Nitric Oxide Attenuates Vascular Smooth Muscle Cell Activation by Interferon-γ

THE ROLE OF CONSTITUTIVE NF-κB ACTIVITY*

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Atherosclerosis involves cellular immune responses and altered vascular smooth muscle cell (SMC) function. Cytokines such as interleukin (IL)-1α and interferon-γ (IFN-γ) may contribute to this process by activating SMC. To determine whether the anti-atherogenic mediator, nitric oxide (NO), can modulate cytokine-induced SMC activation, we investigated the effects of various NO-generating compounds on the expression of intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1). Induction of ICAM-1 expression by IL-1α and VCAM-1 expression by IFN-γ was attenuated by NO donors but not by cGMP analogues. Nuclear run-on assays and transfection studies using various VCAM-1 promoter constructs linked to the chloramphenicol acetyltransferase reporter gene showed that NO repressed IFN-γ-induced VCAM-1 gene transcription, in part, through inhibition of nuclear factor-κB (NF-κB). Electrophoretic mobility shift assay revealed that SMC possess basal constitutive NF-κB activity, which was augmented by treatment with IL-1α. In contrast, IFN-γ induced and activated interferon regulatory factor (IRF)-1 but had little effect on basal constitutive NF-κB activity. NO donors had no inhibitory effect on IRF-1 activation but did inhibit basal and IL-1α-stimulated NF-κB activation. These findings suggest that the induction of ICAM-1 and VCAM-1 expression requires NF-κB activation and that NO attenuates IFN-γ-induced VCAM-1 expression primarily by inhibiting basal constitutive NF-κB activity in SMC.

Atherosclerotic lesions contain proliferating intimal smooth muscle cells (SMC)† and cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-1α. Although the involvement of cytokines in atherosclerosis is well established, their signaling events leading to SMC activation and proliferation are still poorly understood. Recent studies have suggested that many cytokines activate the oxidant-sensitive transcription factor, nuclear factor-κB (NF-κB) (4, 5), which may be important in mediating SMC activation and proliferation (6, 7). Activated SMC express proinflammatory genes such as intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) (8, 9). Indeed, we have shown that cytokines such as IL-1α and TNF-α can activate NF-κB and induce the expression of VCAM-1 in human vascular endothelial cells (10). It is not known, however, whether NO can similarly modulate cytokine-induced NF-κB activity in SMC.

SMC responds to endothelium-derived nitric oxide (NO), which emerges as an important modulator of vascular tone via stimulation of soluble guanylyl cyclase (11, 12). However, NO may have other important effects on SMC such as inhibition of SMC activation and proliferation (13, 14). Supplementation of L-arginine, the precursor of NO, lessens the extent of atherosclerosis in diet-induced hypercholesterolemic rabbits (15). In vivo transfer of the type III NO synthase gene into balloon-injured vessels decreases intimal SMC proliferation in rat carotid arteries (16). These studies demonstrate that NO can antagonize the effects of cytokines and growth factors, in part, by attenuating SMC activation and proliferation. Although the mechanism(s) by which NO exerts its inhibitory effect(s) on SMC is not presently known, recent studies from our laboratory have indicated that NO can modulate endothelial activation via cGMP-independent inhibition of cytokine-induced NF-κB activation (17, 18). Thus, NO production in the vessel wall may influence SMC not only in their vasomotor functions, but also perhaps in their more prolonged transcriptional responses to NF-κB activation by cytokines.

