Laminar Shear Stress Modulates Phosphorylation and Localization of RNA Polymerase II on the Endothelial Nitric Oxide Synthase Gene

Jeffrey P. Moore, Martina Weber, Charles D. Searles

Objective—In endothelial cells exposed to unidirectional laminar shear stress, endothelial nitric oxide synthase transcription (eNOS), mRNA stability, and protein levels are enhanced. We have previously demonstrated that these changes are associated with increased 3’ polyadenylation of eNOS mRNA. Here, we investigated the effect of laminar shear stress on the phosphorylation and localization of RNA polymerase (Pol) II, the enzyme primarily responsible for coordinating transcription and posttranscriptional processing.

Methods and Results—Using Western and chromatin immunoprecipitation analyses, Pol II phosphorylation and localization on the eNOS gene were assessed in bovine aortic endothelial cells exposed to laminar shear stress. Total Pol II (phosphorylated and unphosphorylated) levels were increased 65% in response to laminar shear stress. This was associated with an increase in Pol II phosphoserine 2, but no change in levels of the unphosphorylated or phosphoserine 5 isoforms. Quantitative chromatin immunoprecipitation analysis showed that laminar shear stress enhanced binding of Pol II phosphoserine 2 to the 3’ end of the eNOS gene, particularly exon 26, which encodes the 3’ UTR. Treatment of cells with DRB attenuated laminar shear stress-induced Pol II phosphorylation, eNOS 3’ polyadenylation, and eNOS expression.

Conclusion—These data suggest that laminar shear stress enhances eNOS mRNA 3’ polyadenylation by modulating phosphorylation and localization of Pol II. (Arterioscler Thromb Vasc Biol. 2010;30:561-567.)

Key Words: endothelial nitric oxide synthase • gene expression • laminar shear stress • mRNA processing • RNA polymerase II

Mechanical forces created by pulsatile pressure and blood flow are important modulators of cellular function in the cardiovascular system. In the blood vessel wall, endothelial cells are particularly adept at changing their properties in response to mechanical forces.1 One of these forces is unidirectional laminar shear stress (LSS), the dragging frictional force created by blood flow that acts parallel to the luminal surface of the vessel. Endothelial cells respond to LSS by modulating gene expression, cell metabolism, cell morphology, and the release of vasoactive substances,2 including nitric oxide (NO). NO is produced by endothelial NO synthase (eNOS), and LSS has been shown in in vitro and in vivo studies to increase the activity and expression of eNOS.3-5

LSS-induced upregulation of eNOS occurs by 2 mechanisms: a transient increase in eNOS transcription, followed by a prolonged stabilization of eNOS mRNA.6 Although both mechanisms are dependent on the activity of c-Src, downstream signaling events for transcriptional and posttranscriptional regulation of eNOS differ.6 Previously, we described a posttranscriptional mechanism by which LSS induces enhanced eNOS mRNA stability and translation that involves enhanced 3’ polyadenylation. Under static conditions, eNOS transcripts have short 3’ poly(A) tails, but these tails dramatically lengthen in response to LSS.7

In eukaryotic cells, mRNA synthesis is performed by a transcription elongation complex whose main component is RNA polymerase II (Pol II). This enzyme plays a critical role in coordinating chromatin remodeling and posttranscriptional mRNA processing, including 5’ capping, splicing, and 3’ polyadenylation. The processing functions of Pol II are mediated primarily by its carboxy-terminal domain (CTD),8,9 which serves as a platform for factors required for mRNA processing. Pol II CTD contains repeats of a highly conserved 7-amino-acid consensus sequence, YSPTSPS,10 and serines at positions 2 and 5 are targets for phosphorylation.11 Transcription initiation is preferentially associated with binding of unphosphorylated Pol II to promoter sequences, whereas Pol II phospho-serine 5 is associated with release of the transcription complex from the promoter, 5’ capping and splicing.12

