Nitric Oxide Regulates Shear Stress–Induced Early Growth Response-1 Expression via the Extracellular Signal–Regulated Kinase Pathway in Endothelial Cells


Abstract—Endothelial cells (ECs) subjected to shear stress constantly release nitric oxide (NO). The effect of NO on shear stress–induced endothelial responses was examined. ECs subjected to shear stress induced a transient and shear force–dependent increase in early growth response-1 (Egr-1) mRNA levels. Treatment of ECs with an NO donor, $S$-nitroso-$N$-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN-1), inhibited this shear stress–induced Egr-1 expression. Conversely, an NO synthase inhibitor to ECs, $N$-$G$-monomethyl-$L$-arginine, augmented this Egr-1 expression. NO modulation of Egr-1 expression was demonstrated by functional analysis of Egr-1 promoter activity using a chimera containing the Egr-1 promoter region (~698 bp) and reporter gene luciferase. In contrast to the enhanced promoter activity after $N$-$G$-monomethyl-$L$-arginine treatment, shear stress–induced Egr-1 promoter activity was attenuated after ECs were treated with an NO donor. ECs cotransfected with a dominant negative mutant of Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of extracellular signal–regulated kinase (ERK)–2 (mERK) inhibited shear stress–induced Egr-1 promoter activity. NO modulation of the signaling pathway was shown by its inhibitory effect on shear stress–induced ERK1/ERK2 phosphorylation and activity. This inhibitory effect was further substantiated by the inhibition of NO on both the shear stress–induced transcriptional activity of Elk-1 (an ERK substrate) and the promoter activity of a reporter construct containing serum response element. NO-treated ECs resulted in a reduction of binding of nuclear proteins to the Egr-1 binding sequences in the platelet-derived growth factor-A promoter region. These results indicate that shear stress–induced Egr-1 expression is modulated by NO via the ERK signaling pathway in ECs. Our findings support the importance of NO as a negative regulator in endothelial responses to hemodynamic forces. (Circ Res. 1999;85:238-246.)

Key Words: Egr-1 ■ endothelial cell ■ nitric oxide ■ shear stress ■ signaling pathway

Vascular endothelial cells (ECs) are constantly exposed to hemodynamic forces, including shear stress imposed by blood flow. Shear stress exerts significant influences on ECs, many of which reflect alterations in gene expression.1,2 A number of pathophysiologically relevant genes, such as the platelet-derived growth factor (PDGF)-B chain,3,4 intercellular adhesion molecule-1 (ICAM-1),5-8 and monocyte chemotactic protein-1 (MCP-1),9,10 are activated by shear stress. The induction of genes in ECs exposed to shear stress is believed to involve the activation of a variety of transcription factors, including nuclear factor-$\kappa$B (NF-$\kappa$B) and activator protein-1 (AP-1).4,10,11 Khachigian et al12 recently demonstrated that early growth response-1 (Egr-1), an immediate-early gene, is activated by shear stress in ECs. This shear-activated Egr-1 acts as a transcription factor that interacts with the overlapping binding elements for Egr-1 and Sp1 in the promoter region of PDGF-A and subsequently induces its gene expression. Similar consensus binding sites for Egr-1 and Sp-1 are also present in the promoter of various genes such as PDGF-B, transforming growth factor-$\beta$, and tissue factor, the expression of which is also affected by shear stress and cytokines.13-15 Thus, Egr-1 induction has been indicated as a common theme in vascular injury.13

Despite intensive studies on the effects of fluid shear stress on ECs, the detailed mechanisms that transmit the mechanical stimuli to intracellular signaling still remain largely unclear. Various signals, including calcium mobilization,16 inositol triphosphate,17,18 G protein,19,20 and cyclic GMP (cGMP),20 have been shown to be activated by shear stress. Recent studies have demonstrated that signaling-pathway extracellular signal–regulated kinase (ERK) is activated by shear force.2,21 Li et al,22 however, indicated that shear stress primarily activates the c-Jun N-terminal kinase (JNK) pathway. The detailed mechanisms particularly regarding the
synergism and cross talk among different signaling pathways thus remain undefined. Our recent studies demonstrated that reactive oxygen species (ROS) are induced by shear stress or cyclic strain and consequently act as second messengers to stimulate the expression of various genes, including MCP-1, c-fos, and ICAM-1.8,23–25 This hemodynamic force–induced gene expression is inhibited after ECs are pretreated with an antioxidant.8,23–25

Nitric oxide (NO), a relaxing factor derived from endothelium via the activation of endothelial NO synthase (eNOS), plays a protective role during atherogenesis.26–30 Shear stress to ECs increases eNOS mRNA levels and NO production.19,28,31,32 This released NO modulates various gene expressions, including MCP-1,29 vascular cell adhesion molecule-1 (VCAM-1),33–36 and ICAM-136 in cells exposed to various stimuli. Frangos and Baö37 further demonstrated that NO regulates shear stress–induced PDGF-A and MCP-1 gene expressions in ECs. However, the detailed mechanism by which NO modulates endothelial responses to chemical or mechanical stimuli remains unclear. NO may exert its effect by modulating the intracellular redox status via suppressing ROS levels28 or by triggering redox-sensitive mechanisms36 in ECs. NO may modulate the VCAM-1 expression via the elevation of cGMP.37 A recent study35 demonstrated that cytokine-induced VCAM-1 expression was mediated by NO via the inhibition of NF-κB activation. The ERK signaling pathway has been suggested to be involved in the inhibitory effect on smooth muscle cell proliferation by NO.38 NO may inhibit the Ras/Raf/ERK pathway through the activation of cGMP-dependent protein kinase.39 For the induction of Egr-1 in ECs by shear stress, the signaling pathway involving ERK has been demonstrated.40 Moreover, NO inhibits Egr-1 expression in cytokine-treated macrophages.41 Because NO plays a role by inhibiting key events that promote atherogenesis, and Egr-1 induction is involved in the vascular injury, we postulate that shear stress–induced transient expression of Egr-1 is mediated by NO via the inhibition of the ERK signaling pathway. The present study clearly indicates that NO down-regulates shear stress–induced Egr-1 expression via the inhibition of the ERK pathway in ECs. This inhibition consequently results in a reduction of binding of nuclear Egr-1 proteins to the corresponding binding sequences in the promoter region of PDGF-A. Our findings thus support the notion that NO serves as a negative regulator in endothelial responses to hemodynamic forces.

Materials and Methods

Materials

The Egr-1 cDNA probe was a gift from Dr Y.J. Shyy (University of California at San Diego). The catalytically inactive mutant of ERK2 (mERK) was a gift from Dr R.J. Davis, University of Massachusetts Medical School (Worcester, MA), RasN17, RasL61, and Raf310 were previously described.42,43 The Elk-1 trans-reporting system and serum scavenging cDNA plasmids were obtained from Stratagene (catalog numbers 219005 and 219079, respectively). S-Nitroso-N-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and Nε-monomethyl-L-arginine (L-NMMA) were purchased from Calbiochem. All other chemicals, of reagent grade, were obtained from Sigma.

EC Culture

ECs were isolated from human umbilical cords as previously described.44 ECs were grown in Petri dishes for 3 days and then seeded onto glass slides (75×38 mm, Corning) to reach confluence. The culture medium was then exchanged with medium that was identical except that it contained only 2% FBS, and the cells were further incubated 24 hours before the experiment.

Flow Apparatus

The slide with cultured ECs was mounted in a parallel-plate flow chamber, which has been characterized and described in detail elsewhere.45 The chamber was connected to a perfusion loop system, kept in a constant-temperature–controlled enclosure, and maintained at pH 7.4 by continuous gassing with a mixture of 5% CO2 in air. The flow channel width (w) was 1 cm, and the channel height (h) was 0.025 cm. The Reynolds number, defined by the average inlet velocity and the channel height, was 30. The fluid shear stress generated on the ECs by flow was calculated as 20 dyne/cm2, using the formula τ = μQ/wh2, where τ is the shear stress, μ is the dynamic viscosity of the perfusate, and Q is the flow rate. In some experiments, ECs were pretreated with SNAP (100 μmol/L) or SIN-1 (100 μmol/L) for 30 minutes or L-NMMA (250 μmol/L) for 1 hour. These ECs were then subjected to shear flow in the presence of the same reagent. The static control cells were incubated and changed to new culture medium while the experimental cells were placed under flow conditions.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from ECs by the guanidine isothiocyanate/phenoxychloroform method as previously described.46 The RNA (10 μg/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nyt-ran, Schleicher & Schuell, Inc) by a vacuum blotting system (VacuGene XL, Pharmacia). After hybridization with the 32P-labeled cDNA probes, the membrane was washed with 1 SSC containing 1% SDS at room temperature for 30 minutes and then exposed to x-ray film at ~70°C. Autoradiographic results were analyzed by using a densitometer (Computing Densitometer 300S, Molecular Dynamics).

