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Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells

(antioxidant/adhesion/oxidation/endothelium/reactive oxygen species)

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ABSTRACT **Decreased nitric oxide (NO) activity, the formation of reactive oxygen species, and increased endothelial expression of the redox-sensitive vascular cell adhesion molecule 1 (VCAM-1) gene in the vessel wall are early and characteristic features of atherosclerosis. To explore whether these phenomena are functionally interrelated, we tested the hypothesis that redox-sensitive VCAM-1 gene expression is regulated by a NO-sensitive mechanism. In early passaged human umbilical vein endothelial cells and human dermal microvascular endothelial cells, the NO donor diethylamine-NO (DETA-NO, 100 μM) reduced VCAM-1 gene expression induced by the cytokine tumor necrosis factor α (TNF-α, 100 units/ml) at the cell surface level by 65% and intracellular adhesion molecule 1 (ICAM-1) gene expression by 35%. E-selectin gene expression was not affected. No effect on expression of cell adhesion molecules was observed with DETA alone. Moreover, DETA-NO suppressed TNF-α-induced mRNA accumulation of VCAM-1 and TNF-α-mediated transcriptional activation of the human VCAM-1 promoter. Conversely, treatment with N^G-monomethyl-L-arginine (L-NMMA, 1 mM), an inhibitor of NO synthesis, augmented cytokine induction of VCAM-1 and ICAM-1 mRNA accumulation. By gel mobility shift analysis, DETA-NO inhibited TNF-α activation of DNA binding protein activity to the VCAM-1 NF-κB like binding sites. Peroxy-fatty acids such as 13-hydroperoxydodecanoeic acid (linoleyl hydroperoxide) may serve as an intracellular signal for NF-κB activation. Using thin layer chromatography, DETA-NO (100 μM) suppressed formation of this metabolite, suggesting that DETA-NO modifies the reactivity of oxygen intermediates in the vascular endothelium. Through this mechanism, NO may function as an immunomodulator of the vessel wall and thus mediate inflammatory events involved in the pathogenesis of atherosclerosis.**

Oxidative signals in the form of oxidized lipoproteins and reactive oxygen species such as superoxide, lipid peroxides, and nitric oxide (NO) are early and characteristic features in the pathogenesis of atherosclerosis and other inflammatory diseases (1–4). These signals may function in part through oxidation-reduction (redox) sensitive regulation of vascular endothelial genes such as vascular cell adhesion molecule 1 (VCAM-1) which plays an important role in mediating the mononuclear leukocyte selective inflammation characteristic of early atherosclerosis (5–8). Regulation of VCAM-1 gene expression is coupled to oxidative stress through specific reduction-oxidation sensitive transcriptional factors such as nuclear factor κB (NF-κB) (6, 9, 10). Cytokine-activated endothelial expression of VCAM-1 is inhibited by antioxidants such as pyrrolidine dithiocarbamate (PDTC) (6, 8), while oxidative metabolites of polyunsaturated fatty acids serve to regulate VCAM-1 expression (7, 11). These studies suggest that cellular analogues to these exogenous oxidants and antioxidants may function to control redox-sensitive vascular gene expression. The precise identity and relative functional roles of these intracellular signals remain unknown.

Early events in the atherosclerotic process include loss of endothelium-derived NO modulation of vasomotion, increased production of oxygen-derived radicals, and the expression of VCAM-1 (1, 12). NO is produced by a variety of mammalian cells, including endothelium, macrophages, smooth muscle cells, platelets, and fibroblasts (12). NO produced by the endothelium modulates vasomotor tone, inhibits platelet aggregation, and inhibits smooth muscle cell proliferation, properties that have been shown to be anti-atherogenic (13). NO may also exhibit a dual redox function based on its interaction with reactive oxygen species (14). Depending on the concentrations present, NO has been shown to both augment (15, 16) and inhibit (17, 18) oxygen-radical mediated tissue damage and lipid peroxidation.

It has recently been demonstrated that inhibition of NO production markedly enhances the development of atherosclerosis in cholesterol-fed rabbits (19). Additionally, dietary L-arginine, a precursor of NO, improves endothelium-dependent vasorelaxation in hypercholesterolemic rabbits and reduces atherogenesis (20). As lipid peroxidation plays an important role in mediating VCAM-1 gene expression (7, 11), we tested the hypothesis that NO is a redox sensitive signal that inhibits cytokine activation of VCAM-1 gene expression in vascular endothelial cells. To this end, we demonstrated that NO suppresses generation of oxidative modified polyunsaturated fatty acids, cytokine activation of endothelial VCAM-1 gene expression, and VCAM-1 promoter transactivation through an NF-κB-like transcriptional regulatory factor. Conversely, treatment with N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis, augments cytokine induction of VCAM-1 gene expression. These studies suggest that NO may function as an important cellular mediator of redox-sensitive gene expression in the vasculature.