Received October 22, 2008; revision accepted November 30, 2009.
From Division of Cardiology, Emory University School of Medicine (J.P.M., M.W., C.D.S.), Atlanta, Ga, and the Atlanta Veterans Administration Hospital, Atlanta, Ga (C.D.S.).
J.P. Moore and M. Weber contributed equally to this study.
Correspondence to Charles D. Searles, MD, 1639 Pierce Drive, WMB 319, Atlanta, GA 30322. E-mail csearle@emory.edu
© 2010 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBAHA.109.199554

561
Pol II phosphoserine 2 is responsible for transcript elongation, termination, and 3’polyadenylation.\textsuperscript{13}

Transcript elongation and posttranscriptional mRNA processing are highly regulated,\textsuperscript{14} and distinct signal transduction pathways have been shown to modulate Pol II activity through CTD phosphorylation.\textsuperscript{5,16} We hypothesized that mechanotransduction of LSS in endothelial cells leads to changes in Pol II phosphorylation, which in turn enhances eNOS mRNA 3’polyadenylation. In this study, we focused on changes in Pol II phosphorylation and localization on the eNOS gene in endothelial cells exposed to LSS. We found that LSS enhanced expression of Pol II phosphoserine 2 and its localization to sequences encoding the eNOS 3’UTR, adjacent to the transcription termination site. These findings provide further insight into mechanisms by which transduction of mechanical forces can modulate endothelial gene expression.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (Cell Systems) were cultured in Media 199 (Mediatech) containing 10% fetal calf serum (Hyclone), penicillin-streptomycin, glutamine, and nonessential amino acids. Postconfluent bovine aortic endothelial cells between passages 4 and 8 were used for all experiments. A cone-in-plate viscometer with a 1-degree angle was used to create unidirectional laminar shear stress of 15 dynes/cm\(^2\) for 6 hours.\textsuperscript{17} The magnitude and 6-hour period of shear stress was selected based on previous experiments, in which eNOS polyadenylation was found to be maximally increased after 6 hours of LSS.\textsuperscript{7}

Western Analysis

Western analysis was performed as previously described.\textsuperscript{18} Antibodies against the different Pol II phosphoisoforms were obtained from Covance. These include H5 (Pol II phosphoserine 2, 1:500), H14 (phosphoserine 5, 1:500), and SWG16 (unphosphorylated Pol II CTD, 1:1000). Antibodies against total Pol II (targeting N-terminal, 1:10000) and eNOS (1:2500) were from BD Pharmingen. The antibody against cyclin-dependent kinase 9 was purchased from Santa Cruz (1:500). Anti-α-actin antibody (1:1000; Sigma) was used as a loading control. All antibodies were prepared in 3% blocking milk.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total cellular RNA was isolated from cells using TRIZol-Reagent (Invitrogen). Light Cycler (Roche) was used for real-time polymerase chain reaction, as described earlier.\textsuperscript{19} Before polymerase chain reaction, DNA was purified with Micro Bio-Spin 30 Chromatography Columns (Bio Rad). Polymerase chain reaction was performed on 2 μL of cDNA using Platinum Taq Polymerase (Invitrogen) and buffer provided with the enzyme. For continuous fluorescence monitoring of RNA-specific binding, 0.5% SYBR green dye (Roche) was used.

Chromatin Immunoprecipitation

Bovine aortic endothelial cells were sheared for 6 hours (15 dynes/cm\(^2\)), with or without DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; 60 μmol/L; Sigma) pretreatment (1 hour), or treated with cytochalasin D (5 μmol/L; Sigma) for 6 hours. Control cells were kept under static conditions or treated with DMSO. Protein was cross-linked to DNA with 1% formaldehyde for 10 minutes at 37°C. The chromatin immunoprecipitation (ChIP) assay was performed following the Upstate ChIP Assay protocol with slight modifications. Briefly, cells were washed with 1× phosphate-buffered saline and lysed for 10 minutes on ice in SDS Lysis-Buffer (Upstate). Cells were sonicated using a Sonic Dismembrator 60 (Fisher Scientific). The DNA was sheared 3 times (4 watts, 10 pulses at 1 second each) into ~200-bp-long fragments (Figure IA, available at http://atvb.ahajournals.org). Protein was immunoprecipitated overnight using Pol II phospho-serine 2 antibody (H5) or IgG as control, and then pulled down with Salmon sperm DNA/protein G/A-agarose (Upstate). Cross-linking was reversed overnight and protein was digested with proteinase K (Invitrogen) for 1 hour. DNA was extracted using phenol/chloroform and ethanol precipitation. Primers for polymerase chain reaction are listed online. Primers were subjected to Basic Local Alignment Search Tool analysis (National Center for Biotechnology Information), and none had 100% homology with any other bovine genomic sequence besides eNOS. In fact, exon 26 primers did not have homology with any other sequence in the bovine genome. Primer efficiency was determined using 0 to 0.6 ng/μL genomic DNA standard curves, analyzed by linear regression. Sample values were calculated by subtracting the y-intercept and then dividing by the slope. Each sample was normalized to the individual amount of input DNA.