Reporter Gene Construct, DNA Plasmids, Transfection, and Luciferase Assay

The Egr-1 promoter construct (Egr-1-Luc) contains 698 bp of Egr-1 5′-flanking DNA linked to the firefly luciferase reporter gene of plasmid pGL2 (Promega, Inc). This fragment of the Egr-1 promoter contains multiple SREs.46 The Elk-1 trans-reporting system contains plasmids GAL4/ELK1-(307–428) and GAL4-Luc. GAL4/ELK1-(307–428) encodes the fusion protein of the GAL4 DNA binding domain fused to the activation domain of Elk-1. GAL4-Luc is a chimeric construct consisting of 5 copies of the GAL4 binding sequence and the luciferase reporter. The SRE cis-reporting system contains plasmid pSRE-Luc, which consists of 5 repeats of the SRE. DNA plasmids were transfected into bovine aortic ECs (BAECs) at their 60% confluence level by using the lipofectamine method (GIBCO-BRL). The pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency. After transfection, cells were incubated with DMEM (GIBCO) containing 10% FBS overnight and then seeded onto slides. The medium of the cultured BAECs was exchanged with medium that was identical except that it contained only 0.5% FBS, and the cells were further incubated overnight before being subjected to shear flow treatment. Luciferase activity was measured by using the Biotec assay system (Promega). β-Galactosidase activity was assayed by adding the substrate o-nitrophenyl-β-d-galactopyranoside to 20 μL of cell lysate and incubating at 37°C before recording at 420 nm.

Assay of ERK Activity and Phosphorylation

ERK activity was assayed according to the method previously described.22 Briefly, ECs were lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease
inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). Cells were disrupted by repeated aspiration through a 21-gauge needle. The same amount of protein from each sample was incubated with anti–ERK1/ERK2 antibody (Santa Cruz Biotechnology) for 2 hours at 4°C with gentle shaking. The immune complex was then incubated with protein A/G agarose for 1 hour. This agarose-bound immune complex was then incubated with kinase reaction buffer containing myelin basic protein (MBP). The kinase reaction was carried out for 20 minutes at 30°C in buffer containing 0.3 mg/mL MBP, 50 μM ATP, and 1 μCi [γ-32P]ATP. The reaction was stopped by adding an equal volume of sample buffer containing SDS and boiling for 3 minutes. The samples were electrophoresed on a 15% polyacrylamide gel. After drying, the gel was exposed to x-ray film. For detection of ERK phosphorylation in sheared ECs, the cell lysates were collected and boiled. Total cell lysates (100 μg of protein) were separated by SDS-PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with anti–active ERK1/ERK2 antibody (Promega Inc). Immunodetection was performed by using the Western-Light chemiluminescent detection system (Tropix, Inc).

Electrophoretic Mobility Shift Assay (EMSA)

To prepare nuclear protein extracts, ECs were washed with cold PBS and then immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (containing, in mmol/L, KCl 10, EDTA 0.1, DTT 1, and PMSF 1) for 15 minutes. The cells were lysed by adding 10% NP-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing, in mmol/L, HEPES 20, EDTA 1, DTT 1, and PMSF 1, and 0.4 mol/L NaCl), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at −70°C until used. Double-stranded oligonucleotides (30 bp) containing the Egr-1 binding site in the proximal region of the PDGF-A promoter were prepared as described.12–14 The oligonucleotides were end labeled with [γ-32P]ATP. Extracted nuclear proteins (10 μg) were incubated with 0.1 ng 32P-labeled DNA for 15 minutes at room temperature in 25 μL binding buffer containing 1 μg poly(dI-dC). In the antibody supershift assay, anti–Egr-1 antibody (1 μg, Santa Cruz Biotechnology) was incubated with the mixture for 10 minutes at room temperature followed by the addition of the labeled probe. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. Gels were dried and imaged by autoradiography.

Statistical Analysis

Results are expressed as mean±SEM. Significance was determined by using the Student t test, and the level of statistical significance was defined as P<0.05.

Results

Shear Stress Induces a Transient and Force-Dependent Increase in Egr-1 mRNA Levels in ECs

To examine the effects of shear stress on Egr-1 gene expression in ECs, cultured ECs were exposed to shear stress of 20 dyne/cm² for 0.5, 1, 2, 3, and 6 hours, and their Egr-1 mRNA expression was determined by Northern blot analysis. As shown in Figure 1A, ECs subjected to 30 minutes of shear flow significantly increased their Egr-1 mRNA levels as compared with the Egr-1 mRNA levels in static controls. The Egr-1 mRNA levels in ECs remained at an elevated level for 1 hour and then returned to the basal level 3 hours after the onset of shear stress. The shear stress–induced Egr-1 expression was shear force dependent (Figure 1B). There was no apparent increase of Egr-1 mRNA levels in ECs exposed to shear stress of 3 dyne/cm² for 30 minutes. However, ECs exposed to shear stress of 10 dyne/cm² for 30 minutes significantly increased their Egr-1 mRNA expression. As shear stress increased to 40 or 100 dyne/cm², the Egr-1 levels were further augmented. These results suggest that shear stress–induced Egr-1 gene expression in ECs is time and shear force dependent.