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Abbreviations: NO, nitric oxide; 13-HPODE, 13-hydroperoxydodecanoeic acid (linoleyl hydroperoxide); DETA, diethylamine; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor α; L-NMMA, N^G-monomethyl-L-arginine; PDTC, pyrrolidine dithiochabamate; TMB, 3,3′,5,5′-tetramethylbenzidine; HUVEC, human umbilical vien endothelial cells; HMEC, human dermal microvascular endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase.

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MATERIALS AND METHODS

Materials. The cytokine tumor necrosis factor α (TNF-α) was obtained from Boehringer Mannheim. Sodium nitrate, diethylamine (DETA), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma. L-NMMA was obtained from Bachem. The reporter plasmids p85 VCAMCAT and pSV_2CAT were a generous gift of Douglas Dean (Washington University).

Cell Culture. Human umbilical vein endothelial cells (HUVEC), obtained from Clonetics (San Diego), were cultured in M199 medium containing 20% fetal bovine serum, 2 μg of epidermal growth factor per ml, 50 μg of endothelial cell growth supplement per ml (Biomedical Technologies, Stoughton, MA), and 2 mM L-glutamine. Human dermal microvascular endothelial cells (HMEC) were derived from a cell line and obtained from the Emory University School of Medicine (21) and grown using the above conditions. Cells were grown at 37°C on tissue culture plates and used for experimentation at passages 4–8. HUVECs were grown on plates coated with 0.1% gelatin.

Preparation of Compounds. DETA and sodium nitrate were complexed for the preparation of diethylamine-NO (DETA-NO), as described (22). The kinetics and release of DETA-NO have been previously defined (23). DETA-NO was used for experimentation on the day of preparation. Either DETA or DETA-NO was dissolved in M199 medium and added to HUVEC or HMEC at the indicated concentrations.

ELISA of Cell Adhesion Molecule Expression. HUVEC were plated in 96-well plates and grown to confluence. Where indicated, TNF-α (100 units/ml) was added 9 h before ELISA. HUVECs were pretreated with either DETA or DETA-NO (1–1000 μM) for 30 min. Cell surface expression of VCAM-1, intercellular adhesion molecule 1 (ICAM-1), and E-selectin was determined by primary binding with specific mouse antibodies [VCAM-1 from Southern Biotechnology Associates, ICAM-1 from Emory University (Atlanta), and E-selectin from Barry Wolitsky, Hoffmann–La Roche (Nutley, NJ)] followed by secondary binding with a horseradish peroxidase-tagged goat anti-mouse IgG. Quantitation was performed by determination of colorimetric conversion at 450 nm of TMB and compared to the maximal response for TNF-α (100 units/ml) at 9 h. Studies were performed at least in triplicate.

Northern Blot Analysis. To determine whether NO regulates the cell adhesion molecule at the mRNA level, HUVECs were plated in 150-mm plates at near confluence and pretreated with either DETA or DETA-NO (100 μM) for 30 min and then incubated with TNF-α (100 units/ml) for 4 h. In separate experiments, HMEC were pretreated with L-NMMA (1 mM) for 30 min and then incubated with TNF-α (100 units/ml) for 4 h. Total cellular RNA was isolated by a single extraction, as described (6), and size-fractionated by using 1% agarose formaldehyde gels in the presence of 1 mg of ethidium bromide per ml. RNA was hybridized to ^{32}P-labeled human VCAM-1, ICAM-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA, as described by Marui *et al.* (6). Visualization was performed by using autoradiography at −70°C for 24 h.

Transcriptional Analysis of Cell Adhesion Molecules. HMEC were split at the ratio to give ≈60% confluence in 100-mm tissue culture plates. HMEC were transfected with either 10 μg of p85VCAMCAT or pSV_2CAT plasmid by the calcium phosphate coprecipitation technique for 8 h (24). After a 24-h recovery period, HMEC were pretreated or not with 100 μM DETA or DETA-NO and after 30 min exposed to TNF-α (100 units/ml) directly added to the plates. After 18 h, cell extracts were prepared by rapid freeze-thaw in 0.25 M Tris (pH 8.0). Protein was assayed for chloramphenicol acetyltransferase (CAT) activity as described (25).

Gel Mobility Shift Assay. HUVEC, plated in 150-mm plates, were pretreated with DETA or DETA-NO (100 μM) for 30 min and exposed or not to TNF-α (100 units/ml) for 3 h. Nuclear extracts were prepared as described by Dignam *et al.* (26). Protein (5 μg) was mixed with a double-stranded oligonucleotide corresponding to the NF-κB binding motif located −57 bp from the initiation site in the VCAM-1 promoter (27). Oligonucleotides were synthesized according to the published sequence (cs-5′-TGAAGGGATTTCCC-3′) and labeled with [^{32}P]dATP by using Klenow DNA polymerase (8). Binding was performed as described (6). Samples were separated on a nondenaturing 4% polyacrylamide gel and exposed to autoradiography at −70°C for 24 h.

