IκBα-dependent regulation of low-shear flow-induced NF-κB activity: role of nitric oxide

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results (17, 18) and data from other investigators (19, 25, 27) have also shown that activation of NF-κB in endothelial cells by low shear results in the increased expression of the vascular cell adhesion molecule VCAM-1. Endothelial cell-derived VCAM-1 mediates increased adhesion of circulating monocytes (6, 26) and the enhanced recruitment of monocytes to the subintimal space, both hallmarks of early atherosclerosis.

NF-κB, in its active binding form, is a collection of homo- and heterodimers (13, 21) composed of various combinations of members of the NF-κB/Rel family. The NF-κB/Rel family of proteins includes NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel, RelA (p65), and RelB. The NF-κB complex is maintained in an inactive form by sequestration in the cytoplasm through interaction with the inhibitory protein inhibitor κB (IκB) that sterically hinders binding of import proteins to the nuclear localization sequence of the NF-κB subunits. The IκB family includes IκBα, IκBβ, IκBγ, IκBe, Bcl-3, and the precursors of NF-κB1 (p105) and NF-κB2 (p100). Members of the IκB family of proteins are characterized by the presence of six or more ankyrin repeats, an NH2-terminal regulatory domain, and a COOH-terminal domain that contains a PEST motif involved in basal turnover. On stimulation, an activation cascade results in the phosphorylation of IκB, which leads to polyubiquitination and degradation by the 26S multicatalytic proteosomes (13). The released NF-κB translocates to the nucleus where it binds to κB sites in the promoters and enhancers of target genes. The signal is eventually terminated through cytoplasmic reseques- tration of NF-κB, which depends on IκBα synthesis, a process itself requiring NF-κB transcriptional activity. Of all inhibitor subunits, IκBα is the one subunit that is best characterized.

Recently, Bhullar et al. (3) demonstrated the involvement of upstream kinases in the phosphorylation of IκBα and the activation of NF-κB in high shear stress (12 dynes/cm²)-exposed vascular endothelial cells (EC). However, the mechanism of activation of these up-
stream mediators in low shear-exposed EC where, unlike high shear exposed cells, NF-κB activation is persistent is unclear. In other studies, it has been shown that nitric oxide (NO) can inhibit the activation of NF-κB by stabilizing the NF-κB/IkBα complex (14, 20, 24). It has been further suggested that NO may also attenuate NF-κB activation by mediating an as yet unknown signaling pathway that leads to the increased transcription of the IkBα gene (23).

In this study, we have investigated the effect of low shear on the expression of IkBα in human aortic endothelial cells (HAEC) and whether this is influenced by an altered generation of NO. A reduced level of IkBα would lead to persistent activation of NF-κB. In support of this observation, we have also demonstrated downregulation of NF-κB activation, as well as κB-dependent VCAM-1 expression and endothelial-monocyte adhesion under conditions of low shear stress by forced overexpression of IkBα.

MATERIALS AND METHODS

Cell culture. HAEC (Clonetics, San Diego, CA) were cultured in MCDB131 medium (Sigma, St. Louis, MO) containing 10% bovine calf serum (BCS, Hyclone, Kansas City, KS) and enriched with 250 ng/ml fibroblast growth factor (Pepro Tech, Rocky Hill, NJ), 1 mg/ml of epidermal growth factor (Pepro Tech), 1 mg/ml of hydrocortisone (Sigma), 100 units/ml of penicillin, and 100 mg/ml streptomycin (Mediatech, Herndon, VA). Cells from passages 4 to 7 were used in all experiments.

Shear stress. HAEC were seeded on polyester film (10 × 19 cm Mylar sheets; Regal Plastics, San Antonio, TX) precoated with 2% gelatin and grown to near confluence within 2–3 days. The cells were subsequently incubated in complete MCDB-131 medium supplemented with 2% BCS alone for 20 h before the initiation of the flow shear to avoid any influence of growth factors on the induced responses. Flow experiments were performed using the closed loop flow system described previously (17). The cone and the plate model (4) were used in shear stress experiments involving NO measurements to limit the volume of culture medium to be used.