NO Modulates Shear Stress–Induced Egr-1 Gene Expression in ECs

Shear stress to ECs increases the production and release of NO.19,28 To explore whether NO modulates the induction of Egr-1 gene in sheared ECs, ECs were preincubated with an NO donor, ie, SNAP or SIN-1, for 30 minutes before being subjected to shear flow in the presence of the NO donor. As shown in Figure 2A, SNAP treatment of ECs at a concentration of 100 or 400 μmol/L significantly suppressed shear stress–induced Egr-1 mRNA expression. Similarly, treatment of ECs with another NO donor, SIN-1 (100 μmol/L), also significantly attenuated shear stress–induced Egr-1 expression. In separate experiments, ECs were pretreated with L-NMMA, an NO synthase (NOS) inhibitor, for 1 hour and then subjected to flow in the presence of the agent. In contrast to the inhibitory effect by the NO donor, L-NMMA treatment of ECs at a concentration of 250, 500, or 1000 μmol/L significantly augmented shear stress–induced Egr-1 mRNA levels (Figure 2B). L-NMMA treatment had no effect on Egr-1 mRNA expression in static control cells (data not shown). These results suggest that shear stress–induced Egr-1 expression is modulated by NO. Thus, shear stress–induced NO may serve as a negative regulator for shear stress–induced Egr-1 expression.

To further determine whether the modulation of NO in shear stress–induced Egr-1 expression is a transcriptional event, an Egr-1 promoter construct containing the Egr-1 promoter region (−698 bp) and the reporter gene luciferase were transiently transfected into ECs. As shown in Figure 3,
ECs exposed to 6 hours of flow significantly increased Egr-1 promoter activity by ≈2.4-fold compared with static cells. The addition of both SNAP and SIN-1 to ECs completely abolished this increased Egr-1 promoter activity. Conversely, treatment of ECs with L-NMMA enhanced this promoter activity. Pretreatment of ECs with KT5823, a cGMP-dependent protein kinase inhibitor, did not interfere with the inhibitory effect of NO on shear stress–induced Egr-1 promoter activity. This finding indicates that the effect of NO on Egr-1 induction by shear stress is not mediated via the cGMP-dependent protein kinase pathway. ECs treated with PMA (100 μg/L), as a positive control, greatly increased their Egr-1 promoter activity. These results together suggest that NO modulation of Egr-1 induction by shear stress involves transcriptional regulation.

**Shear Stress–Induced Egr-1 Gene Expression Is Mediated via the Ras/Raf/ERK Pathway**

Egr-1 induction by shear stress reportedly involves activation of the ERK signaling pathway. To further confirm the involvement of this ERK pathway, we used the dominant negative mutants of Ras (RasN17) and Raf-1 (Raf301) and a catalytically inactive mutant of ERK2 (mERK), all of which are associated with the Ras/Raf/ERK pathway, to examine the effect of these mutants on the induction of Egr-1 by shear stress. As shown in Figure 4, ECs that were cotransfected with the empty vector control PSRα, or an expression plasmid encoding the dominant negative mutant mERK, Raf301, or RasN17 (3 μg), was cotransfected with an Egr-1 promoter construct (10 μg) into BAECs. The DNA-transfected cells were seeded onto culture slides to confluence and then either subjected to fluid shear of 20 dyne/cm² for 6 hours or kept as static controls. ECs transfected with Egr-1 promoter construct were pretreated with PD98059 (30 μmol/L) for 30 minutes before being subjected to flow in the presence of this agent (S+PD98059). ECs cotransfected with an expression plasmid encoding MEK1 (0.5 μg) or RasL61 (1 μg) were used as positive controls. Induction was measured as the luciferase activity in the experimental cells relative to those in static controls. Data are mean±SEM from 4 or 5 separate experiments. *P<0.05 vs static control ECs, #P<0.05 vs sheared untreated ECs.