The cellular immune response in atherosclerotic lesions is evidenced by the marked infiltration of T-lymphocytes (19, 20). Although the precise role of T-lymphocytes in the vessel wall has not been established, recent findings suggest that T-lymphocytes can modulate SMC activation, in part, through the lymphokine, interferon-gamma (IFN-γ) (21). In contrast to cytokines such as TNF-α and IL-1α, IFN-γ is not known to activate NF-κB or induce VCAM-1 expression in endothelial cells (22). IFN-γ, however, can potentiate the expression of VCAM-1 and major histocompatibility complex class II antigens and activators of transcription; GAF, γ-activating factor; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; SIN-1, 3-morpholino sydnonimine; SNP, sodium nitroprusside; GSNO, 5-nitrosoglutathione; EMSA, electrophoretic mobility shift assay; ISGF, interferon-stimulated gene factor.
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gen in SMC (21, 23, 24). The signaling pathway for IFN-γ-stimulated responses involves the protein tyrosine phosphorylation of signal transducers and activators of transcription (STATs) and γ-activating factor (GAF) (25, 26). Activation of GAF, in turn, induces the expression of another transcription factor, interferon regulatory factor (IRF)-1, which binds to the promoters of target genes containing the interferon-stimulated response element (ISRE) (27). The VCAM-1 promoter contains two tandem NF-κB sites located in close proximity to an ISRE site (28, 29). Recent studies have shown that IRF-1 synergizes with NF-κB in transactivating the VCAM-1 gene (30).

Since SMC, but not endothelial cells, possess basal constitutive NF-κB/Rel-like activity (6, 7, 31), we hypothesized that the presence of this basal constitutive NF-κB activity may contribute to the differential responses of vascular wall cells to IFN-γ. The purpose of this study, therefore, was to determine the role of basal constitutive NF-κB activity in mediating the effects of IFN-γ and NO on VCAM-1 expression in SMC. We found that NO can modulate IFN-γ-induced SMC activation through its effects on basal constitutive NF-κB activity.

EXPERIMENTAL PROCEDURES

Materials—All standard culture reagents were obtained from JR Biosciences (Lenexa, KS). Actinomycin D and cycloheximide were obtained from Calbiochem. cDNA probes for human ICAM-1 (Hs/3) and VCAM-1 (E1/6) were provided by M. A. Gimbrone, Jr. (Brigham & Women's Hospital, Boston, MA). The cDNA probes for human VCAM-1 and ICAM-1 were obtained from T. Collins (Brigham & Women’s Hospital, Boston, MA) and T. Springer (Harvard Medical School, Boston, MA), respectively.

Cell Culture—SMC were isolated from outgrowths of tunica media explants derived from human aortic and saphenous vein tissues as described previously (32). Cells were grown to confluence in Dulbecco's modified Eagle's medium containing 20% FBS, 5% L-glutamine (Life Technologies, Inc.), 10% fetal calf serum (HyClone lot 1112288, Logan, UT), penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (1.25 µg/ml). The cells were characterized by phase contrast microscopy (Zeiss ICM 405, × 40 objective) and staining for SMC-specific α-actin. Only SMC of passage level of less than 6 were used.

Before any treatment with cytokines or NO donors, SMC were rendered quiescent by incubating in insulin-transferrin media for 24 h (32).

Cellular confluence was maintained for all treatment conditions. Cellular viability was assessed by morphometry, cell number, DNA content, and trypan blue exclusion. The amount of DNA was measured by a Microfluor reader (Dynatech Laboratories, Inc., Chantilly, VA) using a fluorescent dye ( Hoechst 33258) that binds specifically to DNA (Calbiochem).

Cell Surface Enzyme Immunoassay—Cytokine-stimulated SMC were cultured on 96-well Falcon plates (Lincoln Park, NJ), rinsed with phosphate-buffered saline and 2% fetal calf serum, and incubated with the indicated murine monoclonal antibody to human ICAM-1 and VCAM-1 for 2 h. After rinsing three times with phosphate-buffered saline, cells were incubated with biotinylated secondary antibody (horse anti-mouse IgG, Vector Labs, Inc., Burlingame, CA, 1:1000) for 1 h before incubation with streptavidin-alkaline phosphatase (Zymed, South San Francisco, CA) for 30 min. Cells were then treated with p-nitrophenyl phosphate (1 µg/ml) for 30 min at room temperature. Light absorbance was measured in a plate reader (Dynatech Laboratories) at 405 nm, using cells without primary antibody as a blank.