Metabolism of Linoleic Acid. HUVEC were cultured in 3% fetal bovine serum in 24-well plates and exposed to 100,000 dpm of [1-^{14}C]linoleic acid (New England Nuclear; specific activity of 693,243 dpm/μM) for 30–45 min in the presence or absence of TNF-α (100 units/ml) and in the presence or absence of DETA or DETA-NO (100 μM). The radiolabeled linoleic acid preparation was dissolved in ethanol (EtOH) and added to the wells for a final concentration of 0.05% EtOH The cells and media were separated, and the cells underwent a chloroform/methanol (2:1) extraction. Lipid extracts were placed on a thin layer chromatography plate and separated by using a 90:10:0.5:0.5 (chloroform/methanol/acetic acid/deionized water) solvent system. Commercially pure linoleic acid and 13-hydroperoxydodecanoeic acid [linoleyl hydroperoxide (13-HPODE), a lipid peroxide of linoleic acid] were used as standards (both compounds available from Cayman Chemical, Ann Arbor, MI). Zones were identified by fluorescence, and the zones of the cellular lipid extract corresponding to the linoleic acid and 13-HPODE standards were counted by using radioactive scintillation spectrometry.

Statistics. Values presented are the means ± SEM. Statistics were performed by by using an unpaired Student's *t* test. Significance was determined at the 95% level.

RESULTS

NO Suppresses Cytokine-Induced Cell Surface Expression of VCAM-1 and ICAM-1 But Not E-Selectin in HUVEC. To explore whether NO inhibits cytokine induction of cell adhesion molecules, TNF-α was incubated in passaged HUVEC in 96-well plates for 9 h (following pretreatment with DETA or DETA-NO for 30 min). Cell surface expression of VCAM-1, ICAM-1, and E-selectin was determined by using an ELISA assay. In a dose-dependent manner, DETA-NO but not DETA suppressed TNF-α-induced cell surface expression of VCAM-1 (Fig. 1 *Top*) by at least 65%. At concentrations of 100 and 1000 μM, DETA-NO also significantly reduced TNF-α-induced ICAM-1 (Fig. 1 *Middle*) by 35%. E-selectin was not affected by DETA-NO (Fig. 1 *Bottom*). Similar results were obtained from HMEC, another endothelial cell type; DETA-NO (100 μM) suppressed TNF-α-induced VCAM-1 by 52% and ICAM-1 by 43% (Table 1).

NO Regulates Cytokine-Induced VCAM-1 mRNA Accumulation in HUVEC. To determine whether NO regulates VCAM-1 gene expression at the mRNA level, HUVEC in 150-mm plates were pretreated with either DETA or DETA-NO (both 100 μM) for 30 min and then incubated with TNF-α (100 units/ml) for 4 h. The cytokine TNF-α induced VCAM-1 mRNA accumulation by 12-fold. VCAM-1 mRNA accumulation induced by TNF-α was suppressed by DETA-NO but not by DETA (Fig. 2). Neither DETA nor DETA-NO alone had any effect on VCAM-1 mRNA accumulation. In separate experiments, HMEC were pretreated with L-NMMA (1 mM), an inhibitor of NO synthesis for 30 min, and then incubated with TNF-α (50 units/ml) for 4 h. L-NMMA augmented cytokine-induced VCAM-1 and ICAM-1 mRNA accumulation by ≈50% above TNF-α (50