Figure 2. NO modulates shear stress–induced Egr-1 gene expression in ECs. A, NO donor attenuates shear stress–induced Egr-1 mRNA levels in ECs. ECs were untreated or pretreated with SNAP or SIN-1 for 30 minutes at a concentration of 100 or 400 mmol/L and then subjected to shear stress of 20 dyne/cm² for 30 minutes in the presence of respective NO donors. B, L-NMMA augments shear stress–induced Egr-1 mRNA levels in ECs. ECs were untreated or preincubated with L-NMMA for 1 hour at a concentration of 250, 500, or 1000 mmol/L before being subjected to shear stress of 20 dyne/cm² for 30 minutes in the presence of L-NMMA. Autoradiographic results were analyzed by a densitometer. Data are presented as a percentage change to static controls in band density normalized to 18S RNA levels and are shown as mean±SEM from 3 independent experiments. *P<0.05 vs static control ECs, #P<0.05 vs shear treated ECs with empty control PSRα.

Figure 3. NO attenuating shear stress–induced Egr-1 expression is a transcriptional event. Chimera containing 698 bp of the Egr-1 promoter region and reporter gene luciferase were transfected into BAECs and then exposed to shear stress of 20 dyne/cm² for 6 hours (S). Before flow treatment, transfected ECs were pretreated with SNAP (100 μmol/L; S+SNAP) or SIN-1 (100 μmol/L; S+SIN-1) for 30 minutes or L-NMMA (250 μmol/L) (S+L-NMMA) for 1 hour. ECs were pretreated with KT5823 before being subjected to shear flow in the presence of SNAP (KT5823+S+SNAP). ECs treated with PMA (100 μg/L) for 6 hours were used as positive controls. Data are shown as fold induction relative to static control cells. Data are represented as mean±SEM from 4 or 5 separate experiments. *P<0.05 vs static control ECs, #P<0.05 vs sheared untreated ECs.

Figure 4. Shear stress–induced Egr-1 gene expression is mediated via the Ras/Raf/ERK signaling pathway. Empty vector PSRα, or an expression plasmid encoding the dominant negative mutant mERK, Raf301, or RasN17 (3 μg), was cotransfected with an Egr-1 promoter construct (10 μg) into BAECs. The DNA-transfected cells were seeded onto culture slides to confluence and then either subjected to fluid shear of 20 dyne/cm² for 6 hours or kept as static controls. ECs transfected with Egr-1 promoter construct were pretreated with PD98059 (30 μmol/L) for 30 minutes before being subjected to flow in the presence of this agent (S+PD98059). ECs cotransfected with an expression plasmid encoding MEK1 (0.5 μg) or RasL61 (1 μg) were used as positive controls. Induction was measured as the luciferase activity in the experimental cells relative to those in static controls. Data are mean±SEM from 4 or 5 separate experiments. *P<0.05 vs static control ECs, #P<0.05 vs sheared untreated ECs.

NO Regulates Shear Stress–Induced ERK Phosphorylation and Activity in ECs

Given that we showed that the ERK signaling pathway is involved in shear-induced Egr-1 expression and that NO...
rapidly induced ERK activity by 6.7-fold, as indicated by an
stress–induced ERK activity. ECs exposed to shear stress
(Figure 5B). NO modulation of the ERK signaling pathway
stress only, whereas it showed no effect in static control cells
L-NMMA treatment of ECs augmented shear stress–induced
phosphorylation of ERK1/ERK2 by 5.6-fold. ECs preincu-
Figure 5A, shear stress to ECs for 10 minutes induced rapid
pathway in ECs.
these findings imply that NO modulates shear-induced Egr-1
expression via its inhibitory effect on the ERK signaling
pathway in ECs.
modulates shear-induced Egr-1 expression, we further inves-
tigated whether NO modulates the activation of ERK in
sheared ECs. We first examined the ERK1/ERK2 phosphor-
ylation in ECs exposed to shear stress of 20 dyne/cm² in the
presence of the NO donor or NOS inhibitor. As shown in
Figure 5A, shear stress to ECs for 10 minutes induced rapid
phosphorylation of ERK1/ERK2 by 5.6-fold. ECs preincu-
bated with an NO donor, SNAP or SIN-1, significantly
attenuated this ERK activation by 55% or 67%, respectively. In separate experiments, L-NMMA treatment of ECs augmented shear stress–induced ERK phosphorylation in ECs. In separate experiments, ECs were pretreated with L-NMMA (250 μmol/L) for 1 hour and then either subjected to shear (S+L-NMMA) or kept as static controls (C-L-NMMA). ECs treated with PMA (100 μg/mL) for 10 minutes were used as positive controls. C, NO inhibits shear stress–induced ERK activity in ECs. After shearing, ERK was immunoprecipitated and a kinase activity assay was performed in the presence of MBP and [γ-32P]ATP as described in Materials and Methods. The phosphorylated MBP was as indicated. D, L-NMMA treatment enhances shear stress–induced ERK activity in ECs. Data are representative of duplicate experiments with similar results.