Treatment conditions. Cells were treated with 300 μM of 5-nitrosoglutathione (GSNO; Sigma) or 1 mM of sodium nitroprusside (SNP; Sigma) or Nω-nitro-L-arginine (500 μM L-NNA) (Sigma) for 30 min before shear stress. NO donors at the same concentration were added to the circulating medium while the cells were kept under shear stress. Care was taken to avoid photosensitization of SNP by performing the experiment under yellow light. Stock solutions were flushed with nitrogen gas and stored in amber-colored bottles at −20°C.

Transient transfections. The VCAM-1 promoter fragment was generated by PCR using human genomic DNA as the template and the following nested primers: (forward-forward) 5’-TGC GGT TAA ATC TCA CAG CCC-3’, (forward-inner) 5’-AGA GAT TTG CCA CTT CAG ATG G-3’, (reverse-out) 5’-GTA ATG TTG AGG CGC CAA G-3’, and (reverse-inner) 5’-GAG CAT CTT CCC AGG CAT TTT AAG-3’ derived from published sequences (28). A PCR product of ~900 base pairs (bp) (VCAM-1 5’-flanking sequence from −755 to +143 containing the two NF-κB binding sites) was obtained and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). The insert was then subcloned into the pGL3-Basic luciferase reporter vector at the SacI and XhoI sites. For the eNOS overexpression studies, full-length cDNA of eNOS (provided by Dr. Philip A. Marsden, Renal Division and Department of Medicine, University of Toronto, Toronto, ON, Canada) was subcloned into the pCl-neo-expression vector (Promega, Madison, WI) and used for transient transfections. Plasmid pcDNA 3.1 containing the full-length cDNA for IkBα provided by Dr. John Morris (Lineberger Comprehensive Cancer Center, Chapel Hill, NC) was used for IkBα overexpression studies. The transfection efficiency was periodically checked by cotransfecting the cells with pEGFP (enhanced green fluorescent protein) luciferase construct (Clontech Laboratories, along with other plasmids of interest. Transfected cells were subjected to FACS analysis. The fluorescent intensity of pEGFP-positive cells indicated a transfection efficiency of ~30%.

Transfections of IkBα vector and VCAM-1-pGL3 Basic-luciferase reporter system and eNOS expression vectors were performed as follows: cells seeded on plastic slips for shear experiments were incubated in MCDB-131 medium containing 2% serum devoid of growth factors for 24 h. Full-length cDNA (2 μg) for IkBα, VCAM-1-pGL3 Basic-luciferase vector, or the eNOS expression construct was transfected using the Effectene reagent (Qiagen, Valencia, CA) following the manufacturer’s protocol. The DNA-lipid complex was then added to the cells slowly and incubated in a limited volume (~8–10 ml) of medium. Cells were subjected to shear stress (2 dynes/cm2) for 24 h posttransfection.

Electrophoretic mobility shift analysis. After application of flow shear stress for the indicated time periods, cells were washed in ice-cold PBS and harvested. Nuclear extracts were prepared as reported by Mohan et al. (17). The total protein concentrations were measured using the bicinchonic acid (BCA) method following the manufacturer’s protocol (Pierce, Rockford, IL). For electrophoretic mobility shift analysis (EMSA) analysis, a double-stranded oligonucleotide containing a tandem repeat of the consensus NF-κB binding sequences 5’-GGG GAC TTT CC-3’ was end-labeled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP (Amer sham, Arlington Heights, IL). Free unbound radioisotope was separated on a push column (Stratagene, La Jolla, CA). The binding reaction was performed by mixing nuclear extract (8 μg of total protein), 0.1 μg of poly (dl-dc) (Pharmacia Fine Chemicals), and 32P-labeled NF-κB probe (0.5 ng DNA; ~50,000 cpm) in binding buffer containing 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 20% (vol/vol) glycerol. Estimation of NF-κB activation was performed by quantitative analysis of autoradiograms using a phosphor imager, and statistical analysis was carried out using StatView 5.0 software.

RNase protection assay. Total cellular RNA was isolated from HAEC using the Ultra spec reagent following the manufacturer’s protocol (Biotech, Houston, TX). Total RNA (10–15 μg) was used for the RNase protection assay. The antisense RNA probe for VCAM-1 was obtained from Harlingen (San Diego, CA). Following the manufacturer’s protocol, the in vitro transcription and the protection assays were performed.

Monocyte adhesion assay. Whole blood from healthy volunteers was collected into a vacutainer tube containing EDTA by vein puncture and used within 4 h of collection. Enriched monocytes were isolated by standard buoyant density centrifugation technique using NycoPrep 1.068 (Nycomed Pharma AS, Oslo, Norway), as reported earlier (18). Cells obtained were further purified on Optiprep (Nycomed Pharma) to eliminate platelet contamination. Cell viability was determined by the trypan blue dye exclusion method.
Purity of monocyte preparation was determined by labeling the cells with mouse monoclonal antimacrophage antibodies (Enzo Diagnostics, Farmingdale, NY), followed by Texas red-conjugated goat anti-mouse antibodies (Calbiochem, San Diego, CA). Typically, >95% of the cells showed positive staining. For adhesion studies, the purified monocytes (1.67 × 10⁶ cells/ml) were introduced into 150 ml of circulating medium and allowed to circulate at low shear for an additional 1 h after completion of the designated experimental flow regimen. The slips were then washed in PBS solution, fixed in methanol for 5 min, and stained with Giemsa stain (Sigma), which allows light microscopic identification of adherent monocytes. Under high-power light microscopy (400×), total adherent monocytes per high-power field were visualized and counted. No-shear control slips were subjected to 1 h of low shear with the monocytes in the circulation and used for comparison.

**Immunoblotting.** The nuclear and cytoplasmic extracts of shear stress-exposed HAEC were subjected to SDS polyacrylamide gel electrophoresis, and the proteins were transferred to PVDF membrane by electrotransfer. The membranes were blocked with 5% blotto (Amersham) and probed with a 1:1,000 dilution of goat anti-human IκBα antibody (Santa Cruz Biotech, Santa Cruz, CA) or 1:2,000 of anti-eNOS antibody (BIOMOL Research Laboratories, Plymouth Meeting, PA), followed by horseradish peroxidase-conjugated secondary antibody. For loading control, the blots were probed with mouse monoclonal anti-α-tubulin provided by Dr. Asok Banerjee (UT Health Science Center, San Antonio, TX). The blots were developed with ECL reagent (Amersham) following the manufacturer’s protocol. Quantitative analysis of the IκBα and eNOS protein expression was performed using the NIH 1.58b19 image analysis software package with an integrated density program.

**Measurement of NO in HAEC exposed to fluid shear stress.** Confluent monolayers of HAEC grown in 100-mm dishes were exposed to the laminar shear stress of 16 dynes/cm² (high shear) or 2 dynes/cm² (low shear) using the cone and plate system, as described previously (4). This system was used in this experiment because it allows accurate measurements of NO levels (as accumulating levels of nitrite) in small volumes of culture medium. Aliquots (200 μl) of shear media were taken from the dishes at different time intervals to measure accumulating levels of nitrite using an NO sensor in NO-T nitric oxide measurement system; Harvard Apparatus, Holliston, MA).

**Statistical analyses.** Data were analyzed with the randomized block analysis of variance (ANOVA), treating experiments as blocks and treatments as repeated measures for the experiment. These two factors were considered in each analysis. In addition, both change from control and ratio to control were considered to determine which change score better met the assumptions for a valid analyses. The ratio to control better satisfied the assumptions of the analysis of variance, so the results from that analyses are presented. In fact, with this ratio, the experimental variability is small and insignificant. Both this ratio to control and the use of experiment in the ANOVA helped to provide valid comparison of the treatment conditions adjusting for both between and within experimental variability. After the ANOVA, comparisons of the treatments with each other and with the static control condition were done with means from the ANOVA and t-tests using these ANOVA results, accounting for experimental variability.