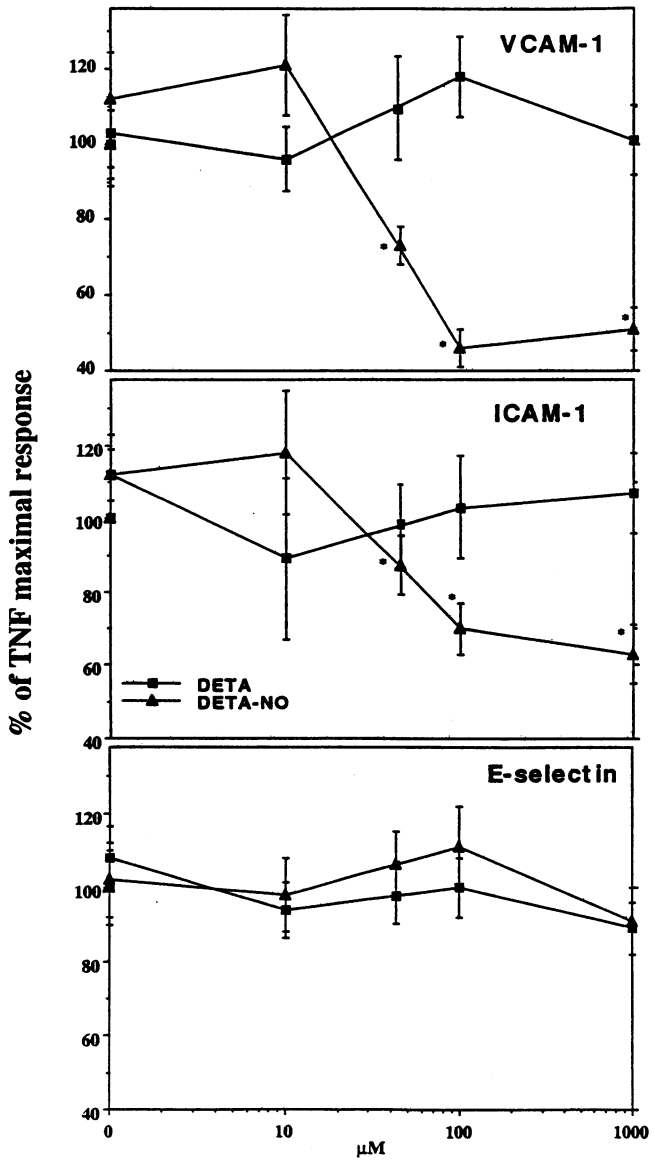

FIG. 1. NO inhibits cytokine induction of cell adhesion molecules through an antioxidant-sensitive mechanism in HUVEC. In early passaged HUVEC in 96-well plates, DETA or DETA-NO were incubated for 9 h at doses from 1 to 1000 μM. Cell-surface expression of VCAM-1, ICAM-1, and E-selectin was determined by primary binding with specific mouse antibodies followed by secondary binding with a horseradish peroxidase-tagged goat anti-mouse IgG. Quantitation was performed by determination of colorimetric conversion at 450 nm of TMB and compared to the maximal response obtained for HUVEC incubated with TNF-α (100 units/ml) at 9 h. Studies were performed in triplicate (n = 3 for each experimental value). *, Value differs from maximal TNF-α (100 units/ml) response.

units/ml) only (Fig. 3). Similar findings were obtained at the cell surface level by ELISA (data not shown). L-NMMA alone had no effect on VCAM-1 or ICAM-1 mRNA accumulation.

DETA-NO Suppresses Cytokine Induced Transcriptional Activation of VCAM-1 in HMEC. To determine whether NO regulates transcriptional activation of the human VCAM-1 promoter, a chimeric reporter gene, p85 VCAMCAT (containing coordinates −85 to +12), was transiently transfected into HMEC (28, 29). This construct contains two NF-κB-like DNA binding elements at −77 and −63 that play an important role in redox-sensitive cytokine and noncytokine activation of the VCAM-1 promoter (6, 27–29). As shown in Fig. 4, TNF-α but not DETA or DETA-NO significantly induced VCAM-1

Table 1. NO inhibits cytokine induction of cell adhesion molecules through an redox-sensitive mechanism in HMEC

	VCAM-1	ICAM-1
Control (n = 3)	0.008 ± 0.003	0.149 ± 0.022
TNFα (n = 3)	0.697 ± 0.122	0.774 ± 0.123
TNFα + DETA (n = 3)	0.646 ± 0.099	0.804 ± 0.088
TNFα + DETA-NO (n = 3)	0.333 ± 0.083*	0.401 ± 0.059*

Early passaged HMEC in 96-well plates were pretreated with DETA or DETA-NO (100 μM) for 30 min and then with TNFα (100 units/ml) for 9 h. Cell surface expression of VCAM-1 and ICAM-1 was determined by primary binding with specific mouse antibodies followed by secondary binding with a horseradish peroxidase-tagged goat anti-mouse IgG. Quantitation was performed by determination of colorimetric conversion at 450 nm of TMB and compared to the maximal response obtained for HMEC incubated with TNF-α (100 units/ml) at 9 h. Values presented are the optical density readings at 450 nm. Studies were performed in triplicate.
*Value differs from TNF-α (100 units/ml).

promoter activity using the p85 VCAMCAT construct. Similar to its effect on VCAM-1 protein and mRNA expression, DETA-NO but not DETA inhibited the transcriptional activation of both VCAM-1 promoter constructs induced by TNF-α.

DETA-NO But Not DETA Suppresses VCAM-1 Promoter Activity Through Inhibition of NF-κB-Like DNA Binding Activity. To explore whether NO regulates VCAM-1 promoter activity through an NF-κB-like transcriptional regulatory factor, confluent HUVEC were pretreated or not with DETA or DETA-NO (100 μM) for 30 min and then exposed to TNF-α (100 units/ml) for 3 h. Using gel mobility shift assay, DETA-NO but not DETA inhibited NF-κB-like DNA binding activity induced by TNF-α, correlating well with its effect on VCAM-1 promoter activity (Fig. 5).