Figure 5. NO regulates shear stress–induced ERK phosphoryla-
tion and activity in ECs. A, NO attenuates shear stress–induced
ERK phosphorylation in ECs. After application of shear stress, ECs were lysed, and the phosphorylation of ERK was deter-
mimed by using Western blot analysis as described in Materials and Methods. Antibody to the active form of ERK1/ERK2 was used. ECs were subjected to shear stress of 20 dyne/cm² for 10 minutes (S). Before shear treatment, ECs were preincubated with SNAP (100 μmol/L; S+SNAP) or SIN-1 (100 μmol/L; S+SIN-1) for 30 minutes. B, L-NMMA treatment augments shear stress–induced ERK phosphorylation in ECs. In separate experiments, ECs were pretreated with L-NMMA (250 μmol/L) for 1 hour and then either subjected to shear (S+L-NMMA) or kept as static controls (C-L-NMMA). ECs treated with PMA (100 μg/mL) for 10 minutes were used as positive controls. C, NO inhibits shear stress–induced ERK activity in ECs. After shearing, ERK was immunoprecipitated and a kinase activity assay was performed in the presence of MBP and [γ-32P]ATP as described in Materials and Methods. The phosphorylated MBP was as indicated. D, L-NMMA treatment enhances shear stress–induced ERK activity in ECs. Data are representative of duplicate experiments with similar results.

NO Regulates Shear Stress–Induced Transcriptional Activity of Elk-1 and the Promoter Activity of a Reporter Gene Construct Containing SRE

NO modulation of shear stress–induced ERK phosphoryla-
tion and activity in ECs led us to speculate that NO may
regulate the transcriptional activity of the ternary complex
factor proteins such as Elk-1, an ERK1/ERK2 substrate. To
test this hypothesis, the plasmid GAL4/ELK1-(307–428),
which encodes the GAL4 DNA binding domain fused to the
C-terminal activation domain of Elk-1, was cotransfected with
GAL4-Luc into ECs to test their shear inducibility. As shown in Figure 6A, shear stress caused a 5.4-fold increase in
luciferase activity. This induction, however, was significantly
inhibited after ECs were treated with an NO donor (SNAP or
SIN-1). Conversely, L-NMMA treatment augmented this
shear stress–induced transcriptional activity of Elk-1. As a
positive control, ECs cotransfected with a plasmid pFC-MEK1, an Elk-1 upstream kinase MEK1 expression vector,
dramatically increased the transcriptional activity of Elk-1.
The activated Elk-1 has been recognized as a transcriptional
factor that cooperatively interacts with the serum response
factor and binds to the SRE in the promoter region of various
genes such as c-fos and Egr-1 to induce their expression.47
In the present study, a reporter gene construct, pSRE-Luc, containing 5 repeats of SRE was transfected into cells to test
the shear inducibility of the construct. As shown in Figure
6B, ECs subjected to 3 hours of flow induced a 2.7-fold
increase in promoter activity. Not surprisingly, treatment of
cells with an NO donor (SNAP-1 or SIN-1) or NOS inhibitor
(L-NMMA) down-regulated or up-regulated this shear stress–
duced promoter activity, respectively. These results indi-
cate that NO inhibits shear stress–induced ERK activity
followed by a decrease of transcriptional activity of Elk-1 and
SRE binding in the promoter region of Egr-1.

NO Regulates Shear-Induced Egr-1 Binding to the Promoter Region of PDGF-A

Activation of Egr-1 by shear stress contributes to an induction
of PDGF-A in sheared ECs.12 The promoter region
of PDGF-A consists of multiple Egr-1 binding sites, and the
increase of this binding enhances PDGF-A gene expres-
sion.12–14 To elucidate the functional role of NO in modulat-
ing shear-induced Egr-1 expression, we further investigated
whether this NO effect results in an inhibition of binding of
extracted nuclear proteins to the proximal region of PDGF-A
promoter. We used the Egr-1 binding sequences in the
PDGF-A promoter region for an EMSA. As shown in Figure
7, when nuclear proteins extracted from sheared ECs were
incubated with oligonucleotides corresponding to the Egr-1
binding sequences, increased binding activity occurred. SNAP
or SIN-1 treatment of ECs caused an attenuation of
this binding activity. Conversely, L-NMMA treatment of
the ECs augmented this binding activity. This binding was
obviously specific to Egr-1, because it was abolished by coinubcation of nuclear proteins with 20-fold unlabeled
oligonucleotide. This specificity was further substantiated by
the supershifting in gel mobility of the Egr-1-oligonucleotide
complex after preincubation of nuclear proteins with Egr-1.
antibody. These results indicate that NO inhibits the Egr-1 protein induced by shear stress that subsequently results in a decrease of the expression of later genes, including PDGF-A, which requires Egr-1 binding for its induction.

