Shear Stress Regulates Endothelial Nitric-oxide Synthase Promoter Activity through Nuclear Factor κB Binding

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We have previously demonstrated that shear stress increases transcription of the endothelial nitric-oxide synthase (eNOS) by a pathway involving activation of the tyrosine kinase c-Src and extracellular signal-related kinase 1/2 (ERK1/2). In the present study sought to determine the events downstream of this pathway. Shear stress activated a human eNOS promoter chloramphenicol acetyl-CoA transferase chimeric construct in a time-dependent fashion, and this could be prevented by inhibition of the c-Src and MEK1/2. Studies using electromobility shift assays, promoter deletions, and promoter mutations revealed that shear activation of the eNOS promoter was due to binding of nuclear factor κB (NF-κB) subunits p50 and p65 to a GAGACC sequence −990 to −984 base pairs upstream of the eNOS transcription start site. Shear induced nuclear translocation of p50 and p65, and activation of the eNOS promoter by shear could be prevented by co-transfection with a dominant negative IκBα. Expression of endothelial cells to shear resulted in IkκB kinase phosphorylation, and this was blocked by the MEK1/2 inhibitor PD98059 and the cSrc inhibitor PP1, suggesting these signaling molecules are upstream of NFκB activation. These experiments indicate that shear stress increases eNOS transcription by NFκB activation and p50/p65 binding to a GAGACC sequence present in the human eNOS promoter. While NFκB activation is generally viewed as a proinflammatory stimulus, the current data indicate that its transient activation by shear may increase expression of eNOS, which via production of nitric oxide could convey anti-inflammatory and anti-atherosclerotic properties.

Unidirectional laminar shear stress, the frictional force of blood over the surface of the endothelium, exerts atheroprotective effects by preventing adhesion molecule expression, reducing platelet aggregation, and inhibiting both smooth muscle cell proliferation and endothelial cell apoptosis (1). In contrast, areas of the vasculature exposed to low levels of shear stress are prone to atherosclerotic lesion formation (2, 3). At least a portion of the beneficial effects of laminar shear stress is due to modulation of nitric oxide (NO) production via two mechanisms. Immediately after the onset of shear, there is an acute activation of the endothelial NO synthase (eNOS) leading to NO- release within seconds thereafter (4). Over several hours, shear stress stimulates an increase in eNOS mRNA and protein expression (5). Recent work from our laboratory has shown that this latter effect occurs by two distinct pathways regulating eNOS transcription and mRNA stability, with transcription peaking at 1 h and returning to baseline levels shortly thereafter. Both of these pathways share a need for activation of c-Src; however, eNOS transcription, as measured by nuclear run-on analysis, involves c-Src activation of the mitogen-activated protein kinases Raf, MEK1/2,1 and extracellular-signal-related kinase 1/2 (ERK1/2) (6).

The precise nuclear events that lead to an increase in eNOS transcription in response to shear stress remain poorly defined. Endothelial cells respond to increases in shear stress by activation of a variety of signaling molecules, including c-Src, PKA, PKB, PKC, and mitogen-activated protein kinases (7, 8). ERK1/2 is phosphorylated shortly (within 5 min) following onset of shear stress and subsequently stimulates binding of a variety of transcription factors, including AP-1 and SP-1 (9). These could both potentially mediate the increase in eNOS transcription by shear stress, as potential binding sites for these are present in the eNOS promoter. Several early response genes are also induced by shear stress including c-myc, c-fos, and c-jun (10). At early time points shear stress increases transcription of several proatherogenic genes including platelet-derived growth factor B (PDGF-B) and endothelin-1 (11). Laminar shear stress activates the PDGF-B promoter through an NFκB-like element with the core sequence GAGACC, which has been characterized as a shear stress response element (SSRE) (12). NFκB is a heterodimer consisting of p50 and p65 subunits (13). Under basal conditions, the subunits are sequestered in the cytosol by binding to an inhibitory molecule IκB. Following phosphorylation of IκB, the p50 and p65 subunits are released and translocate to the nucleus where they can bind specific DNA sequences (14). Finally, shear stress has been shown to increase NFκB/DNA binding during the first hour of flow stimulation (9, 13).