Immunocytochemistry—SMC were cultured on 4-well Nunc culture plate (Naperville, IL) before treatment with IFN-γ (1000 units/ml) in the presence and absence of NO donors, SNP (10−7 m) or SIN-1 (10−5 m), for 24 h. After fixation with cold acetone, cells were incubated with the primary antibody (anti-human VCAM-1) at room temperature for 2 h, washed, and then incubated with biotinylated secondary antibody (horse anti-mouse IgG) for 1.5 h. Antibody detection was accomplished with avidin-biotin peroxidase complex (Vectorstain ABC kit, PK 6100, Vector Labs, Inc.), 3-amino-9-ethyl carbazole, and Gill's hematoxylin. The primary antibody (anti-human VCAM-1) at room temperature for 2 h, washed, and then incubated with biotinylated secondary antibody (horse anti-mouse IgG) for 1.5 h. Antibody detection was accomplished with avidin-biotin peroxidase complex (Vectorstain ABC kit, PK 6100, Vector Labs, Inc.), 3-amino-9-ethyl carbazole, and Gill's hematoxylin.

Stimulation of GMP-dependent Kinases—Confluent SMC (5 × 104) were incubated with cGMP (50 µCi) for 1 h prior to the addition of 8-bromo-cGMP at the indicated concentrations and incubated for an additional 1 h. The study was terminated by the addition of sodium phosphate (50 mm), trichloroacetic acid (20%), and sodium vanadate (1 mm). The supernatants and known molecular weight markers (Life Technologies, Inc.) were separated by SDS/polyacrylamide gel electrophoresis (10% running, 4% stacking gel). The gels were then fixed with Carnoy's Blue (0.4%), methanol (20%), and glacial acetic acid (10%) and dried by a gel dryer before autoradiography at ~70 °C for 12–24 h.

Northern Blotting—Equal amounts of total RNA (20 µg) from approximately 5 × 105 SMC were separated by 1% formaldehyde-agarose gel electrophoresis, transferred overnight onto nylon membranes by capillary action, and baked at 80 °C. Radiolabeling of VCAM-1, (1.4-kilobase pair fragment from Sall/BglII digest), VCAM-1 (2.1-kilo-base pair fragment from HindIII/Sphl digest), or full-length α-actin cDNA probe was performed using random hexamer priming, [α-32P]CTP, and DNA polymerase I (Klenow fragment, Pharmacia Biotech, Inc.). The membranes were hybridized with the probes overnight at 45 °C in a solution containing 50% formamide, 5 × SSC, 2.5 × Denhardt’s solution, 25 mCi sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 µg/ml salmon sperm DNA. All Northern blots were subjected to stringent washing conditions (0.2 × SSC, 0.1% SDS at 65 °C before autoradiography with an intensifying screen for 24–72 h at 80 °C).

Northern Blots—Confluent SMC (106 cells) were stimulated with IFN-γ (1000 units/ml) alone or in combination with GSNO (0.2 mm) for 4 h. Cells were subsequently washed twice with phosphate-buffered saline, trypsined, and centrifuged at 300 × g for 5 min at 4 °C. The cellular pellet was gently resuspended in a buffer containing 10 mm Tris-HCl (pH 7.4), 10 mm NaCl, 3 mm MgCl2, and 0.5% Nonidet P-40, allowed to swell on ice for 15 min, and lysed by a Dounce homogenizer (60–70 strokes) with intermittent inactivation of nucleases. The lysate was recentrifuged at 300 × g, and the resulting nuclear pellet was resuspended in 100 ml of buffer containing 20 mm Tris-HCl (pH 8.1), 75 mm NaCl, 0.5 mm EDTA, 1 mm dithiothreitol, and 50% glycerol. In vitro transcription using the nuclear pellet (100 µl) was performed in a solution containing 5 × SSC, 2.5 × Denhardt’s solution, 25 mCi sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 mg/ml salmon sperm DNA. Hybridization of radiolabeled transcripts to the hybridoma membranes was carried out at 45 °C for 46 h. The membranes were then washed with 1 × SSC, 0.1% SDS for 1 h at 65 °C prior to autoradiography at 72 °C for 80 h.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described (34). Oigonucleotides corresponding to the κB (5′-TGCGCGTGTACCCCTTGAAGGATTCCCTC-3′) and ISRE (5′-AGGGTG-AGTGGATTAGAGGCTGACTTGTACTGCAAGTAGAGAGG-3′) sites at the human VCAM-1 promoter were labeled with [α-32P]ATP and T4 polynucleotide kinase (New England Biolabs) and purified by G-50 Sephadex columns (Pharmacia). Nuclear extracts (10 µg) were added to 32P-labeled oligonucleotides (−20,000 cpm, 0.2 ng) in a buffer containing 4 µg of poly(dI-dC) (Boehringer Mannheim), 10 µg of bovine serum albumin, 10 mm Tris-HCl (pH 7.5), 50 mm NaCl, 1 mm dithiothreitol, 1 mm EDTA, and 5% glycerol (total volume of 20 µl). DNA-protein complexes were resolved on 4% non-denaturing polyacrylamide gel electrophoresed at 12 V/cm for 3 h in low ionic strength buffer (0.5 × TBE) at 4 °C. For supershift assays, the
indicated antibody (15 μg/ml) was added to the nuclear extracts for 10 min before the addition of radiolabeled probe. To determine the specificity of shifted bands, excess unlabeled oligonucleotide (20 ng) was added directly to the nuclear extracts for 10 min before the addition of corresponding radiolabeled probe.

