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Prominent role of NF-κB in the induction of endothelial activation by endogenous nitric oxide inhibition

Guido Lazzerini [a], Serena Del Turco [a], Giuseppina Basta [a], Ana O'Loghlen [a,b], Antonella Zampolli [c], Raffaele De Caterina [c,*]

[a] C.N.R. Institute of Clinical Physiology, Pisa, Italy
[b] Department of Biochemistry, Hospital Ramón y Cajal, Madrid, Spain
[c] Institute of Cardiology and Center of Excellence on Aging, "G. d'Annunzio" University, Chieti, Italy

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ABSTRACT

Decreased endothelial nitric oxide (NO) production and increased expression of vascular cell adhesion molecule-1 (VCAM-1) are early features of atherosclerosis. We investigated the effects of suppressing endogenous NO production by the NO synthase inhibitor L-mono-methyl-arginine (L-NMMA), given alone or in combination with interleukin(IL)-1α, on VCAM-1 expression by human umbilical vein endothelial cells (HUVEC). VCAM-1 expression (by enzyme immunoassay), barely detectable at baseline, was significantly increased by L-NMMA (by no more than 20% over control compared with IL-1α induction). This was paralleled by an increase in U937 monocytoid cell adhesion. When HUVEC incubated with L-NMMA were stimulated with low concentrations of IL-1α (0.05–0.5 ng/mL), these determined a higher VCAM-1 expression than in the presence of L-NMMA or IL-1α alone. Northern analysis indicated that VCAM-1 mRNA was induced by L-NMMA alone, and that the effects of L-NMMA and IL-1α were, again, at least additive. Nuclear factor-κB (NF-κB), GATA, activator protein-1 (AP-1) and interferon regulatory factor-1 (IRF-1), transcription factors all involved in VCAM-1 gene expression, were all activated at electrophoretic mobility shift assay and at chromatin immunoprecipitation assay by L-NMMA, but additive effects with the combined administration of L-NMMA and IL-1α only occurred for NF-κB. These results support the view that endogenous NO mantains a normal endothelial non-reactivity towards circulating monocytes, and that suppression of this endogenous brake for endothelial activation results in the activation of multiple transcription factors even in the absence of other endothelial activators, with a prominent role of NF-κB in the presence or absence of other inflammatory mediators.

Introduction

The initial phases in atherogenesis involve the adhesion of leukocytes to a dysfunctional endothelium through the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin. VCAM-1, an inducible molecule expressed on the vascular endothelium upon stimulation with inflammatory cytokines [1], appears to be crucial in atherogenesis, since low-density-lipoprotein (LDL)-receptor knockout mice expressing a hypomorphic variant of VCAM-1 are protected from lesion development [2]. Functional analysis of the human VCAM-1 promoter has shown that VCAM-1 gene expression in endothelial cells is regulated by various transcription factors, including nuclear factor-κB (NF-κB), GATA, activator protein-1 (AP-1) and interferon regulatory factor-1 (IRF-1), in response to pro-inflammatory cytokines [3,4].

Nitric oxide (NO) is a vasodilatory mediator that plays a prominent role in vascular homoeostasis, maintaining blood vessels in a condition of tonic vasodilation, as well as inhibiting smooth muscle cell proliferation and the release of proliferative stimuli [5]. However NO also acts as an anti-inflammatory mediator, affecting endothelial cell function in an autocrine fashion and preventing leukocyte adhesion to the endothelium through the inhibition of adhesion molecule expression [6–8]. NO is synthesized by NO synthases (NOS), which catalyze the production of NO and L-citrulline from L-arginine, O_2 and NADPH-derived electrons [9]. In endothelial cells, a constitutive endothelial NOS (eNOS, NOS-III) produces small amount of NO, preserving physiological homeostasis [10]. A variety of stimuli, including growth factors, shear stress, hypoxia, estrogens and thrombin, increase eNOS expression and activity leading to increased NO production [11–15].

Experimental evidence of the role of NO in the vasculature can be obtained using both the *in vitro* and *in vivo* administration of

* Corresponding author. Address: Institute of Cardiology, "G. d'Annunzio" University, Chieti, C/o Ospedale SS. Annunziata, Via dei Vestini, 66013 Chieti, Italy. Fax: +39 0871 402817.
E-mail address: rdecater@unich.it (R.D. Caterina).

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exogenous NO by NO donors, which inhibit endothelial activation [6,16,17], as well as by structural analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro L-arginine methyl ester (L-NAME), which inhibit, as false substrates, eNOS production in a competitive and reversible manner [9,18,19]. While a number of reports have investigated the role of NO on mechanisms of endothelial activation through the administration on NO donors [6,18,20], there is no systematic study of the effects of inhibiting endogenous NO on the various transcription factors involved in this process. The purpose of this study was therefore to characterize the effects of the inhibition of endogenous NO by the administration of L-NMMA, on the inducible expression of VCAM-1, and to analyze transcriptional mechanisms that regulate such effects.

Materials and methods

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as described [21]. Experiments were conducted on confluent cells between passages 2 and 3, activated by IL-1α (Sigma–Aldrich, St. Louis, USA, at concentrations of 0.05, 0.1, 0.5 ng/mL) and, in selected experiments, by TNF-α (Sigma–Aldrich, 20 ng/mL). The effects of inhibition of endogenous NO synthesis on endothelial activation were assessed with the use of NG-monomethyl-L-arginine (L-NMMA, Calbiochem-Novabiochem, La Jolla, CA, at concentrations of 0.1, 0.5, 1, 5, 10 mmol/L) and with the inert enantiomer NG-monomethyl-D-arginine (D-NMMA) (Sigma–Aldrich). The effects of such treatments on cell survival was monitored by assessing total cellular proteins by the amido-black assay [6]. Cell viability was evaluated by assessing the surface expression of the endothelial-specific constitutive and non-inducible E1/1 antigen [22].

Determination of nitrite/nitrate production

The concentration of inorganic nitrites (NO_2) and nitrates (NO_3), stable end products of nitric oxide, was assayed by a colorimetric method using a NO_2/NO_3 Kit (Cayman Chemical Co., Ann Arbor, MI, USA), based on the Griess reaction [23], applied to media of control (without L-NMMA) and of L-NMMA-treated samples (1 and 5 mmol/L, for 18 h). The determination of NO_2/NO_3 was then corrected for the number of endothelial cells (10^5 cells) and expressed as μmol/L/10^5 cells.

Detection of VCAM-1 surface protein

VCAM-1 expression was assayed by cell surface enzyme immunoassay (EIA), as previously described [24]. Since obtained on confluent monolayers, resulting in similar total cell counts, results were here not corrected for total cell number.

Monocytoid cell adhesion assays

HUVEC were grown to confluence in 6-well tissue plates and treated with various concentration of IL-1α (0.05, 0.1, 0.5 ng/mL) in the presence or absence of L-NMMA (5 mmol/L) for 18 h or of D-NMMA (5 mmol/L), as control, to induce VCAM-1 expression. For control, some monolayers were treated with a mouse anti-human blocking monoclonal antibody against VCAM-1 (antibody E1/6). Adhesion assays were performed by adding 10^6 monocytoid U937 cells (American Type Culture Collection, ATCC, Promochem, Milan, Italy), grown in RPMI medium 1640 with 10% fetal bovine serum (FBS), to each monolayer under rotating conditions (63 rpm) at 21 °C, as described [6].

Northern analysis

HUVEC were treated with L-NMMA 5 mmol/L in the presence or absence of IL-1α 0.05 ng/mL and 0.1 ng/mL for 4 h, a time when peak VCAM-1 mRNA steady-state levels are attained. Total RNA was isolated from HUVEC using the RNAzol kit (Biotecs Laboratories Houston, TX, USA). RNA (20 μg) was separated on 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham, Milan Italy), and immobilized by short-wave UV illumination. Hybridization was performed as described [6]. 18S and 28S ribosomal RNA fluorescence intensity of ethidium-bromide-stained membranes served as a control for uniform RNA loading. Quantification of densities of autoradiographic bands at Northern analysis was performed with the NIH Image 1.6 software.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described [6,25]. The sense strand of the double-stranded oligonucleotide probes used, corresponding to binding sites on the VCAM-1 promoter, were: for nuclear factor-κB (NF-κB: 5′-TGCCCTGGGTTTCCCCTTGAAGGGA TTTCCCTCC-3′; for NF-κB mutant (mut): 5′-CCTTGGCACCTTGAA GTGAGGTCCCTCC-3′; for activating protein-1 (AP-1): 5′-AAAAAA TGACTCATCAAAA-3′); for AP-1 mut: 5′-AAAAAATCCCTCATCAAA A-3′; for GATA: 5′-TTATCTTTCCAGTAAAGATAGCCTTT-3′; for GATA mut: 5′-TTATCTTTCCAGTAAATTTAGCCTTT-3′; for interferon regulatory factor-1 (IRF-1): 5′-GGAGTGAAATAGAAAGTCTGTG-3′; for IRF-1 mut: 5′-GGAGTTAAATAGCAAGTCTGTG-3′ (all from MWG Biotech, Milan, Italy). The double-stranded oligonucleotides, obtained after annealing at 95 °C for 5 min, were labeled, and DNA binding reactions were performed as described [6].

Immunofluorescence for the p65 (RelA) NF-κB subunit

HUVEC grown on fibronectin-coated coverslips (3 μg/cm^2) in 24-well plates were stimulated with L-NMMA (5 mmol/L) and IL-1α (0.05 ng/mL) for 1 h, and the nuclear translocation of p65, detected by the binding of a specific antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was visualized by immunofluorescence. Images were processed with a custom-made software to measure nuclear fluorescence of each cell in the field, as described [26,27].

Chromatin immunoprecipitation (ChIP) assay

To detect the association of transcription factors with the human VCAM-1 promoter, we also used a ChIP assay Enzymatic Kit (Active Motif, Rixensart, Belgium, catalog No. 53007), according to the manufacturer's instructions. Briefly, confluent HUVEC (2×10^7 cells) were incubated with L-NMMA 5 mmol/L in the presence or absence of IL-1α 0.05 ng/mL. Protein-DNA complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sheared by enzymatic digestion. DNA, immunoprecipitated by anti-NF-κB p65 (sc-372), anti-c-Jun (sc-1694), anti-GATA-1 (sc-1233), or anti-IRF-1 (sc-497) antibodies (all from Santa Cruz), was purified. DNA was subjected to polymerase-chain reaction (PCR) amplification (PuReTaq™ Ready-To-Go, Amersham Bioscience, UK) with two sets of primers, one (forward primer 5′-GCTTCATTCTGCAATCAGCA-3′; reverse primer 5′-CCAAGGA TCACGACCATCTT-3′) specifically designed to amplify the VCAM promoter region including GATA, IRF-1 and NF-κB binding sites, and the other (forward primer 5′-GGTCCTGACCATGAGAGGAA-3′; reverse primer 5′-AAATTGCTGCAAAACAAGG-3′), to amplify the

VCAM promoter region with the AP-1 binding site. The PCR conditions for both primer sets were 95 °C for 15 min and 40 cycles of 94 °C for 20 s; 58 °C for 20 s for the first primer set and 55 °C for the second primer set; then 72 °C for 20 s. PCR products were analyzed on ethidium bromide-stained agarose gels. The use of equal amounts of input DNA, obtained incubating DNA without antibody, was controlled by gel electrophoresis.

Statistics

Two-group comparisons were performed by the Student *t* test for unpaired values. Comparisons of means of $\geqslant 3$ groups were performed by ANOVA, and the existence of individual differences, in case of significant F values at ANOVA, tested by Scheffé's multiple contrasts.

Results

The inhibition of endogenous NO synthesis by L-NMMA increases endothelial VCAM-1 expression

To test the effect of NO synthesis inhibition by L-NMMA on VCAM-1 expression, we performed cell surface EIA incubating HUVEC with increasing concentrations of L-NMMA (0.1, 0.5, 1, 5 and 10 mmol/L) for 18 h, in the absence or presence of IL-1α (0.05, 0.1, 0.5 ng/mL). As shown in Table 1, VCAM-1 expression increased in the presence of L-NMMA alone compared with control conditions, with maximum effects attained at 5 mmol/L (4.8-fold-increase). The co-stimulation with L-NMMA (5 mmol/L) and increasing concentrations of IL-1α (0.05, 0.1, 0.5) further increased VCAM-1 expression compared with either agent alone (Fig. 1A). These reactions were enantiomer-specific, since D-NMMA (5 mmol/L), alone or in combination with IL-1α, was ineffective to induce VCAM-1 expression (Fig. 1B). L-NMMA treatment (5 mmol/L for 18 h) did not produce detectable toxic effects, as shown by the total protein content (L-NMMA 97 ± 16 *vs* control 109 ± 10) and the expression of the constitutive antigen E1/1 (L-NMMA 1936 ± 154 *vs* control 1889 ± 210) (all results as mean ± SD of O.D. mU; comparisons of the conditions with L-NMMA with the conditions without L-NMMA always N.S.).

The inhibition of endogenous NO synthesis by L-NMMA, both in the absence and in the presence of IL-1α, causes increased adhesion of monocytoid cells to the endothelium

To evaluate the functional consequences of NO depletion by L-NMMA on endothelial cells, we performed rotational adhesion assays with human monocytoid U937 cells. U937 did not adhere to unstimulated HUVEC (control, no IL-1α stimulation, Fig. 2, upper panels), while the exposure to L-NMMA 5 mmol/L for 18 h increased U937 adhesion to HUVEC (Fig. 2, upper panels). This stimulated adhesion was almost totally inhibited by the anti-VCAM-1 monoclonal antibody E1/6 (Fig. 2, upper panels). Treatment with IL-1α for 18 h significantly enhanced U937 adhesion in a concentration-dependent manner (Fig. 2, lower panels). The co-administration of IL-1α and L-NMMA 5 mmol/L resulted in significantly higher adhesion compared with IL-1α alone (Fig. 2, lower panels). In the same conditions, D-NMMA, either alone or in the presence of IL-1α, did not increase U937 adhesion (data not shown).

Since culture conditions affect e-NOS activity and, consequently, NO production [28], we analyzed the extent of NO production evaluating nitrite/nitrate levels. Under baseline conditions, HUVEC generate nitrites/nitrates, and treatment with L-NMMA at 5 mmol/L decreased this baseline production in a time-dependent fashion (Table 2).

Table 1
Effect of L-NMMA on cell surface VCAM-1 expression.

Treatment	VCAM-1 (OD ± SD)
Control (no L-NMMA)	60 ± 22
L-NMMA 0.1 mmol/L	89 ± 19
L-NMMA 0.5 mmol/L	94 ± 8
L-NMMA 1 mmol/L	108 ± 5*
L-NMMA 5 mmol/L	140 ± 11*
L-NMMA 10 mmol/L	125 ± 16*

HUVEC were treated with L-NMMA (0.1, 0.5, 1, 5, 10 mmol/L) for 18 h and VCAM-1 cell surface expression was determined by EIA. Values are reported as mean ± SD of optical density arbitrary by at 405 nm, and are representative of four independent experiments. (*=$P < 0.05$ *vs* control, at Scheffé's multiple contrasts after ANOVA). L-NMMA: N^G-monomethyl-D-arginine.

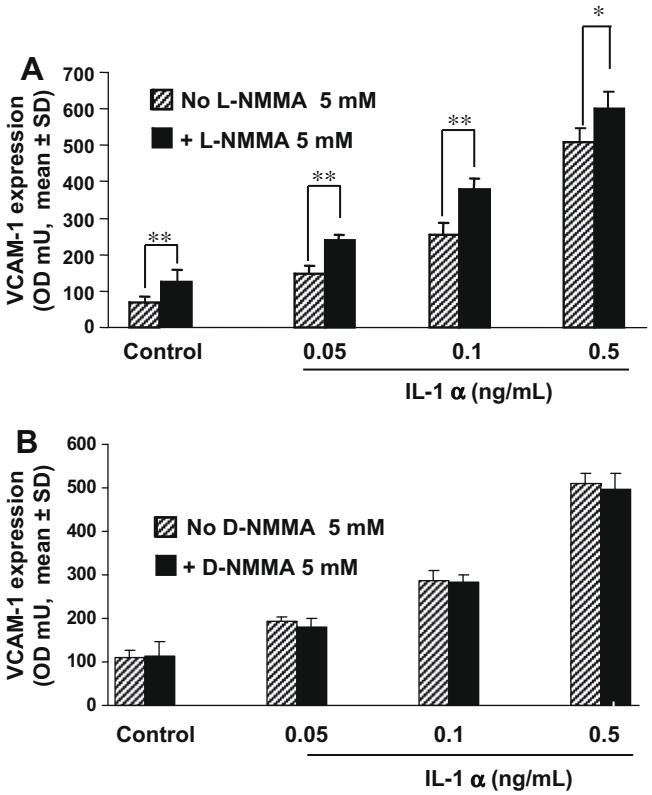

Fig. 1. Effects of endogenous NO synthesis inhibition by L-NMMA or D-NMMA on endothelial VCAM-1 surface expression. Confluent HUVEC were treated with L-NMMA (5 mmol/L) (Fig. 1A) or D-NMMA (B) for 18 h in the absence or presence of IL-1α (0.05, 0.1, 0.5 ng/mL). At the end of the incubation time, VCAM-1 surface expression was quantified by EIA as described in Materials and methods. Values are mean ± SD of optical density arbitrary units at 405 nm, and are representative of four independent experiments, each with four replicates (**=$P < 0.001$, *=$P < 0.05$ at Scheffé's multiple contrasts after ANOVA). Values of VCAM-1 expression in the presence of different concentration of IL-1α are also all significantly different from control ($P < 0.001$ at ANOVA).

The inhibition of endogenous NO synthesis by L-NMMA increases endothelial VCAM-1 mRNA steady-state levels

To determine whether the increase of VCAM-1 protein observed at EIA with L-NMMA treatment is due to increased VCAM-1 transcription (minimum in control conditions), we assessed VCAM-1 steady-state levels at Northern analysis. HUVEC were treated with L-NMMA (5 mmol/L) for 4 h in the absence/presence of IL-1α. As shown in Fig. 3, very low levels of VCAM-1 mRNA were detected in control conditions (in the absence of L-NMMA and IL-1α). VCAM-1 mRNA transcripts, conversely, were clearly detectable

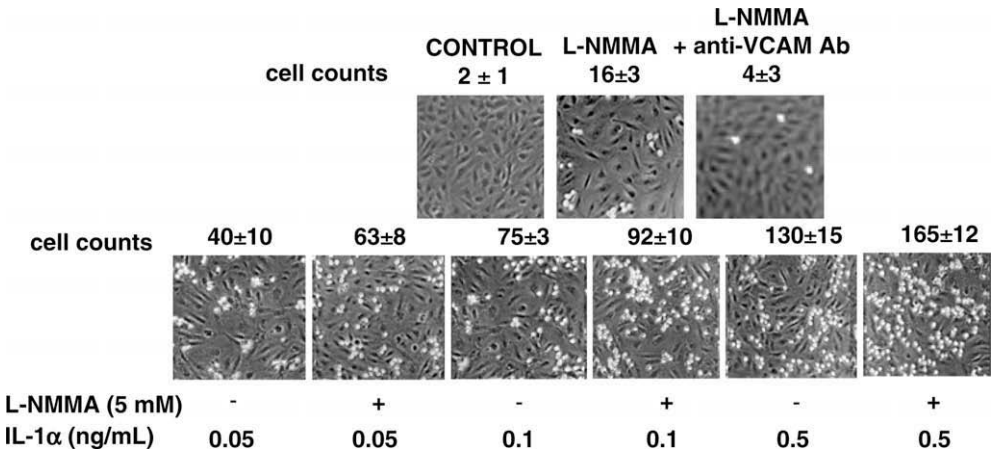

Fig. 2. Effects of endogenous NO synthesis inhibition by L-NMMA on the adhesion of U937 cells to the endothelium. Confluent HUVEC were incubated with L-NMMA 5 mmol/L alone for 18 h and in the presence of increasing concentrations of IL-1α (0.05–0.1–0.5 ng/mL). Cells were then washed and incubated with 1 mL of a concentrated U937 cell suspension (2×10^6 cells) for 15 min at 37 °C under rotating conditions. The photographs show representative optical fields at half-radius distance from the center of the plate. The upper panels show, from left to right, HUVEC monolayers in control conditions, after treatment with L-NMMA, and with L-NMMA in the presence of the blocking anti-VCAM-1 E1/6 antibody. Lower panels show HUVEC monolayers after treatment with IL-1α in the presence or the absence of L-NMMA. Values of adhering cell counts reported here, expressed as mean ± SD, are representative of three independent experiments, and are all significantly different from each other ($P < 0.01$ with Scheffé's multiple contrast analysis after ANOVA, in both the upper and the lower settings). Adhering cells were counted in 16 randomly selected high-powered fields (0.0144 mm^2).

Table 2
Time course of nitrite/nitrate (NO_2^-)/(NO_3^-) production in the supernates of HUVEC treated with L-NMMA.

	(NO_2^-)/(NO_3^-) (μmol/L/10^5 cells)
Control (no L-NMMA), 18 h	17.5 ± 2.5
L-NMMA, 30 min	9.0 ± 1.2*
L-NMMA, 1 h	6.8 ± 0.8*
L-NMMA, 18 h	3.9 ± 0.5*

HUVEC were treated with L-NMMA (5 mmol/L) at different times and nitrite/nitrate levels were measured in cell supernates. Data are mean ± SD and representatives of three independent experiments. (*$P < 0.05$ *vs* control at Scheffé's multiple contrasts after ANOVA). L-NMMA: N^G-monomethyl-D-arginine.

after 4 h of stimulation with IL-1α 0.05 and 0.1 ng/mL, resulting in 2.7–5.8-fold stimulation of VCAM-1 mRNA, respectively, as evaluated by densitometry of the bands (Fig. 3, lanes 3 and 5). VCAM-1 mRNA was however also increased in the presence of L-NMMA alone (by 1.7-fold, Fig. 3, lane 2, in comparison with control, lane 1), and an additive effect of L-NMMA and IL-1α co-stimulation was also observed (2–3-fold increase in VCAM-1 mRNA, lanes 4 and 6, compared with IL-1α 0.05 and 0.1 ng/mL for 4 h alone, respectively). This result is in full qualitative and quantitative agreement with data on VCAM-1 surface expression, suggesting that the inhibition of endogenous NO is sufficient, by itself, to induce endothelial activation, and potentiates cytokine induction of adhesion molecule expression.

Effect of L-NMMA on NF-κB, AP-1, IRF-1, and GATA activation by EMSA

The promoter region of the VCAM-1 gene contains consensus sequences for NF-κB, AP-1, IRF-1 and GATA [4]. We tested the hypothesis that inhibition of the production of endogenous NO by L-NMMA by itself is also sufficient to activate these transcription factors, in turn promoting VCAM-1 gene transcription. We therefore performed EMSA on nuclear extracts from HUVEC treated with L-NMMA (5 mmol/L) for 1 h in the absence and presence of IL-1α (0.05 and 0.1 ng/mL) for 1 h. As shown in Fig. 4A, a significant increase in NF-κB binding activity was detected in nuclear extracts from cells treated with L-NMMA alone compared with the control condition (lane 2 *vs* lane 1), demonstrating that the protein-DNA complex was activated by the mere decrease in intracellular NO levels induced by L-NMMA. As expected, NF-κB-DNA binding increased concentration-dependently in response to IL-1α 0.05 and 0.1 ng/mL (lanes 3 and 5), but this was enhanced, by ~1.5- and 1.3-fold, respectively, in the presence of L-NMMA (5 mmol/L) for 2 h (lanes 4 and 6 *vs* lanes 3 and 5, respectively). To identify whether activation of AP-1, IRF-1, and GATA factors are also involved in VCAM-1 expression elicited by L-NMMA, EMSA analysis was performed (data not shown). After treatment with L-NMMA alone, EMSA showed a significant increase in the levels of nuclear DNA binding proteins corresponding to AP-1, IRF-1 and GATA, suggesting that all these transcription factors can cooperate in the expression of the VCAM-1 gene by L-NMMA. IL-1α treatment also significantly upregulated each of these transcription factors. However, co-treatment with L-NMMA did not further augment AP-1, IRF-1 or GATA binding activity.

These findings indicate that suppression of endogenous NO synthesis by L-NMMA is able to induce VCAM-1 expression activating GATA, IRF-1 and AP-1 both in the absence and in the presence of IL-1α. Differently from NF-κB, however, GATA, AP-1 and IRF-1 are not likely implicated in the further upregulation of VCAM-1 gene expression occurring when NOS inhibition adds up to IL-1α stimulation.

To further prove that NF-κB is involved in L-NMMA-induced VCAM-1 expression, we monitored the nuclear/cytoplasmic distribution of the p65 (Rel A) NF-κB subunit at fluorescence confocal microscopy. Immunofluorescence staining of p65 was all present in the cytoplasm in control, untreated cells, where no nuclear staining was present. In contrast, as a positive control, after treatment with IL-1α (0.05 ng/mL) for 1 h nuclei became heavily fluorescent. Treatment with L-NMMA 5 mmol/L for 1 h alone also induced a significant, albeit more modest, p65 nuclear translocation, and the co-stimulation with IL-1α (0.05 ng/mL) caused a marked increase in the p65 nuclear accumulation, more than after treatment with IL-1α alone (Fig. 4B).

Effect of L-NMMA on NF-κB, AP-1, GATA, and IRF-1 activation at chromatin immunoprecipitation (ChIP) assay

To further investigate the role of transcription factors and of the VCAM-1 promoter regulatory sites, we evaluated the association of

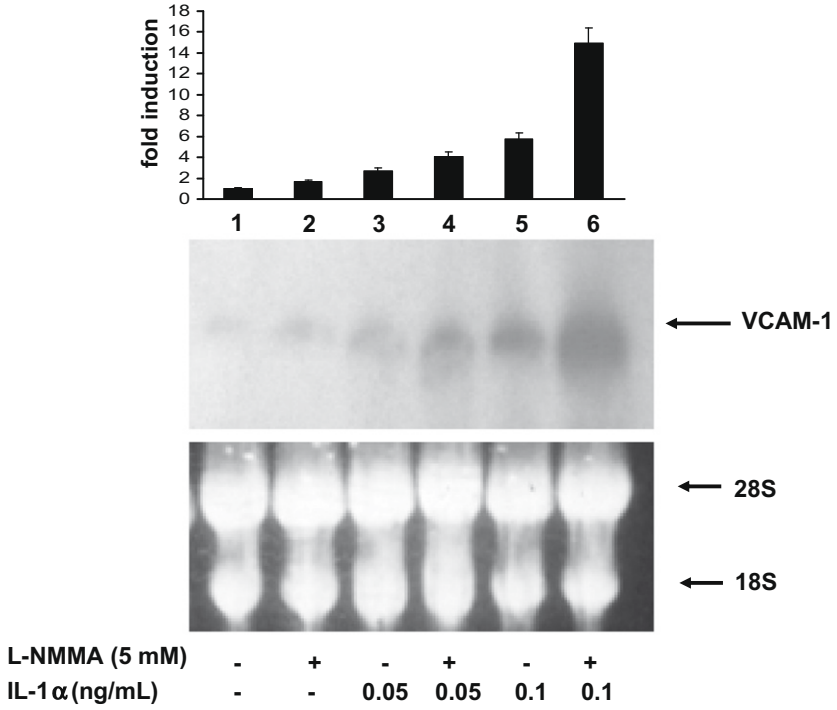

Fig. 3. Effects of endogenous NO synthesis inhibition by L-NMMA, with or without IL-1α, on endothelial VCAM-1 mRNA steady-state levels. Northern analysis (20 μg of total RNA/lane) shows VCAM-1 mRNA steady-state levels after stimulation with IL-1α (0.05, 0.1 ng/mL) for 4 h in the absence or presence of L-NMMA (5 mmol/L) (central panel). The uppermost bar graph shows densitometry analysis of the specific bands as mean ± SD from three representative independent experiments ($P < 0.01$ with Scheffé's multiple contrasts between each IL-1α concentration *vs* L-NMMA 5 mM+IL-1α after ANOVA). The lowermost panel shows ethidium bromide staining for 28S and 18S ribosomal RNAs, to verify equal total RNA loading. This blot is representative of a series of three similar ones run in similar conditions and with equal results.

NF-κB, AP-1, GATA, and IRF-1 with the VCAM-1 promoter region by the innovative ChIP assay. All four transcription factors were thus demonstrated to associate with the VCAM-1 promoter after treatment with L-NMMA or IL-1α (Fig. 5), but only NF-κB binding was increased by the co-stimulation of L-NMMA and IL-1α, thus confirming, by an independent technique, data obtained with EMSA.

Discussion

We [6] and others [18,20] have previously shown (a) that the administration of exogenous NO by various NO donors can potently reduce cytokine-induced endothelial activation, as measured by the surface expression and mRNA levels of endothelial inducible adhesion molecules; (b) that this has functional consequences in terms of monocyte adhesion; and (c) that this is mediated to a large extent by the inhibition of NF-κB activation [6]. Since NO is produced however tonically by the endothelium, through the expression of the constitutive eNOS, it is of interest to understand whether or not the normal endothelium is maintained in its normal anti-inflammatory, anti-atherogenic status through the continued constitutive production of NO. Indeed, another property of NO, vasodilation, has been shown to be tonically active, since the administration of the false substrate NOS inhibitor L-NMMA by itself to humans has been shown to increase blood pressure [9].

We here show that L-NMMA treatment by itself significantly increases monocytoid cell adhesion to endothelial cells. Although quantitatively smaller than the effect of IL-1α, this is the proof-of-concept that the normal vascular endothelium is tonically maintained in an anti-adhesive condition at least in part through the generation of endogenous NO. This effect is largely VCAM-1-dependent, since largely suppressed by treatment with the blocking anti-VCAM-1 E1/6 antibody. We also show here that the abrogation of the endogenous NO production, previously demonstrated to occur by similar concentrations of L-NMMA [6], renders the endothelium more sensitive to the pro-inflammatory effect of IL-1α in terms of a further increase of the pro-adhesive properties of this cytokine, mediated by VCAM-1 expression. On the basis of the effect of L-NMMA, alone or in combination with IL-1α, on endothelial antigenic and functional properties, we here further investigated mechanisms of the increased VCAM-1 expression by L-NMMA.

The molecular mechanisms by which NO modulates gene expression of adhesion molecules involve prevention of the activation of transcription factors that interact with consensus sequences in the promoter region of the corresponding genes [18,29]. VCAM-1 transactivation involves the interaction of NF-κB [3,30], but also of AP-1 [31], GATA [14], and IRF-1 [4]. Previous studies have demonstrated that exogenous NO by the administration of various NO donors inhibits NF-κB-dependent reporter activity [6,32], and that NO donation to animals after lung injury induced by LPS treatment leads to decreased NF-κB-DNA binding *in vivo* [33]. Exogenous NO has been shown to inhibit NF-κB activation mostly by inducing the expression and prolonging the half-life of the NF-κB inhibitory subunit IκBα [20,34–36], and by this mechanism also prevents lipopolysaccharide (LPS)/tumor necrosis factor (TNF)-α-induced NO synthesis [37]. NO donors have also been shown, on the other hand, to inhibit the activation of AP-1 [38]. However neither the effects of exogenous NO donors on GATA and IRF-1, nor the effects of endogenous NO suppression on AP-1, GATA and IRF-1 transcription factors had been previously studied. Our results indicate that L-NMMA treatment alone induces NF-κB translocation into the nucleus and its binding to the target VCAM-1 gene, and that L-NMMA significantly increases the effect of IL-1α on NF-κB activation in human endothelial cells. The induction of VCAM by L-NMMA is here associated also with increased binding activity of AP-1, GATA and IRF-1, suggesting a tonic inhibition by endogenous NO on all

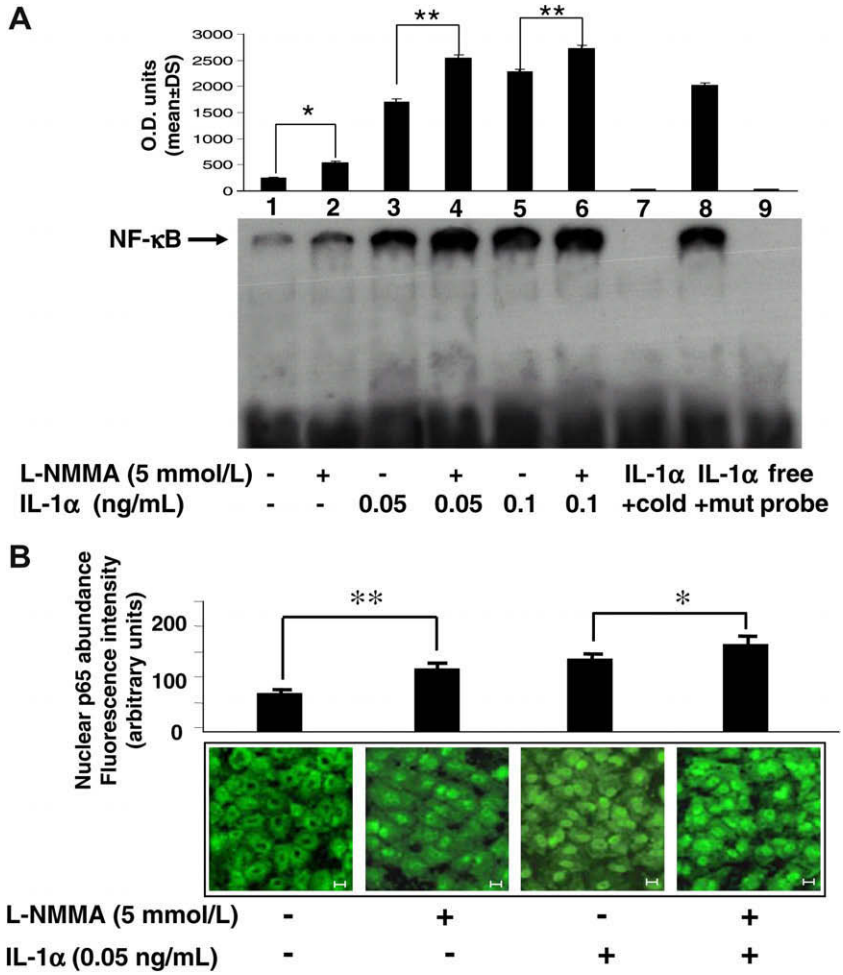

Fig. 4. Effects of endogenous NO synthesis inhibition by L-NMMA, with or without IL-1α, on endothelial NF-κB activation by EMSA. Panel A: Nuclear extracts were obtained from HUVEC subjected to various experimental treatments and electrophoresed (5 μg per lane) on a polyacrylamide gel as detailed in Materials and methods. Compared with nuclear extracts obtained in control, unstimulated conditions, a retardation of electrophoretic mobility (gel shift) of the ^{32}P-labeled oligonucleotide containing the VCAM-1 NF-κB sequence is seen in lane 2, obtained with nuclear extracts from cells incubated with L-NMMA (5 mmol/L) for 1 h. Other conditions tested include treatment of HUVEC with IL-1α (0.05 ng/mL and 0.1 ng/mL) for 1 h in the absence (lanes 3 and 5) and in the presence (lanes 4 and 6) of L-NMMA (5 mmol/L), respectively. Specificity of the retarded band for NF-κB is proven by its absence with nuclear extracts from unstimulated conditions (lane 1), its total abolition with an excess (50×) of cold wild-type oligonucleotide (lane 7), and its preservation with a similar excess of mutant oligonucleotide (lane 8). Lane 9 depicts the run of the free labeled oligonucleotide probe in the absence of nuclear extracts. This EMSA is representative of a series of four, with similar results. The upper bar graph shows densitometry analysis of the specific bands, as mean ± SD representative of four independent experiments (**$P < 0.001$; *$P < 0.05$ between conditions joined by brackets with Scheffé's multiple contrasts after ANOVA among different concentrations of IL-1α). Panel B: HUVEC were incubated in the absence or presence of L-NMMA (5 mmol/L) ± IL-1α (0.05 ng/mL) for 1 h. The images show the immunofluorescence staining for the sub-cellular (nuclear/cytoplasmic) distribution of the p65 (RelA) NF-κB subunit, performed as described in Materials and methods. Photomicrographs, at confocal microscopy, show representative fields, at 40× magnifications, of intact endothelial monolayers. Notice the different nuclear/cytoplasmic distribution of p65 in the various experimental conditions. The upper histogram is a quantitation of nuclear p-65 relative abundance, in arbitrary units of fluorescence. Results are mean ± SD of 3 separate experiments. (**$P < 0.001$; *$P < 0.05$ between conditions joined by brackets with Scheffè's multiple contrasts after ANOVA). Scale bar = 8 μm.

transcription factors involved in VCAM-1 regulation. Since some part of VCAM–1 induction by interleukins is dependent on the stimulatory actions of GATA and AP-1, it was conceivable that L-NMMA induces VCAM-1 in part by upregulating GATA and AP-1 binding activity. Likewise, IRF-1, another inducible transcription factor induced by cytokines and thrombin [39], and contributing to VCAM-1 activation by cytokines [4,40], is an additional potential target of endogenous NO action. Contrary to NF-κB, however, AP-1, GATA and IRF-1 binding activities were not further augmented by L-NMMA in the presence of IL-1α, indicating the absence of their role in the cooperative effect between IL-1α and the inhibition of endogenous NO.

IRF-1 binding activity was also here enhanced by treatments with L-NMMA or IL-1α alone, but was also not increased after co-treatment with both agents compared with IL-1α alone. Altogether, the stimulability of AP-1, GATA and IRF-1 by L-NMMA, but the here-shown non-additivity of such stimulations with the effects of IL-1α suggest that suppression of endogenous NO activates cellular pathways overlapping with those of IL-1α. However, the additive effects of the co-stimulation with L-NMMA and IL-1α on NF-κB activation suggest that these two agents activate distinct intracellular signal transduction pathways involved in NF-κB activation. The differential involvement of such transcription factors in the effects of L-NMMA on VCAM-1 expression has been here shown looking at the binding activity of individual transcription factors by two independent techniques, EMSA and the chromatin immunoprecipitation (ChIP) assay.

The biological relevance of our observations is illustrated by several clinical conditions associated with NO deficiency, which may contribute to endothelial dysfunction. Insulin resistance and chronic cigarette smoking are associated with deficiency of tetrahydrobiopterin (BH_4), an essential cofactor in e-NOS activity, resulting in impaired vascular relaxation [41]. An overproduction of endogenous NOS inhibitors, such as asymmetric and symmetric

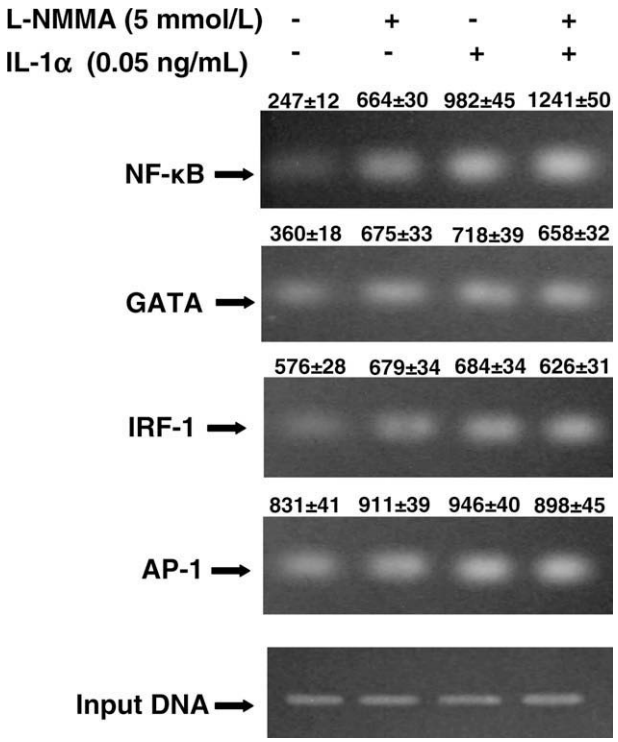

Fig. 5. Effects of endogenous NO synthesis inhibition by L-NMMA, with or without IL-1α, on endothelial NF-κB, AP-1, GATA and IRF-1 activation by chromatin immunoprecipitation (ChIP) assay. Binding of NF-κB, AP-1, GATA and IRF-1 to the VCAM promoter was detected with the ChIP assay. Immunocomplexes of transcription factors associated with DNA were obtained from untreated HUVEC (control), or HUVEC treated with L-NMMA (5 mmol/L) for 1 h, IL-1α (0.05 ng/mL) for 1 h, L-NMMA 5 mmol/L + IL-1α 0.05 ng/mL for 1 h. Specific DNA fragments were quantified by PCR, as detailed in Methods. DNA purified from lysates incubated without antibody was used as input control (Input). The number on top of each strip shows the densitometric quantitation (mean ± SD of optical density mU) of the specific bands obtained in 2 separate similar experiments. All four transcription factors significantly associate with the VCAM-1 promoter after treatment with L-NMMA or IL-1α ($P < 0.01$), but only NF-κB binding is higher with the co-stimulation of L-NMMA and IL-1α ($P < 0.01$). Data are analyzed by Scheffé's multiple contrasts after ANOVA.

dimethylarginine (ADMA and SDMA), found in several disease states and having properties similar to L-NMMA, may enhance atherogenesis through the loss of the chronic anti-atherogenic properties of NO [9]. Many atherogenic factors that contribute to increased oxidative stress, such as hyperlipidemia [42], hypertriglyceridemia [43], hyperglycemia and the accumulation of advanced glycation endproducts (AGEs) [44], have been found to reduce NO bioavailability. All these risk factors for vascular disease may therefore find their ultimate mechanism of action in the here-described effects on the transcription factors involved in endothelial activation as a consequence of the reduced NO bioavailability.

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