Discussion

NO, in addition to being a potent vasodilator, inhibits the expression of a number of pathophysiologically relevant genes, such as MCP-1,29,37 ICAM-1,36 VCAM-1,33–36 and PDGF-A,37 induced by chemical or mechanical stimulation. During atherogenesis, the loss of endothelium-derived NO and the accompanying expression of atherogenesis-related genes are early events.26,27 NO also acts as a negative regulator of vascular smooth muscle proliferation in response to a remodeling stimulus.30 Thus, a primary defect in NO production can promote abnormal remodeling and facilitate pathological changes in vessel walls.

Shear stress increases the production of NO by increasing eNOS expression in ECs.19,28,31,32 One of our recent studies demonstrated that shear stress to ECs for 15 minutes rapidly increases NO release as detected by electron paramagnetic resonance (L.W.L., unpublished observation, 1999). NO release from sheared ECs decreases monocyte adhesion to
ECs. It is conceivable that endogenous NO may exert its effect on signal transduction followed by transcriptional events that ultimately alter gene expression. Among the effects of NO on cells, the elevation of cGMP, change of redox status, and inhibition of NF-κB activation are involved.29,33,35,36 NO inhibits the induction of ERK by growth factors and subsequently suppresses smooth muscle cell proliferation.38 NO may exert its effects through the activation of cGMP-dependent protein kinase.39 The ERK signaling pathway is also involved in shear stress–induced Egr-1 expression.40 Our present study clearly indicates that shear stress–induced Egr-1 expression is mediated through the ERK pathway and the EC-derived NO inhibits the ERK pathway in shear-treated ECs. Several lines of evidence support this notion. First, NO donors attenuated shear stress–induced Egr-1 mRNA levels in ECs. Second, ECs treated with a NOs inhibitor conversely enhanced shear stress–induced Egr-1 gene expression. This result indicates that endogenous NO plays a role in modulating shear stress–induced Egr-1 expression. Third, addition of an NO donor inhibited and an NOs inhibitor to ECs augmented shear stress–induced Egr-1 promoter activity. Fourth, when ECs were cotransfected with a dominant negative mutant of ERK, Raf, or Ras, shear stress–induced Egr-1 promoter activity was abolished. This confirms the involvement of the Ras/Raf/ERK pathway in shear-induced Egr-1 expression. However, this shear-induced ERK phosphorylation and activity were inhibited after ECs were treated with an NO donor. Fifth, the inhibitory effect of NO was further substantiated by the suppression of NO on shear-induced transcriptional activity of Elk-1, a downstream substrate of ERK. Sixth, NO attenuated shear stress–induced promoter activity of a reporter construct containing SRE, which has been shown to be responsible for the Egr-1 induction by shear stress.40 Finally, the inhibitory effect of NO on Egr-1 expression was confirmed by a reduction of binding of nuclear Egr-1 proteins to the corresponding binding sequences in the promoter of PDGF-A.

In addition to the ERK pathway, shear stress to ECs also induces JNK, another mitogen-activated protein kinase.2,22,48 The JNK pathway is involved in the induction of various endothelial genes, including MCP-1.22 Whether ERK and JNK signaling pathways act synergistically or have cross talk in response to mechanical forces that lead to gene induction remains to be clarified. Shear stress exerts differential effects on ERK and JNK in ECs.2,48 Shear stress activates ERK in a time- and force-dependent manner. In contrast, JNK activity is induced by a low shear force (0.5 dyne/cm²) but with a delayed and prolonged response extending from 30 minutes to 4 hours after flow application.48 Consistent with ERK activation, present data indicate that shear stress rapidly induces Egr-1 gene expression in a transient manner, in which the induction peaks at 30 minutes and decreases afterward. These data are consistent with that transient expression reported by Khachigian et al.12 although BAECs rather than human umbilical vein ECs were used in their study. Moreover, ECs exposed to shear stress at ≤10 dyne/cm² did not significantly induce their Egr-1 expression. These observations imply that the activation of ERK, rather than JNK, is critical for shear stress–induced Egr-1 expression. This is in agreement with the observation by Schwachtgen et al.40 who showed that the activation of Egr-1 by shear stress involves Elk-1 but not c-jun activity. We recently demonstrated that Egr-1 induced by cyclic strain is mediated primarily via the ERK pathway.49 The present study further supports the notion that the ERK pathway plays a key role in shear stress–induced Egr-1 expression in ECs.