Transfection and Chloramphenicol Acetyltransferase (CAT) Assay—The human VCAM-1 promoter constructs containing the CAT reporter were previously described by Neish et al. (28). Human SMC were transfected with each reporter plasmid (25 μg) using the calcium phosphate precipitation method (10). As an internal control for transfection efficiency, pSVβ-gal plasmid (10 μg) was co-transfected in all experiments. Preliminary results using β-galactosidase staining indicate that cellular transfection efficiency was approximately 15%. Cells (60–70% confluent) were stimulated 48 h after transfection with IFN-γ (1000 units/ml) to induce VCAM-1 expression more robustly (8.6-fold increase). Both SNPs and SIN-1 were previously described by Neish et al. (11319).

RESULTS

‘NO Donors Inhibit Cytokine-induced Expression of ICAM-1 and VCAM-1—IL-1α (10 pg/ml) produced a 4.2-fold increase in the surface expression of ICAM-1 on vascular SMC as determined by enzyme-linked immunosassay (data not shown). In a concentration-dependent manner, co-treatment with either SNP or SIN-1 decreased IL-1α-induced ICAM-1 expression by 94 ± 5% and 76 ± 6% after 24 h, respectively (Fig. 1A). Because IL-1α only marginally increased surface expression of VCAM-1 (1.8-fold increase), we used IFN-γ (1000 units/ml) to induce VCAM-1 expression more robustly (8.6-fold increase). Both SNP and SIN-1 attenuated IFN-γ-induced VCAM-1 expression in a concentration-dependent manner resulting in a 52 ± 4% and 62 ± 5% reduction after 24 h, respectively (Fig. 1B). To exclude possible nonspecific effects of SNP and SIN-1, two other NO donors, GSNO and diethylamine NONOate were used, which similarly decreased IFN-γ-induced VCAM-1 expression after 24 h (data not shown). These results correlated with attenuated IFN-γ-induced VCAM-1 surface expression as assessed by immunohistochemistry (Fig. 2). Interestingly, pre-incubation with SNP (1 μM) or SIN-1 (1 μM) for 1 h inhibited VCAM-1 expression (53 ± 7% and 51 ± 4%) more than did co-incubation (38 ± 6% and 38 ± 4%) (p < 0.01). These findings indicate that the regulation of VCAM-1 expression by NO occurs very early during IFN-γ induction and suggest that NO may affect VCAM-1 expression at the transcriptional level.

To exclude possible cellular toxicity produced by the NO donors, we examined their effects on cell number, DNA content, and trypan blue exclusion. We found that neither SNP nor SIN-1, at concentrations used in our study, significantly affected cellular viability with respect to cell number, DNA content, and trypan blue exclusion (Table I). This result agrees with immunohistochemical analyses showing that treatment with NO donors did not appreciably affect SMC morphology (Fig. 2).