**RESULTS**

Low shear stress stimulates IκBα degradation and induces NF-κB activation. To determine whether low shear stress-induced activation of NF-κB is associated with proteolytic degradation of IκBα, HAEC were subjected to a low shear stress of 2 dynes/cm² for 1 and 6 h. As shown in Fig. 1A, immunoblotting of the cytoplasmic extracts with polyclonal anti-IκBα antibody revealed reduction of IκBα levels after exposure of HAEC to low shear stress. Compared with the static control maintained at 1 h, a decrease (to 83% of control) in the amount of cytoplasmic IκBα could be seen within 1 h of exposure of HAEC to low shear stress. The levels reduced to 70% of control after 6 h of exposure to low shear stress compared with static control maintained for 6 h. No changes were seen in the corresponding levels of α-tubulin used as the loading control. As shown in Fig. 1B, quantitative analysis of the autoradiograms from three independent experiments clearly

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**Fig. 1.** A: time course study showing low shear-induced inhibitor κBα (IκBα) downregulation. Human aortic endothelial cell (HAEC) monolayers were kept as static controls or subjected to shear stress of 2 dynes/cm² for 1 and 6 h. Cytoplasmic extracts were immunoblotted with polyclonal anti-IκBα antibody and anti-goat horseradish peroxidase (HRP)-conjugated second antibody. The decreased IκBα in the cells exposed to shear for 1 and 6 h demonstrated degradation of IκBα. α-Tubulin expression level shows equal loading of each protein sample. B: quantitative analysis performed by densitometric scanning of IκBα protein expression. Autoradiograms were scanned, and changes in IκBα expression levels were quantified using an integrated density program (NIH 1.58b19 image analysis software). Results of 3 independent experiments were summarized and expressed as means ± SD.

*Significant decrease compared with the control, P = 0.003.
demonstrated a significant reduction ($P = 0.003$) of IκBα expression observed at 6 h of low shear stress.

Overexpression of IκBα attenuates low shear-induced NF-κB activation, mRNA expression, and subsequent endothelial-monocyte adhesion. The above data indicate that the degradation of IκBα is a critical intermediate step in the early low shear-induced activation of NF-κB. To obtain further evidence for this important role of IκBα, we examined low shear-induced NF-κB activation in HAEC that were transfected with an IκBα expression plasmid. Immunoblotting performed in the cytoplasmic extracts of the transfected HAEC confirmed that the cells overexpressed IκBα compared with cells transfected with vector alone (Fig. 2A). The IκBα-transfected cells, when subjected to low shear stress for 6 h, showed a complete attenuation of shear-induced NF-κB activation (Fig. 2, B and C). Compared with untransfected HAEC subjected to low shear stress, overexpression of IκBα significantly blocked (63.4%; $P < 0.05$) the NF-κB DNA binding activity. Both control untransfected cells and cells transfected with empty vector exhibited higher levels of low shear-induced NF-κB activity, indicating the functional specificity of the insert.

Next, we determined whether the IκBα overexpression that downregulated shear-induced NF-κB activation also altered κB-dependent downstream target gene expression. In a parallel experiment, VCAM-1 mRNA expression was analyzed by RNase protection assay using a $^{32}$P-labeled antisense VCAM-1-specific probe. Consistent with the observed downregulation of NF-κB DNA binding activity, cells overexpressing IκBα also showed a significant decrease in the induction of VCAM-1 mRNA expression (53.2%; $P = 0.024$), whereas cells transfected with vector alone showed a normal induction (Fig. 3). The fold increase in VCAM-1 mRNA in low shear-induced untransfected and vector transfected HAEC were 2.69 ± 0.66 and 2.25 ± 0.3, respectively, compared with no shear static control.

To address whether overexpression of IκBα also attenuated κB-dependent low shear-induced endothelial-

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Fig. 2. Inhibition of low shear (2 dynes/cm$^2$)-induced nuclear factor-κB (NF-κB) DNA binding activity by overexpression of IκBα in HAEC. Cells incubated for 20 h in serum-deficient medium (2% serum) in the absence of growth factors were transfected with either pcDNA 3.1 IκBα expression vector or empty vector using Effectene reagent (Qiagen). At 24 h posttransfection, the cells were subjected to (A) immunoblotting to confirm the overexpression of IκBα in the cytoplasm (NS indicates nonspecific bands). B: HAEC overexpressing IκBα were exposed to shear stress for 6 h. Static cultures were used as negative controls. The total amount of NF-κB binding activity in nuclei from at least 3 independent experiments (means ± SD) was determined by subjecting the autoradiograms to PhosphorImager for quantitation using BioRad Image Software program. *Significant inhibition ($P < 0.05$) compared with vector only transfected cells. C: nuclear proteins were extracted, and electrophoretic mobility shift analyses were performed. A representative autoradiogram of at least 3 independent experiments is shown.