While abundant data indicate that eNOS gene expression is increased by shear stress, the exact binding region in the eNOS promoter and the transcription factor(s) involved remain poorly defined. In this study, using a chimeric human eNOS promoter—

1 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-related kinase; eNOS, endothelial nitric-oxide synthase; PDGF, platelet-derived growth factor; SSRE, shear stress response element; CAT, chloramphenicol acetyltransferase; BAECs, bovine endothelial cells; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; PP1, 4-amino-5-(4-methylphenyl)-7-(4-butyryl)pyrazolo[3,4-d]pyrimidine; ANOVA, analysis of variance.

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chloramphenicol acetyltransferase (CAT) construct, we defined a 25-base pair region that is responsible for eNOS promoter activity in response to laminar shear stress. Transactivation of this element was found to be regulated by c-Src and ERK1/2 activity as PP1 and PD98059 completely inhibited shear-induced eNOS-CAT activity. Our studies indicate the NFκB subunits p50 and p65 are translocated to the nucleus and bind to this site in response to shear stress. This phenomenon seems to be a critical mediator for eNOS transcription in response to laminar shear stress.

EXPERIMENTAL PROCEDURES

Cell Culture—BAECs (Cell Systems, Kirkland, WA) were cultured in Media 199 (M199; Invitrogen) containing 10% FCS (Hyclone Laboratories, Logan, UT) as described earlier (14). Post-confluent BAECs between passages 4 and 9 were used for experiments.

Shear Apparatus—A cone-in-plate viscometer with a 1° angle was used to shear cells (15). All shear studies used a shear rate of 15° at 37°C in this study.

Cloning of the Human eNOS Promoter—The human eNOS promoter was cloned by polymerase chain reaction using Elongase (Invitrogen) with human genomic DNA (50 ng, Invitrogen) as a template. This approach used a forward primer from the 5′-upstream genomic sequence with a XhoI site at the 5′-end (underlined) (5′-GCGCTCGAGATGCACTCTG-3′) and a reverse primer from downstream prior to the initiator methionine codon with a HindIII site at the 5′-end (underlined) (5′-CAGGCTCTTCAAGCTTCC-3′). The resultant PCR product was sequenced by the dye-oxide chain termination method using the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH). The sequence obtained was similar to that reported previously (16).

For generation of deletion mutants, the following forward primers with human genomic DNA (50 ng, Invitrogen) as a template. This was done because in preliminary experiments, antibodies to specific DNA-binding proteins were incubated for gel shift electrophoretic mobility shift assay (EMSA) kit (Pierce). In some complexes were then separated on a 6% acrylamide-GTG non-denaturing gel and transferred to a nylon membrane for detection using the LightShift chemiluminescent ECL detection kit (Pierce). In some studies, antibodies to specific DNA-binding proteins were incubated for 30 min prior to addition of biotinylated DNA.

Materials—PD98059, UO126, and PPM-18 were obtained from Calbiochem. PP1 was obtained from Biomol (Plymouth Meeting, PA). All drugs were dissolved in Me2SO, and the resulting stock solutions were filtered (0.2 μm) before use. All antibodies used in EMSA experiments were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Effect of Shear on eNOS Promoter Activity and Identification of the Region Responsive to Shear—BAECs transfected with the full-length eNOS-CAT promoter construct were sheared for 0, 1, 2, 4, 6, 12, and 24 h and CAT activity measured. We found significant increases in shear-induced eNOS promoter activity at 12 and 24 h of shear stress (Fig. 2A).

We have previously shown that shear-induced eNOS transcription, as measured by nuclear run-on analysis, occurs
through c-Src activation of the MEK/ERK pathway (6). In keeping with these findings, pretreatment of BAECs with the Src inhibitor PP1 (10 μM) completely prevented eNOS-CAT activity in response to shear stress (Fig. 3). Furthermore, pretreatment of BAECs with the MEK1/2 inhibitors PD98059 (50 μM) and UO126 (50 μM) also abolished shear induction of eNOS promoter activity. Taken together, these data indicated that the activity of the chimeric eNOS promoter/CAT construct is regulated by shear stress in response to signaling events that also modulate activation of the of the endogenous eNOS gene by shear.