RNase Protection Assay

RNA was isolated using TRIZol (Invitrogen). To detect the length of Polyadenylated eNOS mRNA, we used a special riboprobe generated from a 3’ RACE-PAT of eNOS as described previously.\textsuperscript{7}

Results

LSS Preferentially Increases Expression of Pol II Phosphoserine 2

Bovine aortic endothelial cells subjected to LSS (6 hours, 15 dynes/cm\(^2\)) had a 65% increase in total Pol II protein expression compared to static control cells (Figure 1A). The antibody used to assess total Pol II expression targets the N-terminal domain of the enzyme and therefore detected both phosphorylated and unphosphorylated Pol II. Because the mRNA processing activities of Pol II are linked to phosphorylation of its CTD, further Western analysis was performed to assess expression of the three specific Pol II phosphoisoforms in response to LSS. Endothelial cells exposed to LSS had a 40% to 50% increase in expression of Pol II phosphoserine 2 (Figure 1B) compared to control cells. In contrast, there was no significant effect of LSS on expression of unphosphorylated Pol II (Figure 1C) or Pol II phosphoserine 5 (Figure 1D). Thus, the LSS-induced upregulation of total Pol II appeared to be largely associated with enhanced expression of the Pol II phosphoserine 2 isoform.

The 6-hour time point was used for these studies because after 6 hours of LSS, maximum 3’ polyadenylation of eNOS mRNA was observed.\textsuperscript{7} A shear time course (1–6 hours) showed that expression of Pol II phosphoserine 2 is increased over baseline levels as early as 1 hour after the cells are subjected to LSS and remains increased for the duration of LSS (Figure II A). In comparison, eNOS protein is increased after 4 to 6 hours of LSS.

Localization and Quantitative Assessment of Pol II Phosphoserine 2

ChIP analysis was performed to determine the effect of LSS on Pol II phosphoserine 2 binding to different regions of the eNOS gene. Chromatin from sheared and static control cells was immunoprecipitated with the antibody against Pol II phosphoserine 2, and quantitative polymerase chain reaction was performed with primers targeting
sequences in the promoter area, exons 19 to 26, and sequences downstream of the transcription termination site (Figure 2A). The different efficiencies of the primer pairs were determined and subsequently used to quantify binding of Pol II phosphoserine 2 to the eNOS gene (Figure 1B). The DNA sequences that were studied were chosen because of the importance of their interaction with Pol II in demonstrating the different activities of Pol II during the

Figure 1. Pol II levels in bovine aortic endothelial cells exposed to LSS or nonsheared conditions. Western blot and densitometric analysis for (A) total Pol II, (B) Pol II phosphoserine 2; (C) Pol II phosphoserine 5; and (D) unphosphorylated Pol II (n=4–6). *P=0.01 vs static, unpaired t test.

Figure 2. Pol II localization on the eNOS gene. A, Location of ChIP polymerase chain reaction primer pairs. B, Quantitative ChIP analysis of Pol II phosphoserine 2 binding to promoter area and later exons (n=3–8). *P=0.038 vs static.
transcription cycle. These activities include transcription initiation, demonstrated by Pol II binding to promoter sequences, and transcript elongation, termination, and 3’ processing, demonstrated by Pol II binding to exonic sequences at the 3’ end of the gene as well as sequences downstream of the polyadenylation site.

