Shear Stress Regulates Endothelial Nitric Oxide Synthase Expression Through c-Src by Divergent Signaling Pathways

Michael E. Davis, Hua Cai, Grant R. Drummond, David G. Harrison

Abstract—In this study, we defined the signaling cascade responsible for increased eNOS mRNA expression in response to laminar shear stress. This pathway depends on the tyrosine kinase c-Src because shear induction of eNOS mRNA is blocked by the c-Src inhibitors PP1 and PP2, as well as an adenovirus encoding kinase inactive c-Src. After activation of c-Src, this pathway diverges. One arm is responsible for the short-term (6 hour) increase in eNOS mRNA. This involves a transient, 1-hour increase in eNOS transcription, as detected by nuclear run-on, that is dependent on activation of Ras and is blocked by adenoviral infection with dominant negative Ras. Downstream of Ras, MEK1/2 and ERK1/2 are important in this pathway, as 2 inhibitors of MEK1/2, PD98059 and UO126, completely prevented this early increase in eNOS mRNA. ERK1/2 was rapidly phosphorylated in response to shear, and this was prevented by c-Src and Ras inhibition. Further, Raf is phosphorylated in response to shear stress, and this is prevented by c-Src inhibition, suggesting that Raf may transduce the signal between Ras and ERK1/2. The second arm of the pathway linking activation of c-Src to eNOS expression involves stabilization of eNOS mRNA by shear stress. This response to shear is completely abrogated by the c-Src inhibitor PP1 but not altered by Ras or MEK1/2 inhibition. Thus, c-Src plays a central role in modulation of eNOS expression in response to shear stress via divergent pathways involving a short-term increase in eNOS transcription and a longer-term stabilization of eNOS mRNA. **(*Circ Res*. 2001;89:1073-1080.)**

Key Words: endothelial nitric oxide synthase ■ c-Src ■ ERK1/2 ■ Raf ■ mRNA stability

When first identified, eNOS was considered a constitutively expressed enzyme. During the past several years, however, it has become evident that its expression is regulated by a variety of stimuli.[1] One of the most physiologically important of these is laminar shear stress, the tangential force exerted by flow over the surface of the endothelium. