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

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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

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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