Identification of the Region of the Human eNOS Promoter Responsive to Shear Stress—To examine the region of the eNOS promoter sensitive to shear, several truncation mutants were generated (Fig. 1). Deletion of the first 600 bases resulted in no significant reduction in eNOS-CAT activity (2.7-fold). Deletion of the next 25 bases (−1000 to −975) completely inhibited shear stress activation of the eNOS promoter. Deletion of the following 25–75 bases maintained the inhibition of shear-induced eNOS-CAT activity (Fig. 2B). In keeping with prior studies, there was no difference in the basal activities of these various constructs (p = 0.4, ANOVA, data not shown). These data define a 25-base pair region in the eNOS promoter between −1000 and −975 as the region responsible for shear responsiveness. Using the Transfac sequence analysis, we found that this region contained potential binding sites for several transcription factors, including NFκB, Sp-1, Elk-1, and GATA (19).

Determination of Transcription Factor Binding to the Shear-responsive Promoter Sequence—To determine the factors binding to the shear responsive element, EMSAs were performed. Using the 25-base pair sequence and flanking 6 bases on either side as a probe, we found that shear stress resulted in the binding of several complexes in a time-dependent manner (Fig. 4). Using the 25-base pair sequence encompassing the 25 bases of interest defined by CAT binding of several complexes in a time-dependent manner (Fig. 4), correlating with the time course we have previously reported for eNOS mRNA transcription by shear stress (6).

To determine the composition of the complex induced by shear stress that binds to −1000 to −975 base pairs, we used cold competitor oligonucleotides and antibodies for supershift analysis. As seen in Fig. 5A, preincubation of the binding reaction with 50× excess of cold probe completely prevented binding of the complex. Furthermore, preincubation with an NFκB unlabeled consensus probe abrogated binding (Fig. 5A).

**Fig. 3.** Chimeric eNOS-CAT promoter activity in BAECs demonstrating a requirement for c-Src and MEK1/2. BAECs were transfected with the full-length promoter construct, as well as a β-galactosidase construct, and sheared at 15 dynes/cm² for 24 h. 1 h prior to shear stress experiments is shown. Asterisks indicate significant differences from non-sheared cells (*, p < 0.05; Tukey-Kramer test following one-way ANOVA).

**Fig. 4.** Two representative EMSAs showing binding of nuclear complexes to the 25-base pair region of interest in a time-dependent manner. Nuclear protein was incubated with a biotinylated DNA probe encompassing the 25 bases of interest defined by CAT studies and 6 flanking bases on each end in the presence of 2× binding buffer. Complexes were run on a non-denaturing gel and transferred to a positively charged nylon membrane. The membrane was probed using streptavidin-conjugated to horseradish peroxidase and detected with Lightshift kit (Pierce).

**Fig. 5.** EMSA analysis identifying NFκB as the complex binding to the 25-base pair region of interest. A, complexes were preincubated with 50-fold excess of cold probe or an unlabeled consensus NFκB oligonucleotide (5′-AGTTGAGGGACTTTCCCAGGC-3′). B, antibodies directed against p50 and p65 subunits were preincubated for 30 min prior to addition of biotinylated probe.

**Fig. 6.** A, EMSA analysis identifying NFκB as the complex binding to the 25-base pair region of interest. BAECs were pretreated with PPM-18 (10 μM) for 1 h and subjected to 15 min of shear stress. B, Western analysis showing inhibition of p50 and p65 nuclear translocation in response to shear stress. 12 μg of nuclear protein extracts from A were loaded on a 12.5% polyacrylamide gel, and membranes were probed with antibodies for p50 and p65.
In contrast, binding was unaffected by AP-1, SP-1, and CREB cold consensus oligonucleotides (data not shown). Additionally, the antibodies against p65 and p50 diminished binding of both complexes, suggesting that they are both comprised of p65 and p50 subunits (Fig. 5B). Finally, pretreatment of BAECs with the NFκB inhibitor PPM-18 completely prevented binding to the probe (Fig. 6A) and also preventing nuclear translocation of p50 and p65 in response to shear (Fig. 6B). These data implicate a role for NFκB binding to the 25 base pair segment that regulates shear stress induction of eNOS transcription.