Stimulation of SMC with either IL-1α or IFN-γ did not induce type II ‘NO synthase expression by Northern analyses or result in increased ‘NO production by SMC as measured by nitrite production (data not shown). In addition, activation of soluble guanylyl cyclase by exogenous NO did not contribute to the observed decrease in cytokine-induced ICAM-1 and VCAM-1 expression, since neither 8-bromo-cGMP (1 μM) nor dibutyryl-cGMP (1 μM) inhibited ICAM-1 or VCAM-1 surface expression (Table II). In fact, there was a slight increase in ICAM-1 and VCAM-1 expression with higher concentrations of 8-bromo-cGMP (0.1 μM to 1.0 μM), 8-bromo-cGMP (1 μM), however, did stimulate cGMP- and probably cAMP-dependent protein kinase activity (Fig. 3).

‘NO Donors Decrease Cytokine-induced mRNA Expression of ICAM-1 and VCAM-1—Vascular SMC under basal culture conditions express low levels of ICAM-1. Treatment with IL-1α (10 pg/ml) augmented steady-state ICAM-1 mRNA levels by 5.1-fold (Fig. 4). In a concentration-dependent manner, SNP and SIN-1 inhibited both basal and IL-1α-induced ICAM-1 mRNA levels. After 6 h, SNP and SIN-1 reduced IL-1α-induced ICAM-1 mRNA levels by 98 ± 5% and 76 ± 5%, respectively.

Under basal culture conditions, SMC express little or no VCAM-1. Exposure to IL-1α weakly induces and exposure to IFN-γ strongly induces VCAM-1 (3.7-fold and 17.4-fold, respectively). In a time- and concentration-dependent manner, both SNP (1 μM) and SIN-1 (1 μM) decreased IFN-γ (1000 units/ml) induced VCAM-1 mRNA level, resulting in 83 ± 6% and 70 ± 5% reduction after 6 h, respectively (Fig. 5, A and B). Another ‘NO donor, GSNO, also decreased IFN-γ-induced VCAM-1 mRNA levels by 79 ± 4% and 61 ± 4%, respectively (Fig. 5, B and C).

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Fig. 1. The concentration-dependent effects of ‘NO donors, SNP and SIN-1, on IL-1α (10 pg/ml)-induced ICAM-1 (A) and IFN-γ (1000 units/ml) induced VCAM-1 surface expression (B) after 24 h as measured by an enzyme immunofluorescent assay (percentage of expression relative to cytokines alone). All experiments were performed three different times with at least six replicates.
mRNA levels in a concentration-dependent manner (73, 53, and 35% reduction with GSNO concentrations of $10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M, respectively). Neither glutathione (0.2 mM) nor sodium nitrite (0.2 mM) alone significantly affected IFN-γ-induced VCAM-1 mRNA levels (46 and 86% reduction, respectively).

NO Represses IFN-γ-induced VCAM-1 Gene Transcription—Actinomycin D studies showed that GSNO (0.2 mM) did not significantly affect VCAM-1 mRNA stability (half-life of 6.5 ± 2.1 h versus 7.1 ± 1.8 h, $p > 0.05$) (Fig. 6A). To confirm that GSNO decreases IFN-γ-induced steady-state VCAM-1 mRNA levels by transcriptional repression, we performed nuclear run-on experiments using SMC stimulated with IFN-γ (1000 units/ml) for 4 h in the presence or absence of GSNO (0.2 mM) (Fig. 6B). Preliminary studies using different amounts of radiolabeled RNA transcripts demonstrate that under our exper-

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**TABLE I**

Measurements of cellular viability after treatment with IFN-γ (1000 units/ml) in the presence and absence of SNP (1 mM) or SIN-1 (1 mM) for 24 h

| Condition       | Basal   | IFN-γ   | IFN-γ + SNP | IFN-γ + SIN-1 |
|-----------------|---------|---------|------------|--------------|
| Cell number ($× 1000/cm^2$) | 121 ± 8 | 113 ± 4 | 114 ± 6    | 107 ± 6      |
| DNA amount (ng/cm²)   | 107 ± 9 | 92 ± 2  | 97 ± 2     | 92 ± 11      |
| Trypan blue (% exclusion) | 97 ± 1  | 97 ± 1  | 97 ± 1     | 96 ± 1       |

Values are mean ± S.E. for each condition performed three times in quadruplicate.