The present study implies that NO regulates shear stress–induced Egr-1 expression in ECs via the inhibition of the ERK signaling pathway. However, the exact target molecule(s) of this NO modulation has not been defined. NO activates guanylate cyclase to produce cGMP, which affects various targets, including cGMP-dependent protein kinase.50 The Ras/Raf/ERK signaling pathway is inhibited by the cGMP-dependent protein kinase via the phosphorylation of c-Raf kinase on Ser43.59 However, NO inhibits the activation of NF-κB via non–cGMP-dependent mechanisms.34,35 In the present study, ECs treated with KT8253, a cGMP-dependent protein kinase inhibitor, did not attenuate the inhibitory effect of NO on shear-induced Egr-1 promoter activity. This implies that the inhibitory effect of NO on Egr-1 induction in sheared ECs is not mediated via the cGMP-dependent protein kinase.

The protection effect of NO has been suggested to be caused by the attenuation of intracellular oxidative stress in cells exposed to various stimuli, including reperfusion.51,52 Evidence suggests that NO may reduce intracellular ROS, including superoxide anion, via direct action on the NADPH oxidase.53 Khan et al.36 suggested that such an inhibitory effect is responsible for the NO modulation of cytokine-induced VCAM-1 expression in ECs. The inhibition of NO on MCP-1 induction by cytokines or oxidized lipoproteins is mediated via the suppression of the superoxide levels and NF-κB activity.29 Egr-1 is activated by ROS.54,55 Furthermore, ROS modulate various signaling pathways, including ERK.56 We previously demonstrated that shear stress to ECs increases intracellular ROS levels, and this increased ROS is involved in shear stress–induced ICAM-1 and c-fos expression.8,25 Whether shear induces endothelial Egr-1 expression as a result of increased ROS levels remains to be determined. However, our previous study demonstrated that the Egr-1 promoter region contains a common SRE that is shared by cyclic strain as well as H₂O₂ stimulation.49 Sheared ECs pretreated with NO, however, resulted in a decrease of superoxide levels (H.J.H., unpublished observation, 1999). Thus, the intracellular levels of NO and ROS and the consequence of their interplay may affect signaling pathways and then determine the endothelial responses under hemodynamic conditions. The precise molecular mechanism(s) by which NO modulates endothelial responses to mechanical forces remains a complicated issue that warrants further investigation.

Initial studies by Resnick et al.3 defined a shear stress–responsive element in the PDGF-B promoter region that was required for its induction by shear stress. Later studies4 indicated that NF-κB is the responsible transcriptional factor binding to the shear stress–responsive element. Shy et al.10 identified another shear-responsive element in MCP-1 gene that corresponds to the activator protein-1 binding site.
Recent studies have suggested that the overlapping consensus binding elements for Sp1 and Egr-1 in the promoter of various genes may be crucial for shear inducibility.\textsuperscript{12,15} For the Egr-1 induction in sheared ECs, the SRE is the responsible element for Egr-1 expression.\textsuperscript{40} The transcriptional factors, including Elk-1, cooperatively interact with the serum response factor and bind to the SRE in the promoter region of various genes, including c-fos and Egr-1, and trigger their gene expression.\textsuperscript{47} The present study demonstrated that NO regulates Egr-1 expression by inhibiting Elk-1 transcriptional activity, which results in a decrease of SRE activity as demonstrated by the promoter activity of a reporter construct containing 5 repeats of SRE. Taken together, our data support the notion that the decrease of SRE activation by NO contributes to the inhibition of Egr-1 expression in NO-treated ECs.

In summary, the present study demonstrates that shear stress–induced Egr-1 expression is inhibited by NO via the inhibition of the ERK signaling pathway in ECs. This Egr-1 inhibition consequently leads to a decrease of expression of later genes, including PDGF-A. Because ECs under shear stress constantly produce greater NO levels, this released NO later genes, including PDGF-A. Because ECs under shear stress constantly produce greater NO levels, this released NO

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Nitric Oxide Regulates Shear Stress–Induced Early Growth Response-1: Expression via the Extracellular Signal–Regulated Kinase Pathway in Endothelial Cells


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