**Determination of the Importance of Binding-regulating eNOS Promoter Activity**—Transfac analysis indicated that a consenus NFκB site exists in the human eNOS promoter at nucleotides −994 to −988 and that this is adjacent to an SSRE site between nucleotides −987 to −981. Because both of these sites could potentially bind NFκB and regulate eNOS promoter activity in response to shear stress, we generated mutations of the NFκB site alone, the SSRE site alone, as well as both sites and performed EMSAs (Fig. 7A). Mutation of the consensus NFκB site had no effect on complex binding, whereas mutation of the GAGACC abolished binding of the lower band. Mutation of both sites completely abolished all complex binding (Fig. 7B).

The above data indicate that either the putative NFκB consensus site or the GAGACC sequence might be responsible for increasing eNOS transcription in response to shear stress. To determine which of these are involved, mutations identical to those describe above were made in the full-length human eNOS promoter and not NFκB mutation. C, BAECs were transfected with either the F1 or mutant NFκBm, SSREM, or DBLm promoter constructs, as well as β-galactosidase and sheared for 24 h. CAT activity was determined using scintillation counting and normalized to β-galactosidase activity and expressed as fold over static control. Mean ± S.E. from four separate experiments is shown. Asterisks indicate significant differences from F1 and NFκBm (**, $p < 0.01$; Tukey-Kramer test following one-way ANOVA).

**Fig. 7.** A, sequences of mutations used for EMSA and promoter activity studies. **Bold** sequences are sequences of interest (underline = consensus NFκB; *italics* = SSRE; NFκBm = NFκB mutant; SSREM = SSRE mutant; DBLm = double mutant). B, representative EMSA showing loss of complex binding with SSRE mutation and not NFκB mutation. C, mean S.E. from four separate experiments is shown.
**Laminar Shear Stress and eNOS Promoter Activity**

**Determination of NFkB Activation in Regulating eNOS Promoter Activity**—To determine whether NFkB binding is critical for eNOS promoter activation in response to unidirectional shear stress, BAECs were transfected with full-length eNOS/CAT chimeric construct and a dominant negative IkBα (20). In resting cells, p50 and p65 are sequestered to IkBα and can only be released by phosphorylation of IkBα on serine 32 (21). Mutation of the serine to lysine prevents phosphorylation and subsequent dissociation (20). Co-transfection of F1-CAT and IkBα dominant negative resulted in complete inhibition of eNOS promoter activity in response to shear stress (Fig. 8). In contrast, dominant negative IkBα had no effect on eNOS promoter activity in response to lysophosphatidylcholine, which increases eNOS expression by enhancing Sp-1 binding to the eNOS promoter (22). These data clearly show NFkB binding regulates eNOS promoter activity following unidirectional shear stress.

**Determination of NFkB Pathway Activation by Laminar Shear Stress**—To determine whether shear stress could activate the NFkB pathway, phosphorylation of IkK, the major kinase involved in IkB phosphorylation, was examined using Western analysis. Shear stress caused a time-dependent phosphorylation of IkK, with no effect on the total levels of the protein (Fig. 9A). Furthermore, IkK phosphorylation by shear stress was prevented by preincubation with the Src inhibitor PP1 and the MEK1/2 inhibitor PD98059 (Fig. 9B). These data demonstrate NFkB pathway activation in response to shear stress and that this pathway is regulated through c-Src, MEK1/2 and ERK1/2.