DETA-NO Inhibits the Oxidative Modification of Fatty Acid to a Hydroperoxide Form. We have obtained data (11) indicating the similarity in redox-sensitive activation of the transcriptional factor NF-κB by the polyunsaturated fatty acid linoleic acid (18:2) and TNF-α, raising the possibility that cytokines may transactivate the VCAM-1 promoter through a polyunsaturated fatty acid or its metabolic product (i.e., a fatty acid hydroperoxide). We next determined whether a fatty acid metabolic product signal could be regulated by cytokines through an NO-dependent mechanism. HUVEC were exposed to [1-^{14}C]linoleic acid for 30–45 min. By using lipid extraction and thin layer chromatography analysis with purified linoleic acid and 13-HPODE as standards, we observed that TNF-α markedly increased incorporation of [1-^{14}C]lino-

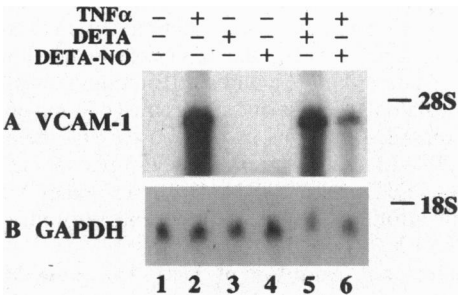

FIG. 2. NO regulates cell adhesion molecule expression at the mRNA level in HUVEC. HUVEC in 150-mm plates were pretreated with either DETA or DETA-NO (both 100 μM) for 30 min and then with TNF-α (100 units/ml) for 4 h. Total RNA was isolated and 20 μg size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, and hybridized to ^{32}P-labeled human VCAM-1 or GAPDH-specific cDNA and visualized by autoradiography. Lanes are described using the following concentrations: TNF-α (100 units/ml), DETA (100 μM), and DETA-NO (100 μM). Hybridization analysis for TNF-α + DETA and TNF-α + DETA-NO is shown.

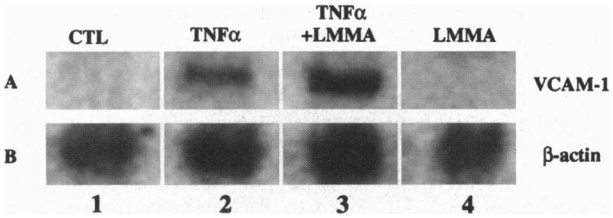

FIG. 3. The inhibitor of NO synthesis L-NMMA augments cytokine-induced VCAM-1 and ICAM-1 mRNA accumulation in HMEC. HMEC in 150-mm plates were pretreated with L-NMMA (1 mM) for 30 min and then incubated with the cytokine TNF-α (50 units/ml) for 4 h. Total RNA was isolated and 20 μg size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, and hybridized to ^{32}P-labeled human VCAM-1, ICAM-1, or GAPDH-specific cDNA and visualized by autoradiography. Lanes are described using the following concentrations: TNF (50 units/ml) and L-NMMA (1 mM).

leic acid into a modified form comigrating with 13-HPODE (Table 2), suggesting that oxidation of polyunsaturated fatty acids in endothelial cells is enhanced by cytokines. Pretreatment with DETA-NO for 30 min before cytokine exposure markedly inhibited accumulation of this 13-HPODE-like metabolic product, similar to previously reported findings with PDTC (11). Pretreatment with DETA had no significant effect. Neither cytokines nor DETA analogues effected any appreciable change in cellular loading of linoleic acid.

DISCUSSION

The present experiments demonstrate that exogenous NO administered in the form of DETA-NO prevents the induction of VCAM-1, and to a lesser extent ICAM-1, in response to cytokine stimulation. We provide evidence that NO prevents cytokine induction of the VCAM-1 promoter, and this likely occurs via inhibition of NF-κB-like activation. Recent evidence suggests that an important intermediate that modulates cytokine activation of NF-κB and induction of VCAM-1 is a lipid hydroperoxide (11). In the present experiments, we found that the formation of such an intermediate in response to TNF-α was prevented by exogenous NO. Thus, these studies