The Pol II CTD is phosphorylated at serine 2 during elongation of the primary RNA transcript, and Pol II phosphoserine 2 is responsible for coordinating 3’ polyadenylation by recruiting factors necessary for 3’ processing. High amounts of DNA (~6 ng) were found to bind Pol II phosphoserine 2 on exon 26 (eNOS 3’UTR) in cells exposed to LSS. Compared to control cells, LSS induced a significant 11.4-fold increase in Pol II binding to exon 26. We did not observe a lot of binding of Pol II phosphoserine 2 in the promoter area; this region of the gene should be associated with unphosphorylated Pol II or Pol II phosphoserine 5, respectively. We detected binding of Pol II phosphoserine 2 to exons 19, 21, and 22, but the values for binding to these sequences were still much lower compared to binding of Pol II phosphoserine 2 to exon 26 (~1 ng DNA). Together, these data indicate that LSS modulates Pol II by increasing phosphorylation of CTD serine 2 and enhancing localization of the serine 2 phosphoisoform to eNOS sequences encoding the 3’ UTR. Further, quantitative ChIP analysis of the actin 3’UTR did not show increased Pol II phosphoserine 2 binding in response to LSS (Figure IIB), suggesting that enhanced binding of Pol II phosphoserine 2 to eNOS 3’UTR in response to LSS is specific.

**DRB Attenuates LSS-Induced Pol II Phosphorylation and eNOS 3’ Polyadenylation**

Positive transcription elongation factor b (P-TEFB) is responsible for the transition of Pol II from the initial phases of transcription into the productive elongation phase. P-TEFB is a heterodimer composed of CDK9 and a regulatory cyclin (T1, T2, or K), and Pol II serine 2 is a specific substrate for phosphorylation by CDK9. We have observed that DRB, an agent that blocks transcription by inhibiting CDK9, attenuated LSS-induced changes in eNOS 3’polyadenylation. Figure 3A shows a ribonuclease protection assay of RNA from control and sheared cells using a riboprobe that targets the 3’ end of eNOS mRNA. The ribonuclease protection assay of control cells shows one protected fragment of 250 nt, which correlates with a short eNOS 3’ poly(A) tail of 25 nucleotides in length. Additional protected fragments were detected for RNA from sheared cells; these fragments correlate with poly(A) tails of 75 to 160 nucleotides in length. Pretreatment of cells with DRB (60 µmol/L) for 1 hour before LSS dramatically reduced eNOS polyadenylation (Figure 3A, right). Subsequently, we examined the effect of DRB pretreatment on LSS-induced changes in Pol II phosphorylation. DRB pretreatment attenuated the observed LSS-induced increase in total Pol II (Figure 3B), Pol II phosphoserine 2 expression (Figure 3C), and eNOS expression (Figure 4B). Together, these data suggest that the LSS-induced increase in Pol II serine 2 phosphorylation and eNOS 3’polyadenylation is mediated by CDK9.

**Figure 3.** Effect of DRB on LSS-induced changes in expression of Pol II. A, Representative ribonuclease protection assay of eNOS transcripts showing inhibition of 3’ polyadenylation after DRB pretreatment (n=3). Western analysis for (B) total Pol II and (C) Pol II phosphoserine 2 (n=3) *P<0.05 vs static.

**Figure 4.** DRB Modulates LSS-Induced Binding of Pol II Phosphoserine 2 to eNOS Gene

To examine the effect of DRB on localization of Pol II phosphoserine 2 on the eNOS gene, we performed additional ChIP analysis on cells that were pretreated with DRB for 1 hour and subsequently subjected to LSS for 6 hours. Quantitative assessment of Pol II phosphoserine 2 binding to exon 26 showed that DRB prevented LSS-induced binding of Pol II phosphoserine 2 (Figure 4A). We hypothesized that a consequence of this effect would be less 3’ processing and, therefore, less eNOS mRNA stability and translation. Western analysis of eNOS protein in static and LSS-treated cells with and without DRB pretreatment demonstrated the well-
known increase of eNOS protein in response to LSS (Figure 4B), and it revealed that this response is prevented in the presence of DRB, most likely because of the lack of Pol II phosphoserine 2 binding to eNOS 3'/H11032 UTR.