Fig. 3. Inhibition of vascular cell adhesion molecule (VCAM-1) mRNA expression in HAEC overexpressing IκBα. Transfected cells were exposed to low shear stress (2 dynes/cm$^2$) for 6 h and harvested for RNA extraction. Total RNA was subjected to RNase protection assay following the manufacturer’s protocol (BD Pharmingen, San Diego, CA). Autoradiogram is representative of at least 3 independent experiments. *Significant decrease ($P = 0.024$) compared with vector only-transfected cells. Quantitative analysis of autoradiograms was performed using PhosphorImager.
monocyte adhesion, we examined monocyte adhesion as described earlier (18). As shown in Fig. 4, HAEC exposed to 6 h of low shear stress showed a 6.1 ± 0.65-fold increase in monocyte adhesion compared with the no-shear static control (P < 0.05). Consistent with results obtained in NF-κB activation and VCAM-1 mRNA expression experiments (Figs. 2 and 3), cells overexpressing IkBα exhibited a significant decrease (77%; P < 0.05) in low shear-induced monocyte adhesion. The empty vector-transfected cells exposed to low shear stress exhibited increased monocyte adhesion similar to the untransfected cells subjected to low shear stress. The fold increases were 6.1 ± 0.65 and 6.8 ± 0.1 in untransfected and empty vector-transfected HAEC, respectively.

Exogenous NO donors inhibit low shear-induced NF-κB activation and VCAM-1 promoter expression by increasing IkBα levels. NO has been shown to induce synthesis of IkBα in TNF-α-stimulated human umbilical vein endothelial cells (20, 24). To investigate the effect of NO on low shear-induced NF-κB activation, HAEC were subjected to 6 h of low shear stress in the absence or presence of SNP (1 mM), an NO donor. As shown in Fig. 5A, EMSA performed with nuclear extracts of HAEC demonstrated a high induction of NF-κB activation after 6 h of low shear stress, as we have shown previously. This low shear-induced NF-κB activation was inhibited by the presence of SNP (1 mM). Furthermore, to confirm the effect of NO on low shear-induced NF-κB DNA binding activity, HAEC were exposed to low shear stress in the presence of l-NNA (500 μM), an eNOS inhibitor that is known to prevent endogenous generation of NO. As demonstrated in Fig. 5A, lane 4, l-NNA enhanced the NF-κB DNA binding activity compared with HAEC exposed to low shear stress in the absence of the inhibitor. These results confirm the involvement of NO in regulating the pathway responsible for low shear-induced NF-κB induction.

To investigate whether the attenuation of low shear-induced NF-κB by SNP (1 mM) is associated with an...
increase of IkBα in the cytoplasm, immunoblotting studies were performed. Quantitative analysis carried out on the immunoblots by densitometric scanning showed that low shear alone reduced IkBα levels in HAEC by 31.6% (P = 0.0008). In contrast, the presence of SNP (1 mM) elevated the cytoplasmic levels of IkBα in the low shear-induced cells by 27% (P < 0.0001).

To determine whether NO donors can also inhibit the low shear-induced transactivation of kB-dependent genes, HAEC transiently transfected with a VCAM-1 promoter/luciferase reporter construct containing the two functional NF-kB binding sites were incubated with the NO donors, SNP (1 mM) or GSNO (300 μM), and subjected to 6 h of low shear stress. As shown in Fig. 6, low shear induced a 0.6-fold increase in luciferase activity (normalized for total cell protein) in HAEC. The addition of the NO donors significantly inhibited the low shear-induced NF-kB-dependent VCAM-1 promoter expression. The inhibition of VCAM-1 promoter activity in the low shear stress-treated cells in the presence of SNP and GSNO was 66% (P < 0.0001) and 23.1% (P < 0.01), respectively.