**TABLE II**

Effects of cGMP analogues (1 mM) on cytokine-induced surface expression of ICAM-1 and VCAM-1 on SMC

The indicated values ($A_{410}$ × 1000) are mean ± S.E. Each condition was performed three times in quadruplicate.

| ICAM-1 | VCAM-1 |
|--------|--------|
| Basal  | 22 ± 7 |
| IL-1α  | 923 ± 25 |
| + 8-bromo-cGMP | 1337 ± 21 |
| $10^{-5}$ M | 1316 ± 33 |
| $10^{-4}$ M | 1407 ± 20a |
| $10^{-3}$ M | 1415 ± 29a |
| + dibutyryl-cGMP | 1336 ± 35 |
| $10^{-5}$ M | 1287 ± 30 |
| $10^{-4}$ M | 1277 ± 46 |
| IFN-γ (1000 units/ml) | 305 ± 17 |
| + 8-bromo-cGMP | 10^{-5} M |
| $10^{-4}$ M | 386 ± 22a |
| $10^{-3}$ M | 381 ± 19a |
| + dibutyryl-cGMP | 10^{-5} M |
| $10^{-4}$ M | 317 ± 7 |
| $10^{-3}$ M | 332 ± 10 |

* $p < 0.05$ in comparison with value obtained with IL-1α or IFN-γ alone.
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![SDS-polyacrylamide gel electrophoresis analysis (50 μg lane) showing the concentration-dependent effects of 8-bromo-cGMP on 32P, labeling of SMC cellular proteins. Two separate experiments yielded similar results.](Image)

![Northern analyses (20 μg total RNA/lane) showing the concentration-dependent effects of SNP and SIN-1 on basal and IL-1α (10 pg/ml) stimulated ICAM-1 steady-state mRNA levels at 6 h. RNA loading was determined by hybridization to SMC α-actin. Each blot is representative of three separate experiments.](Image)

![Northern analyses (20 μg total RNA/lane) showing the concentration-dependent and time-dependent effects of SNP and SIN-1 on IFN-γ (1000 units/ml) induced VCAM-1 steady-state mRNA levels at 6 h. Equal RNA loading for each experiment was verified by hybridization to α-actin. Experiments were performed three times.](Image)

In experimental conditions, hybridization was linear and nonsaturable. The density of each VCAM-1 band was standardized to the density of its corresponding β-tubulin band. The specificity of each band was determined by the lack of hybridization to the nonspecific pGEM cDNA vector. In unstimulated SMC (control), there was little basal VCAM-1 transcriptional activity. IFN-γ augmented VCAM-1 gene transcription by 20-fold. Co-administration with GSNO (0.2 mM) resulted in only a 3-fold induction, indicating repression of VCAM-1 gene transcription by NO.

NO Inhibits Activation of NF-κB, but Not IRF-1—EMSA showed that under our basal culture conditions, there were two constitutive bands corresponding to NF-κB that were both “supershifted” in the presence of antibody to p65, whereas only the lower band was supershifted in the presence of antibody to p50 (Fig. 7A). These findings suggest that the composition of NF-κB binding to the tandem κB sites of the VCAM-1 promoter probably consists of the p65 homodimer (top band) and p50-65 heterodimer (lower band). The anti-c-Rel antibody neither obliterated nor supershifted these basally active NF-κB bands (data not shown). IFN-γ (1000 units/ml) slightly augmented, while IL-1α (10 pg/ml) caused an increase in, basal constitutive NF-κB activation. Higher concentrations of IL-1α (0.1–10 ng/ml) produced an even greater activation of NF-κB (data not shown). Treatment with ‘NO donors inhibited both basal constitutive and IL-1α (10 pg/ml) stimulated NF-κB activation.

Using the VCAM-1 ISRE oligonucleotide, several different antibodies to p91 (STAT-1α) failed to supershift any bands induced by IFN-γ (data not shown), suggesting that interferon-stimulated gene factor-3 (ISGF-3) does not bind to the ISRE of VCAM-1 promoter and, therefore, may play only a limited role in the transcriptional activation of the VCAM-1 promoter by IFN-γ. However, IFN-γ did induce IRF-1 in a cycloheximidesensitive, time-dependent manner (data not shown). The induction and activation of IRF-1 appeared no sooner than 2 h after stimulation with IFN-γ and was not inhibited by treatment with ‘NO (Fig. 7B).