Cytochalasin D Treatment Does Not Alter Expression or Localization of Pol II on eNOS
Cytochalasin D is an agent that directly alters actin cytoskeleton organization and, like LSS, increases eNOS 3' polyadenylation and expression. The effect of cytochalasin D on Pol II phosphorylation and localization in bovine aortic endothelial cells was studied using Western analysis and quantitative ChIP analysis (Figure 4C). We did not observe any change in expression of total Pol II levels or any of the Pol II phosphoisoforms. Quantitative ChIP analysis of Pol II phosphoserine 2 binding to eNOS exon 26 did not reveal any significant differences between cytochalasin D-treated and control endothelial cells (Figure 4D). Because this finding differs from the response to LSS, we conclude that not all stimuli that increase eNOS polyadenylation will necessarily have enhanced Pol II phosphoserine 2 binding to the 3' UTR. Interestingly, stimulus-dependent changes in Pol II serine 2 phosphorylation and localization are not unique to our model system; this phenomenon has been noted in other mammalian model systems.16

Discussion
Shear stress forces generated by blood flow are important modulators of signaling and gene expression in the vessel wall. In the current study, we sought to extend our previous work on LSS-induced changes in eNOS expression by examining how LSS increases 3' polyadenylation of eNOS mRNA. Previous studies of eNOS expression have focused on the early phases of the transcription cycle, such as transcription initiation. However, the cell’s transcription machinery is not solely associated with promoter activity, and other aspects of mRNA synthesis, such as capping, splicing, and 3' polyadenylation, are dynamic and highly regulated. These posttranscriptional processing reactions are influenced by changes in phosphorylation of Pol II, whose CTD coordinates mRNA processing reactions by providing key molecular contacts throughout transcriptional elongation and termination (Figure 5). The central finding of this study is that LSS alters expression, phosphorylation, and localization of Pol II on the eNOS gene. Thus LSS, besides stimulating recruitment of Pol II to the eNOS promoter for transcription initiation, also...
enhances recruitment of Pol II phosphoserine 2 to the 3′ region of the eNOS gene important for transcription termination and polyadenylation.

In quiescent endothelial cells, the expression of eNOS is constitutive and restricted to the endothelial cell layer of medium-caliber to large-caliber vessels. Basal eNOS transcription is controlled by 2 positive regulatory domains (I and II) in the proximal promoter, and the endothelial cell-specific nature of eNOS transcription has recently been linked to the chromatin structure in this region. It has been shown that active elongation and 3′-processing of eNOS transcripts by phosphorylated Pol II in quiescent endothelial cells is relatively hindered, analogous to the elongation block observed in immediate early gene transcription. Interestingly, both eNOS mRNA from human and bovine quiescent endothelial cells have short 3′ poly(A) tails, suggesting that 3′ mRNA processing is impeded in nonstimulated cells.

Consistent with this concept, we found very little Pol II phosphoserine 2 binding to eNOS DNA in nonsheared control cells; however, in response to LSS, there was increased binding of Pol II phosphoserine 2 to DNA on exon 26. This observation supports our hypothesis that mechanotransduction of LSS in endothelial cells leads to upregulation of eNOS expression in part by altering phosphorylation of Pol II, thus relieving the baseline impediment to active elongation and 3′ polyadenylation. We found that LSS specifically enhanced expression of the Pol II phosphoserine 2, the phosphoisoform associated with transcript elongation and 3′ polyadenylation (Figure 5). In contrast, we also found that cytochalasin D, which, like LSS, is known to increase eNOS 3′ polyadenylation and expression, had no effect on Pol II expression, phosphorylation, or localization to the eNOS 3′UTR. This result indicates that LSS and cytochalasin D increase eNOS 3′ polyadenylation by different mechanisms, which may not be unexpected given that LSS also increases transcription initiation, whereas cytochalasin D does not.