Overexpression of eNOS in HAEC inhibits low shear-induced activation of NF-kB. Because it is possible that exogenous NO donors may interact with unrelated cellular components and produce an indirect inhibitory effect on NF-kB activity, we sought supporting evidence that the observed inhibitory activity was due to NO. Thus we examined the effect of increasing endogenous levels of NO by overexpressing full-length eNOS in HAEC by NO donors. HAEC were transfected with a VCAM-1 promoter/luciferase reporter construct containing the two functional NF-kB binding sites were incubated with the NO donors, SNP (1 mM) or GSNO (300 μM), and subjected to 6 h of low shear stress. As shown in Fig. 6, low shear induced a 0.6-fold increase in luciferase activity (normalized for total cell protein) in HAEC. The addition of the NO donors significantly inhibited the low shear-induced NF-kB-dependent VCAM-1 promoter expression. The inhibition of VCAM-1 promoter activity in the low shear stress-treated cells in the presence of SNP and GSNO was 66% (P < 0.0001) and 23.1% (P < 0.01), respectively.

Low shear stress downregulates NO production in HAEC. The above observations suggested that a reduction in NO release by HAEC undergoing low shear stress for 6 h might be one mechanism responsible for some of the changes observed in the cellular levels of NF-kB and IkBα. Studies were undertaken to measure NO release in HAEC exposed to shear stress. Because high shear stress is known to be the potent inducer of NO release in HAEC, high shear stress was used as a positive control. The NO production was significantly reduced in low shear-exposed HAEC, especially during the earlier time point (measured at 5 min; P < 0.01) compared with high shear stress. Although it did not reach a statistical significance, cells exposed to 6 h of low shear stress showed an ~40% decrease in NO release compared with cells subjected to high shear stress of 16 dynes/cm². However, as shown in Fig. 8, compared with no-shear controls, low and high shear stress significantly increased NO release (P < 0.001). Cells incubated with nitro-L-arginine methyl ester (L-NAME; 2 mM) prevented production of NO induced by both low and high shear stress, indicating the specificity of the assay. These experiments were conducted using the cone and plate model (4) because it uses a smaller volume of culture medium and permits the accurate measurement of NO as accumulating concentrations of nitrite.
DISCUSSION

In this study, we have demonstrated that in HAECs, low-shear flow-induced activation of NF-κB, VCAM-1 mRNA expression, and endothelial-monocyte adhesion can be inhibited by IkBα overexpression. We also provide evidence for the involvement of intracellular NO levels in the regulation of low shear-induced IkBα degradation and subsequent activation of NF-κB. Our data suggest that the relative inhibition of NO production during the early phase of low shear stress expo-
sure may be partly responsible for the induced activation of NF-κB.

Most studies exploring the mechanism of NF-κB activation in endothelial cells have focused on the cytokines TNF-α and interleukin-1β (IL-1β) as the stimulating agents (8, 11). However, the mechanisms responsible for NF-κB activation in response to shear stress have not been extensively investigated and may differ from those induced by cytokines. Bhullar et al. (3) demonstrated that a high shear of 12 dynes/cm² induced activation of IkBα kinases and the transient degradation of IkBα. The degradation of IkBα could be detected as early as 10 min, and the degradation was complete within 30 min after the onset of high shear stress. However, the IkBα levels reappeared again within 60 min and thereafter were restored to control levels. This sequence of degradation and resynthesis of IkBα correlated with the transient NF-κB activation in high shear-exposed cells (17). In contrast to high shear stress, in which there was a rapid rebounce of IkBα expression, in the present study in which a shear of 2 dynes/cm² was employed, we observed a prolonged (6 h) absence of cytoplasmic IkBα. This most likely was responsible for the persistent NF-κB activation observed in the HAEC. The transient overexpression of IkBα, which blocked the 6 h low shear-induced NF-κB activity, as well as the κB-dependent VCAM-1 expression and endothelial-monocyte adhesion, confirmed the role of IkBα in the regulation of NF-κB activation.