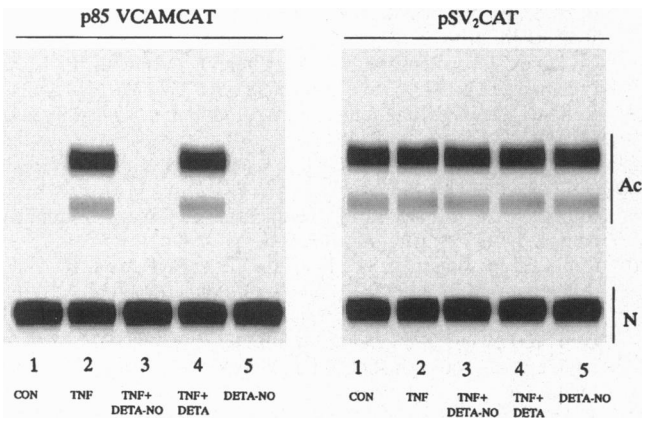

FIG. 4. NO inhibits cytokine transactivation of the VCAM-1 gene promoter. HMEC were split at the ratio to give ≈60% confluence in 150-mm tissue culture plates. HMEC were transfected with either 10 μg of p85VCAMCAT or pSV$_2$CAT plasmid by the calcium phosphate coprecipitation technique for 6 h (24). After an overnight recovery period, HMEC were pretreated with either 100 μM DETA or DETA-NO and after 60 min exposed to TNF-α (100 units/ml) directly added to the plates. After 18 h, cell extracts were prepared by rapid freeze-thaw in 0.25 M Tris (pH 8.0). Protein of each cell extract was assayed for CAT activity as described (24). Ac, acetylated; N, nonacetylated chloramphenicol. Assays were performed twice with similar results, and the best analysis is presented.

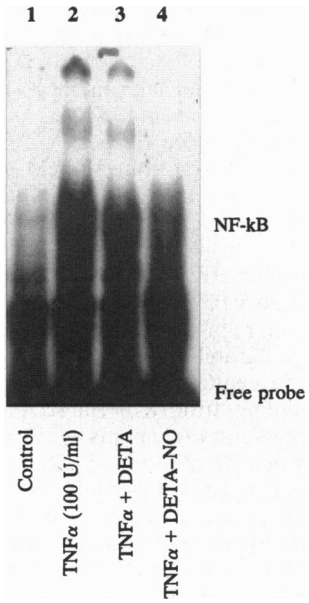

FIG. 5. NO regulates VCAM-1 promoter activity through an NF-κB-like transcriptional regulatory factor. Confluent HUVEC were pretreated or not with DETA or DETA-NO (100 μM) for 30 min and then exposed for 3 h to TNF-α (100 units/ml). Nuclear extracts were prepared, and 5 μg of nuclear extract was incubated with a double-stranded oligonucleotide corresponding to the NF-κB binding motif located −50 bp from the initiation site in the VCAM-1 promoter. Oligonucleotides were synthesized (5′-TGAAGGGATTTCCC-3′ and labeled with [^{32}P]dATP using Klenow DNA polymerase. The extracts were size fractionated on 4% native acrylamide gels and exposed to autoradiography film at −70°C. Lanes: 1, control; 2, TNF-α (100 units/ml); 3, TNF-α + DETA (100 μM); 4, TNF-α + DETA-NO (100 μM). Experiments were performed twice with similar results, and the best analysis is presented.

delineate a potential pathway whereby NO may inhibit important early atherogenic events.

Our findings that DETA-NO suppresses the cytokine-induced transcriptional activation of the VCAM-1 promoter as well as NF-κB-like DNA binding activity in endothelial cells are similar to previous investigations involving structurally diverse antioxidants such as PDTC and *N*-acetylcysteine (6, 8). While the extent of suppression of VCAM-1 cell surface protein appears somewhat less than the decrease in mRNA levels or gene transcription at the time points measured, this

Table 2. NO suppresses oxidative modification of linoleic acid in human umbilical vein endothelial cells through a redox-sensitive pathway

	Linoleic, dpm	Modified linoleic acid, dpm
Control (n = 4)	9867 ± 987	11,498 ± 1839
TNF-α (n = 4)	8976 ± 1233	30,987 ± 4899*
TNF-α + DETA (n = 3)	8342 ± 1922	26,785 ± 3776*
TNF-α + DETA-NO (n = 3)	9777 ± 1022	9,066 ± 1226†

HUVEC were cultured in 24-well plates and exposed to 100,000 dpm of [1-^{14}C]linoleic acid for 30-45 min in the presence or absence of DETA or DETA-NO (100 μM). The cells and media were separated, and the cells underwent a chloroform/methanol (2:1) extraction. The lipid extract from the cells was placed on a thin layer chromatography plate, and lipids were separated using a 90:10:0.5:0.5 (chloroform/methanol/acetic acid/deionized water) solvent system. Linoleic acid and 13-HPODE (commercially available) were used as standards. The corresponding zones on the plate were collected and were counted using radiation scintillation spectrometry. n, Number of experiments.

*Value differs ($P < 0.05$) from control.

†Value differs ($P < 0.05$) from TNF-α.

is likely due to differences in the kinetics of activation of the VCAM-1 gene at these different regulatory levels. Moreover, these studies do not take into account posttranscriptional and/or posttranslational mechanisms involved in the regulation of VCAM-1 gene expression (8, 28).