Induction of VCAM-1 Gene Transcription by IFN-γ Requires κB Enhancer Element—Transient transfection studies using various VCAM-1 promoter constructs (F0, F3, and F4) linked to the CAT reporter gene demonstrated that the two tandem κB enhancer elements in the VCAM-1 promoter are required for transcriptional induction and repression by IFN-γ and ‘NO (Fig. 8). The promoterless p.CAT showed no basal or IFN-γ-stimulated relative CAT activity (32 ± 21 and 34 ± 20). The highly expressed constitutive SV40 promoter (basal relative CAT activity of 1350 ± 210) showed no response to IFN-γ and ‘NO (relative CAT activity of 1360 ± 198 and 1300 ± 254, p > 0.05). The induction and activation of IRF-1 by IFN-γ and its subsequent binding to the ISRE is not sufficient to transactivate −44F4 (basal relative CAT activity of 54 ± 23 and IFN-γ-stimulated CAT activity of 51 ± 22, p > 0.05). However, −98F4 (basal relative CAT activity of 64 ± 28), which contains the κB enhancer elements exhibits responsiveness to IFN-γ and ‘NO (relative CAT activity of 524 ± 34 and 204 ± 27, p < 0.05). A greater transcriptional response to IFN-γ and ‘NO (relative CAT activity of 698 ± 60 and 273 ± 33, p < 0.05) may require other response elements contained in −755F0 (basal relative CAT activity of 99 ± 11) such as AP-1 and GATA. Since IFN-γ does not activate NF-κB (Fig. 7A), these findings indicate that IRF-1 and basal constitutive NF-κB activity are necessary for the transcriptional induction of the VCAM-1 gene by IFN-γ. Furthermore, since ‘NO does not affect IFN-γ-induced
IRF-1 activity (Fig. 7B). NO attenuates IFN-γ-induced VCAM-1 expression via inhibition of basal constitutive NF-κB activity.

DISCUSSION

We have shown that NO can attenuate the surface expression of ICAM-1 and VCAM-1 on SMC in response to stimulation with IL-1α and IFN-γ, respectively. The mechanism for NO’s effect is independent of cGMP production, occurs at the transcriptional level, and involves inhibition of both basal constitutive and IL-1α-stimulated NF-κB activity. These findings agree with our earlier findings that NO decreases cytokine-induced endothelial expression of VCAM-1 and ICAM-1 via inhibition of NF-κB activation (10). However, SMC differ from endothelial cells in exhibiting basal constitutive NF-κB activity (6, 18). Indeed, we observed a small amount of SMC activation under basal culture conditions as exhibited by low levels of VCAM-1 mRNA expression, gene transcription, and promoter activity. The presence of basal constitutive NF-κB activity has also been shown to be important in mediating SMC proliferation (7).

Previous studies have shown that NO inhibits SMC proliferation via a cGMP-dependent mechanism (13, 14). However, the expression of ICAM-1 and VCAM-1 were not affected by increasing concentrations of two different cGMP analogues that are able to stimulate protein kinase activity. Indeed, several groups have shown that NO can exert non-cGMP-dependent effects on other cell types such as platelets (36), fibroblasts (37), and macrophages (38). Interestingly, the inhibitory effects of NO on basal and stimulated NF-κB activation resemble those of antioxidants such as N-acetylcysteine and pyrrolidine dithiocarbamate (39, 40). Antioxidants have been shown to inhibit SMC proliferation, and at higher concentrations they appear to induce SMC apoptosis (41). SMC did not exhibit any signs of cellular toxicity with the concentrations of NO donors used. Furthermore, the actual amount of NO released was probably comparable with the levels achieved by the continuous release of NO from cytokine-induced type II NO synthase (42). Such localized high concentrations of NO are readily achieved within the vicinity of cytokine-activated SMC, endothelial cells, or macrophages in atherosclerotic lesions.