We investigated the possible role of NO to identify the mediators responsible for prolonged absence of IkBα in low shear stress-exposed HAEC on the basis of the following published reports providing supportive evidence. Inhibition of endogenous NO production has been shown to increase NF-κB DNA binding activity in vitro conditions (20) and promote endothelium-leukocyte interactions, probably through the enhanced expression of NF-κB-dependent adhesion molecules in vivo (2, 7). NO donors have been shown to efficiently block TNF-α-induced adhesion molecule expression and enhanced monocyte adhesion in human umbilical and saphenous vein EC (14, 20, 23, 24). Additionally, Spieker et al. (24) have reported that NO inhibits cytokine-induced NF-κB activation, as well as VCAM-1 expression, by increasing cytoplasmic and nuclear levels of IkBα.

In the present study, we have demonstrated the inhibition of low shear flow-induced NF-κB activation by incubating HAEC with exogenous NO donors and by transiently overexpressing eNOS. Incubation with exogenous NO donors, GSNO (300 μM), and SNP (1 mM) strongly inhibited the low shear-induced degradation of IkBα and the concomitant increase in VCAM-1 promoter expression and NF-κB DNA binding activity. Assays performed using the WST-1 reagent confirmed that this was not the result of NO donor-induced cell death (data not shown). However, it should be noted that differences were observed in the potency of these donors in reducing VCAM-1 promoter expression. This may have been due to differences in their stability. Compared with SNP, GSNO is shortlived and, therefore, may be less efficient in inhibiting NF-κB DNA binding activity. Also, the different levels of inhibition caused by different NO donors, as demonstrated in Fig. 6, may suggest the possibility that they may also inhibit endogenous promoter expression with varying efficiency.

Ranjan et al. and Malek et al. (22, 16) and results presented in this study (Fig. 7, D and E) independently demonstrated the downregulation of eNOS protein expression in low shear flow. However, Ranjan et al. (22) observed induction of eNOS mRNA expression in high arterial levels of shear stress (25 dynes/cm²) and low shear stress-exposed cells (4 dynes/cm²) compared with static culture conditions. Results from Ziegler et al. (29) implied that regulation of eNOS expression by mechanical factors occurs by both transcriptional and posttranscriptional mechanisms that still need to be determined. It would be interesting to speculate, on the basis of this evidence, as well as our findings, that low shear stress may initiate degradation of the expressed eNOS protein or attenuate phosphorylation of eNOS.

The requirement for phosphorylation at serine 1179 by phosphatidylinositol 3-kinases (9) and PKA (4) for eNOS activity has recently been reported. The disconnect in the levels of eNOS expression and NO release in HAEC maintained under no-shear controls (shown in Fig. 7, D and E, and Fig. 8) may be due to the lack of phosphorylation of eNOS, which is only initiated at the onset of shear stress. Therefore, under static culture conditions, even though HAEC expresses elevated levels of eNOS compared with low shear exposed HAEC, the NO release is significantly lower due to the absence of shear stress induction. In our attempts to measure NO, a ~40% decrease was observed after 6 h of low shear stress compared with high shear stress, and these results correlate well with the eNOS expression levels that are significantly reduced in low shear stress compared with high shear stress. Because NO can bind superoxide anion with extremely high affinity (12), the bioavailability of NO in low shear regions will be far less than high shear regions under the assumption that both low and high shear stress generate equivalent levels of superoxide anions and other reactive oxygen species. Together, one mechanism by which NO stabilizes IkBα and inhibits NF-κB activation may be through quenching superoxide anion, thereby reducing its dismutation product, hydrogen peroxide, which is known to activate NF-κB in endothelial cells. Nevertheless, whether there is a differential generation of superoxide and other oxygen free radicals occur in low vs. high shear-exposed HAEC remains to be determined. In addition, NO may directly affect upstream protein kinases and phosphatases that regulate IkBα phosphorylation.

In summary, the prolonged absence of IkBα, which in turn may be regulated by changes in NO levels, is responsible for the persistent activation of NF-κB DNA binding activity observed in HAEC exposed to 6 h of low shear stress. Regulating the levels of IkBα may be one of the mechanisms by means of which NO exhibits its antiatherogenic properties.
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