Our present results provide further evidence that NF-κB-like factors are necessary to activate VCAM-1 gene expression in endothelial cells (6, 28–31). Similar to studies utilizing the antioxidant PDTC (6), DETA-NO markedly inhibited cytokine activation of NF-κB-like binding and VCAM-1 expression but had no significant effect on E-selectin expression. This suggests that regulatory factors other than NF-κB may play an important role in the regulation of the endogenous E-selectin gene. Alternatively, the ability of the NF-κB factor to modulate VCAM-1 and gene expression may differ quantitiatively in terms of DNA binding affinity or transcriptional activation. Transcriptional regulatory elements flanking the κB sites interact with activated NF-κB to modulate transcription from distinct promoters (8). The interaction of multiple transcriptional factors in a pattern distinct from VCAM-1 and E-selectin may contribute in part to the observation that NF-κB appears to be essential for VCAM-1 but not sufficient from ICAM-1 or E-selectin induction (32). In addition, different kinetics of induction may reflect distinct mechanisms for the regulation of VCAM-1, ICAM-1, and E-selectin gene expression.

NO is a free radical with both antioxidant and pro-oxidant properties. For example, lipid peroxidation can be enhanced or suppressed by NO, depending on the relative concentrations of NO and reactive oxygen species such as hydrogen peroxide (33) and superoxide (14, 16). In accord with an antioxidant role of NO, we found that exogenous NO suppressed the cytokine-induced conversion of fatty acids to a peroxidized metabolite, suggesting that NO plays a significant role in its regulation (14). Previous investigations suggest that supplementation with polyunsaturated fatty acids enhances the susceptibility of endothelial cells to oxidant stress (34, 35). Oxidants such as oxidized low-density lipoprotein, and its components 13-HPODE and lysophophatidylcholine (lyso-PC), increase production of partially reduced oxygen species, including hydrogen peroxide, superoxide anion, and hydroxyl radical (36) as well as selectively regulating the gene expression of VCAM-1 and ICAM-1 (5, 7). It may be through these products that pro-oxidants promote biological effects such as cytotoxicity (37) and alteration of gene expression in arterial cells (38, 39). Conversely, in sufficient quantities, NO donors inactivate superoxide (17, 40, 41), although the mechanism through which this occurs is incompletely understood (16, 42). It may be that there is formation of the oxidant peroxynitrite (16). Our present findings, as well as others (43), suggest that a relatively large production of intracellular NO may direct some reactive oxygen species through other oxidative pathways and serve to terminate radical-dependent chain propagation reactions, thereby affecting cell adhesion.

It is possible that NO could act via mechanisms other than an antioxidant effect. NO has been reported to inhibit enzyme function by direct protein nitrosylation (15). Theoretically, nitration of tyrosine residues by peroxynitrite may also influence protein function (15). It is unknown if NF-κB contains heme groups that could also serve as a target for NO signaling. One signal transduction pathway by which NO may suppress cytokine-activated gene expression of VCAM-1 is via elevation of cyclic GMP, as described (44). NO activates soluble guanylyl cyclase, leading to increased formation of cyclic GMP and vasorelaxation (12). Further studies are required to elucidate these mechanisms.

NO has been considered an antiatherogenic molecule based on its inhibition of platelet aggregation, vascular smooth muscle cell proliferation, and neutrophil adhesion to the endothelium. The present study provides yet another mechanism through which NO has an anti-atherogenic effect. These results also provide evidence that NO can modulate gene transcription. The precise pathway by which this occurs remains undefined but is mimicked by other antioxidants, suggesting that NO may function through a redox-sensitve mechanism.