**DISCUSSION**

One of the most important physiological stimuli for regulation of the eNOS expression is laminar shear stress. Previously, we have shown that this occurs by a transient increase in transcription and a prolonged increase in eNOS mRNA stability (6). The increase in eNOS transcription, while transient, is likely important as it could underlie the increase in eNOS caused by bouts of exercise training, which via increasing cardiac output enhance eNOS expression (23, 24). In the present study, we defined a specific region of the eNOS promoter that regulates induction of eNOS transcription by shear. We demonstrated that shear stress stimulates binding of the NFkB subunits p50 and p65 to this region and that this phenomenon is crucial for increasing promoter activity.

Our present findings on our keeping with previous studies in which deletion of the eNOS promoter—1600 to −779 base pairs upstream from the transcription start site inhibited induction by laminar shear stress (25, 26). There are numerous potential binding sites for a variety of transcription factors within this large region of DNA (19). Using eNOS-CAT promoter constructs, we identified a more precise region, specifically between −1000 and −975 base pairs, which confers eNOS transcriptional activation by shear stress. Even within this 25-base pair region, there are several putative binding sites for transcription factors such as Sp-1, Elk-1, and GATA. Our studies, however, suggest that these are not involved in modulation of eNOS promoter activity in response to shear but strongly support a role of NFkB in transactivation of the eNOS reporter in response to shear stress. Studies using cold competitor oligonucleotides, antibodies against p50 and p65, and pharmacological treatment with PPM-18 (27) indicated that the proteins responsible for the complexes formed in the electrophoretic mobility shift assays were p50 and p65. Studies with the eNOS promoter further supported a role of NFkB as co-transfection with the dominant negative IkBα prevented transactivation of eNOS by shear stress.

Analysis of the 25-base pair segment indicated that two potential binding sites for NFkB are present within this region, both present on the antisense strand. Interestingly, in electrophoretic mobility shift assays, both of these regions were capable of binding p50 and p65, as mutation of either alone did not completely abolish binding while mutation of both prevented complex formation. In studies of the full-length eNOS promoter, however, only the GAGACC consensus site had no effect on transactivation by shear stress, while mutation of the GAGACC site completely prevented the effect of shear. These seemingly disparate results might be due to differences in secondary structure between the full-length promoter and the simple 27-base pair oligonucleotide used in the EMSAs.

Our data further provide some insight into how shear activates NFkB and eNOS transcription. We have previously found that eNOS transcription is signaled by a pathway involving cSrc, Ras, Raf, MEK1/2, and ERK1/2. In keeping with these findings, pharmacological inhibition of either cSrc (with PP1) or MEK1/2 (with PD98059 or U0126) prevented activation of the eNOS promoter. Likewise, phosphorylation of IkKα was prevented by PP1 and PD98059. These data indicate that cSrc and ERK1/2 are upstream of IkKα activation in response to shear stress. This finding is similar to the situation described recently by Dhawan and Richmond (28) who demonstrated that MEK1/2 and ERK1/2 activation are upstream of NFkB activation in melanoma cells.

Historically, NFkB activation has been implicated in regulation of several proatherogenic related genes including VCAM-1, MCP-1, and PDGF (29). Indeed, in lesion prone areas of the circulation there is increased expression of p65 as well as IkKα and IkKβ (30). Interestingly, NO− has been shown to inhibit NFkB activation via at least two mechanisms. One involves increased expression of the inhibitor subunit IκB (31), while a second involves nitrosylation of p50, which diminishes its nuclear translocation (32). One can therefore envision that this represents a classical negative feedback loop in which shear stress activates NFkB, which in turn increases eNOS expression. The increase in eNOS expression would allow for an increase NO− production, which would ultimately inhibit NFkB activation. This is potentially important, because several common cardiovascular diseases, such as hypercholesterolemia, hypertension, and diabetes, are associated with oxidative inactivation of NO− or oxidation of critical NO− cofactors. In these situations the reduction in the ambient levels of biologically active NO− shear stress could lead to an unmitigated activation of NFkB. Such an interruption of a normal negative feedback situation might dramatically predispose to endothelial inflammation and atherosclerosis.

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