitor L-NIO (0.5 mM) (Fig. 4, lane 3). In contrast, cytokine-induced nitrite formation was below the detection limit in cultures grown under arginine-depleted conditions (Fig. 4, lane 5).

When the effect of arginine on the expression of endothelial stress response genes was analyzed (Fig. 5), we found that endogenous iNOS-derived NO synthesis completely paralleled the expression of the genes bcl-2, HO-1, and VEGF. Compared with nonactivated 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 (Fig. 5, lane 2). Both competitive inhibition by the NOS inhibitor L-NIO (Fig. 5, lane 3) and limitation of arginine supply (Fig. 5, lane 5) significantly decrease the mRNA expression of these three genes.

A direct correlation between iNOS expression and activity and the endothelial expression of the three gene products (Fig. 3, B–D). Of note is that with VEGF inhibition affects all three splice variants that can be detected with the primer pair used (12). 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 experiments with empty lipid vesicles, because these increase the mRNA expression of all three studied genes to the same degree as the iNOS mRNA expression.
genes to control levels. Addition of the NO-donor molecule diethylenetriamine (DETA) NO (1 mM) fully restores the increases in bcl-2, HO-1, and VEGF mRNA expression in the absence of arginine (Fig. 5, lane 6). The effect of AS-mediated knockdown of iNOS expression is comparable with the effect of limited substrate supply as regards the expression of the stress response genes tested.

Role of iNOS activity in increasing endothelial resistance toward \( \text{H}_2\text{O}_2 \)-induced apoptosis. We next examined whether this altered gene-expression profile correlated with endothelial survival during oxidative stress, which was experimentally induced by the addition of \( \text{H}_2\text{O}_2 \). 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 knockdown experiments for such a study.

Resting EC cultured at the high-physiological arginine concentration of 200 \( \mu \text{M} \) in the presence of increasing \( \text{H}_2\text{O}_2 \) concentrations showed a concentration-dependent increase in cell death as determined after 18 h. Cytotoxicity reached a half-maximal level at 0.6 ± 0.05 mM \( \text{H}_2\text{O}_2 \) and maximal death of >80% of the cells at and above 0.8 mM \( \text{H}_2\text{O}_2 \) (Fig. 6A). 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 L-NIO (0.5 mM) (Fig. 6A).

Cell death occurs via apoptosis, as confirmed by detecting DNA strand breaks by using in situ nick translation (Fig. 6, B and C) or by staining with the Hoechst dye (Fig. 6, D and E). Addition of catalase (2,000 U/ml) or butylated hydroxytoluene (10 mM), the latter an inhibitor of lipid peroxidation, blocks this apoptosis (Fig. 6F), indicating that lipid peroxidation represents an initiating event. Indeed, \( \text{H}_2\text{O}_2 \) challenge leads to a marked increase in lipid peroxidation in resting cells, as analyzed by detection of malondialdehyde formation (Fig. 6F, 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 the addition of L-NIO (0.5 mM) again abrogated this protective effect. The effect of arginine on \( \text{H}_2\text{O}_2 \)-induced cell death is demonstrated by the close correlation between the exogenous arginine concentration and the degree of cell survival (Table 2). Thus cytokine-activated ECs grown in the presence of 200 \( \mu \text{M} \) arginine are highly sensitive toward \( \text{H}_2\text{O}_2 \)-induced death, comparable to nonactivated cells at any arginine concentration. Activated cells grown in the presence of 200 \( \mu \text{M} \) arginine are fully protected from \( \text{H}_2\text{O}_2 \)-induced cell death (Table 2). 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.

DISCUSSION

The expression of iNOS has to be considered as an early marker for inflammatory processes (22). Moreover, in a number of chronic inflammatory diseases, high-output NO synthesis is thought to contribute to local tissue destruction (23). When expressed in infiltrating macrophages (24) and in ECs (34), iNOS may trigger apoptotic and necrotic cell death in the neighboring 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 (31). 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 that also displays protective and thus beneficial activity (41). Indeed, inhibition of iNOS activity severely aggravated transplant atherosclerosis (33). Furthermore, in one of the earliest studies on NO-mediated protection, it was
seen that NO protects murine endothelia from TNF-α (9). A second study has shown that either a NO donor or transfection of iNOS into sheep endothelia will protect from lipopolysaccharide-induced death (5). Interestingly, in this latter study, a NO-mediated effect on gene expression caused by 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 antiapoptotic effect (38), and this metal has been repeatedly proven to alter stress response gene expression (20).

The experiments presented here were performed to achieve a successful AS-mediated iNOS inhibition in nontransformed ECs 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-1β expression was not affected, although this de novo induction uses partly identical pathways and follows a similar time pattern. Furthermore, it is impossible that iNOS-targeted ODN interacted with eNOS or neuronal NOS expression since there were no sequence matches between the iNOS-specific AS ODN chosen here and eNOS or neuronal NOS.