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1. Freeman, B. A. (1993) in *Free Radicals in Molecular Biology Aging and Disease*, ed. Armstrong, D. (Raven, New York), pp. 43–52.
2. Rubanyi, G. M. (1988) *Free Radicals Biol. Med.* **4,** 107–120.
3. Lindsay, S. L. & Freeman, B. A. (1990) in *Pulmonary Circulation:Normal and Abnormal*, ed. Fishman, A. P. (Univ. of Pennsylvania Press, Philadelphia), pp. 217–229.
4. Ohara, Y., Peterson, T. G. & Harrison, D. G. (1993) *J. Clin. Invest.* **91,** 2546–2551.
5. Kume, N., Cybulsky, M. I. & Gimbrone, M. A. (1992) *J. Clin. Invest.* **90,** 1138–1144.
6. Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W. & Medford, R. M. (1993) *J. Clin. Invest.* **92,** 1866–1874.
7. Khan, B. V., Parthasarathy, S., Alexander, R. W. & Medford, R. M. (1995) *J. Clin. Invest.* **95,** 1262–1270.
8. Weber, C., Erl, W., Pietsch, A., Strobel, M., Loms Ziegler-Heitbrock, H. W. & Weber, P. C. (1994) *Arterioscler. Thromb.* **14,** 1665–1673.
9. Lenardo, M. J. & Baltimore, D. (1989) *Cell* **58,** 227–229.
10. Schreck, R., Rieber, P., Manner, D., Droge, W. & Baeuerle, P. A. (1992) *J. Exp. Med.* **175,** 1181–1194.
11. Khan, B. V., Olbrych, M. T., Parthasarathy, S., Alexander, R. W. & Medford, R. M. (1994) *Circulation* **90,** I-82.
12. Moncada, S. & Higgs, E. A. (1991) *Eur. J. Clin. Invest.* **21,** 361–374.
13. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
14. Rubbo, H. & Freeman, B. A. (1994) *J. Biol. Chem.* **271,** 21304–21312.
15. White, C. R., Brock, T. A., Chang, L.-Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A. & Tarpey, M. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1044–1048.
16. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. & Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
17. Rubanyi, G. M. & Vanhoutte, P. M. (1986) *Am. J. Physiol.* **250,** H822–H827.
18. Gryglewski, R. J., Palmer, R. M. J. & Moncada, S. (1986) *Nature (London)* **320,** 454–456.
19. Cohen, R. A., Zitnay, K. M., Haudenschild, C. C. & Cunningham, L. D. (1988) *Circ. Res.* **63,** 903–910.
20. Cooke, J. P., Singer, A. H., Tsao, P., Zera, P., Rowan, R. A. & Billingham, M. E. (1992) *J. Clin. Invest.* **90,** 1168–1172.
21. Swerlick, R. A., Lee, K. H., Li, L. J., Sepp, N. T., Caughman, W. T. & Lawley, T. J. (1992) *J. Immunol.* **149,** 698–705.
22. Hrabie, J. A., Klose, J. R. & Wink, D. A. (1993) *J. Org. Chem.* **58,** 1472–1476.
23. Morley, D., Maragos, C. M., Zhang, X. Y., Boignon, M., Wink, D. A. & Keefer, L. K. (1993) *J. Cardiovasc. Pharmacol.* **2,** 670–676.
24. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1991) *Current Protocols in Molecular Biology* (Wiley, New York).
25. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
26. Dignam, J., Lebovitz, R. & Roeder, R. (1983) *Nucleic Acid Res.* **11,** 1476–1489.
27. Wertheimer, S. J., Myers, C. L., Wallace, R. W. & Parks, T. P. (1992) *J. Biol. Chem.* **267,** 12030–12035.
28. Neish A. S., Williams, A. J., Palmer, H. J., Whitley, M. Z. & Collins, T. (1992) *J. Exp. Med.* **176,** 1583–1593.

29. Iademarco, M. F., McQuillan, J. J., Rosen, G. D. & Dean, D. C. (1992) *J. Biol. Chem.* **267,** 16323–16327.
30. Toborek, M., Barve, S., McClain, C. J., Young, V., Barger, S., Mattison, M. P. & Hennig, B. (1995) *FASEB J.* **9,** A322 (abstr.).
31. Bing Shu, H., Agranoff, A. B., Nabel, E. G., Leung, K., Duckett, C. S., Neish, A. S., Collins, T. & Nabel, G. J. (1993) *Mol. Cell. Biol.* **13,** 6283–6289.
32. Whelan, J., Ghersa, P., van Huijsduijnen, R. H., Gray, J., Chandra, G., Talabot, F. & deLamerter, J. F. (1991) *Nucleic Acids Res.* **19,** 2645–2653.
33. Dee, G., Rice-Evans, C., Obeyesekera, S., Meraji, S., Jacobs, M. & Bruckdorfer, K. R. (1991) *FEBS Lett.* **294,** 38–42.
34. Hart, C. M., Tolson, J. K. & Block, E. R. (1990) *Am. J. Respir. Cell. Mol. Biol.* **3,** 479–488.
35. Hart, C. M., Tolson, J. K. & Block, E. R. (1991) *Am. J. Physiol.* **260,** L481–L487.
36. Ohara, Y., Peterson, T. & Harrison, D. G. (1994) *Arterioscler. Thromb.* **14,** 1007–1013.
37. Cathcart, M. K., McNally, A. K., Morel, D. W. & Chisholm, G. M. (1989) *J. Immunol.* **142,** 1963–1969.
38. Liao, F., Berliner, J. A., Mehrabian, M., Navab, M., Demer, L. L., Lusis, A. J. & Fogelman, A. M. (1991) *J. Clin. Invest.* **87,** 2253–2259.
39. Cushing, S. D., Berliner, J. A., Valente, A. J., Territo, M. C., Navab, M., Parhami, F., Gerrity, R. A., Schwartz, R. J. & Fogelman, A. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5134–5138.
40. Rubanyi, G. M. (1991) *J. Cell. Biochem.* **46,** 27–36.
41. Rubanyi, G. M., Ho, E. H., Cantor, E. H., Lumma, W. C. & Parker-Botelho, L. H. (1991) *Biochem. Biophys. Res. Commun.* **181,** 1392–1397.
42. Radi, R. A. & Freeman, B. A.(1995) *Arch. Biochem. Biophys.* **319,** 491–497.
43. Niu, X.-F., Smith, C. W. & Kubes, P. (1994) *Circ. Res.* **74,** 1133–1140.
44. Pober, J. S., Slowik, M. R., deLuca, L. G. & Ritchie, A. J. (1993) *J. Immunol.* **150,** 5114–5123.