The recruitment of P-TEFb (consisting of CDK9 and a cyclin subunit) to the transcription unit is important for stimulus-driven synthesis of mRNA. Fujita et al demonstrated that DRB, an adenosine analogue that specifically blocks Pol II elongation by inhibiting P-TEFb, inhibited phosphorylation of Pol II and downstream progression of Pol II to downstream regions of the MKP-1 gene in neuroendocrine cells. Similarly, we observed that in endothelial cells, DRB inhibited LSS-induced changes in Pol II phosphorylation and localization, as well as eNOS 3′ polyadenylation and protein expression. The relationship between shear stress and P-TEFb has not been studied previously, although CDK9 has been shown to be responsive to stress signals associated with cardiac myocyte hypertrophy, such as aortic banding. These signals did not change expression of CDK9 or cyclin T, but they did alter their association with an inhibitory small nuclear RNA, 7SK. Stress-induced deactivation of CDK9 was mediated by calcineurin and Gq. Whether this regulatory pathway is responsible for LSS-induced changes in endothelial Pol II phosphorylation is unknown. Figure 5 depicts a schema of how LSS-induced changes in Pol II phosphorylation correlate with its location and synthesis of eNOS mRNA. A question mark has been inserted between LSS and P-TEFb because we are not certain whether LSS directly or indirectly modulates CDK9.

Steady-state levels of eNOS mRNA are subject to modest, but likely important, degrees of regulation by different stimuli that have been implicated in vascular pathophysiology. Recent work has demonstrated that expression of this gene is complex and involves multiple different cis and transacting factors, as well as changes in promoter activity, chromatin remodeling, mRNA processing, mRNA stability, and translational activity. In addition, in humans and mice, an antisense mRNA that is complementary to the 3′ end of eNOS has been shown to posttranscriptionally downregulate eNOS in nonendothelial cells. This antisense mRNA, known as sONE, is derived from a transcription unit on the DNA strand opposite eNOS, oriented in a tail-to-tail configuration with eNOS. However, we do not think our ChIP analysis of the bovine 3′UTR is confounded by Pol II binding to the 3′UTR of bovine sONE. We have not been able to detect sONE mRNA in bovine endothelial cells. The predicted bovine sONE does not overlap to the extent that the human sONE does, so the sequences encoding bovine eNOS and sONE 3′UTR appear to be different.

**Conclusion**

In this study, we have used a well-established in vitro model of unidirectional shear stress forces, the cone-in-plate viscometer, to show that transduction of prolonged shear stress (6 hours) in endothelial cells leads to changes in Pol II phosphorylation and localization, which then has an impact on eNOS 3′ polyadenylation and expression. The detailed mechanism of how LSS-induced modulation of the general transcription machinery leads to changes in 3′ processing of specific mRNA is not completely clear, but we believe that this issue is related to cis-acting elements recognized by Pol II, including the polyadenylation signal. Both bovine and human eNOS 3′UTR contain relatively inefficient polyadenylation signals and hexamers (AAUAAA, bovine; ACUAAA, humans), which are associated with short poly(A) tails under nonshear or low-shear conditions. The bovine polyadenylation hexamer/signal is depicted in Figure 5. We hypothesize that phosphorylation of Pol II serine 2 facilitates 3′ polyadenylation of genes, like eNOS, that have inefficient polyadenylation signals. Interestingly, LSS did not induce enhanced localization of Pol II phosphoserine 2 to the β-actin gene, which contains an efficient (AAUAAA, canonical) polyadenylation signal. Importantly, we have observed increased Pol II phosphoserine 2 levels relative to the other Pol II phosphoisoforms in human endothelial cells subjected to LSS for 6 hours, suggesting that the effect of LSS is not species-specific (Figure 1C). Current efforts are focused on identifying other endothelial genes that demonstrate similar LSS-induced changes in 3′polyadenylation and localization of Pol II phosphoserine 2.

**Sources of Funding**

This work was supported by National Institutes of Health grant R01HL077274.
Disclosure

None.

References

Laminar Shear Stress Modulates Phosphorylation and Localization of RNA Polymerase II on the Endothelial Nitric Oxide Synthase Gene
Jeffrey P. Moore, Martina Weber and Charles D. Searles

doi: 10.1161/ATVBAHA.109.199554

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/3/561