Fig. 6. Role of iNOS activity on apoptosis and lipid peroxidation during oxidative stress. A: with resting cells (white bars), H$_2$O$_2$ (0.8 mM for 18 h) leads to endothelial cell death in a concentration-dependent manner. Activated cells (black bars) were fully protected. Incubation in the presence of the NOS inhibitor L-NIO (gray bars; 0.5 mM) completely reversed this protection. Values are means ± SD of 6 individual experiments. *P < 0.001 compared with resting or L-NIO-treated cultures. B–E: apoptosis was monitored by in situ nick translation (C), which detected nuclei with DNA strand breaks or detected pyknotic and shrunken cells with condensed and/or fragmented chromatin/nuclei using the Hoestch-stain (E). B and D are representative micrographs of 3 individual experiments with identical outcome. Magnification: ×650. F: H$_2$O$_2$ challenge of resting cells leads to a strong increase in lipid peroxidation as ascertained by monitoring malondialdehyde (MDA) formation. However, the presence of catalase (2,000 U/ml) or the addition of butylated hydroxytoluene (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 means ± SD of 3 individual experiments. *P < 0.001 compared with only H$_2$O$_2$-challenged cells.
Table 2. Input of iNOS activity on \( H_2O_2 \)-induced cell death of endothelial cells

<table>
<thead>
<tr>
<th>Live Cells, %</th>
<th>200 ( \mu M ) arginine</th>
<th>5 ( \mu M ) arginine</th>
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<tr>
<td>Resting</td>
<td>16 ( \pm ) 4</td>
<td>21 ( \pm ) 9</td>
</tr>
<tr>
<td>Activated</td>
<td>88 ( \pm ) 11*</td>
<td>20 ( \pm ) 3</td>
</tr>
<tr>
<td>Activated + l-NIO</td>
<td>21 ( \pm ) 3</td>
<td>nt</td>
</tr>
<tr>
<td>Activated + DETA/NO</td>
<td>nt</td>
<td>77 ( \pm ) 8</td>
</tr>
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</table>

Values are means \( \pm \) SD of 4 individual experiments. Resting or cytokine-activated (IL-1\( \beta \) + TNF-\( \alpha \) + IFN-\( \gamma \), 500 U/ml each) rat aortic endothelial cells grown at physiological (200 \( \mu M \)) or depleted (5 \( \mu M \)) arginine concentrations were incubated for 16 h with 0.8 mM \( H_2O_2 \). The number of live cells was then determined by 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 \( \sim 80\% \) of the cells. In contrast, high-output NO formation in cytokine-challenged cells grown at 200 \( \mu M \) 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 NO synthase inhibitor l-N\( (1\text{-iminoethyl}) \)ornithine (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 diethylentetriamine NO adduct (1 mM DETA/NO). nt, Not tested. *\( P < 0.001 \).

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 (2).

When the impact of AS ODN on enzyme activity as assessed by accumulation of nitrite in the culture supernatants was investigated, the degree of inhibition did not completely reflect the nearly complete inhibition of protein formation. A similar observation has been made by using iNOS-specific AS inhibition in mouse mixed glial cell cultures (10). The reason for this finding may be the cytokine-mediated induction of proteins with accessory functions on enzyme activity, e.g., increased arginine transport via cytokine-inducible cationic amino acid transporter-2 (6), but lack of feedback inhibition might also be responsible for the few enzyme molecules formed to work at maximal activity. A role for increased eNOS activity can be ruled out as the source of NO production since eNOS is downregulated in these cells during cytokine challenge, as was shown previously (36).

When the impact of AS-mediated inhibition on stress-relevant gene expression is examined, our data demonstrate that the blockade of iNOS expression leads to diminished gene expression of proteins representing protective endothelial responses to various stresses. Thus upregulation of bcl-2 expression prevents apoptosis onset (37), and upregulation of HO-1 has been demonstrated to protect during reactive oxygen intermediates-mediated stress (11). The NO-driven expressional increase of HO-1 might well be the most important effect when the impact in atherosclerosis is considered, because it was recently shown that carbon monoxide, an enzyme product of HO-1, can suppress lesion formation (29). Furthermore, the increased VEGF expression promotes endothelial regeneration after injury (15, 26, 40). 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 (14, 32, 37). Our findings are also in complete agreement with the numerous recent reports demonstrating a protective activity of iNOS-mediated NO synthesis (19, 25, 28, 37, 39). However, the modulating effect of NO on the expression of stress response genes has not been directly linked to endothelial survival. In this study, we show for the first time that this tight regulatory control of NO on these genes mediated by the endogenously 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 knockdown. 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 knockdown cells, because the multiple treatments necessary for successive knockdown alters the cellular functions considerably and renders results difficult to control without affecting the experimental outcome, as highlighted also by the impact of empty vehicles 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. (30)] have found that relatively low arginine serum levels appear to correlate with disease progression in atherosclerosis and that oral arginine supplementation may be beneficial in animal models for hypercholesterolemia as well as in patients (8). Investigators have usually speculated but not shown that the beneficial effects of arginine supplementation on progression of atheroma are due to increases in constitutive eNOS activity (7, 8). In contrast, we here give evidence that disease progression might be associated with inappropriately low endothelial iNOS activity and thus a 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 a NO donor. Although the concentration used here (1 mM of DETA NO) appears high at first glance, calculation of the amount of NO generated shows a concentration that corresponds to reasonable levels. We had determined a half-life 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, 1 mM will thus generate 3.3 μM of NO per minute (1) and a calculation of the steady-state concentration of NO above an actively producing cell monolayer resulted in a value of ~4–5 μM of NO in the immediate vicinity (27). 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, although 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: (1) it helps to downregulate the production of inflammatory mediators (21), and (2) 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.

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DISCLOSURES

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