Atherosclerotic plaques contain a variety of cell types including SMC, macrophages, and lymphocytes (1, 2, 20). Immunohistochemical analyses of cellular subtypes in plaques have revealed that most of the lymphocytes are T-cells (19, 20).
IFN-γ, a major product of activated T-cells, exerts a variety of paracrine effects on neighboring cells and, thus, may modulate the evolution of atherosclerotic lesions. For example, IFN-γ can inhibit collagen production by SMC (43), augment the expression of major histocompatibility complex class I, and induce the expression of major histocompatibility complex class II antigens on endothelial cells and SMC (24, 44), and in combination with other proinflammatory cytokines, it can induce apoptotic death of SMC (45, 46). Consequently, SMC within human and experimental atheroma can express increased levels of ICAM-1 and VCAM-1, indicating a state of activation compared with those in normal vessels (47). However, the expression of these adhesion molecules on SMC in atheroma is quite heterogeneous (48). This may be attributed to the locally produced effects of cytokines and endogenously released NO or to a heterogeneous population of intimal SMC that responds differently to external stimuli. In any case, factors such as cytokines, NO, and antioxidants that can regulate the expression of ICAM-1 and VCAM-1 may modulate the course of atherogenesis.

IFN-γ activates at least two transcription factors, ISGF-3 and IRF-1, which are capable of binding to the ISRE (25, 27). ISGF-3 is a multicomplex DNA binding protein that contains the Janus kinase substrates, STATs (p91/84, p113) (25). Upon phosphorylation, ISGF-3 translocates into the nucleus, where it can bind to the ISRE of target genes. However, phosphorylation of p91 or GAF, but not p113, allows GAF to migrate to the nucleus by itself and participate in DNA-binding complexes that recognize a different DNA binding motif, the γ-activated sequence (26). The IRF-1 gene contains γ-activated sequence elements in its promoter, and the expression of IRF-1 is induced by activated GAF in response to IFN-γ or TNF-α (27, 30). IRF-1 binds to ISRE sites in the promoters of IFN-α/β, inducible type II NO synthase, and IFN-inducible genes such as VCAM-1 (27, 30). The induction and activation of IRF-1 is linked to tumor-suppressive properties and, in some instances, to the induction of apoptosis following DNA damage or in response to serum-depriving conditions (49). In our study, we did not find evidence of ISGF-3 binding to ISRE of the VCAM-1 promoter. However, the induction and binding of IRF-1 to ISRE, although not sufficient by itself, was necessary for the induction of VCAM-1 in response to IFN-γ.

The induction of VCAM-1 expression by IFN-γ also required the two tandem κB motifs in the VCAM-1 promoter constructs, Fω and Fγ, and ‘NO’s inhibitory effect on IFN-γ-induced VCAM-1 expression in SMC depends not on inhibition of IRF-1 induction or activity but on inhibition of basal constitutive NF-κB activity. These results indicate that basal constitutive NF-κB is necessary, but by itself is only modestly sufficient to transactivate the VCAM-1 gene in SMC. A more robust transcriptional induction of the VCAM-1 gene by IFN-γ is mediated by the synergistic effects of basal constitutive NF-κB and IFN-γ-stimulated IRF-1. These results are in agreement with a previous study showing that cooperativity between IRF-1 and NF-κB is necessary and sufficient in transactivating the VCAM-1 gene in vascular endothelial cells (30). Consequently, the inability of IFN-γ to stimulate VCAM-1 expression in endothelial cells may result from the lack of basal constitutive NF-κB activity in endothelial cells (18, 31). Interestingly, endothelial cells, but not SMC, have basal constitutive NO production that may render NF-κB inactive under basal conditions. Indeed, treatment with the type III NO synthase inhibitor, N’-arginine methyl ester, inhibits basal NO production in endothelial cells and leads to the activation of NF-κB (10, 17).

In summary, we have identified an important mechanism by which ‘NO inhibits IFN-γ-induced VCAM-1 expression in SMC. Our findings add to the evidence that ‘NO may be anti-atherogenic through its inhibitory effects on not only cytokine-stimulated NF-κB activation, but also on basal NF-κB activity. These results provide new insights into how ‘NO may modulate SMC inflammatory activation in a manner highly relevant to the evolution of human atheroma.

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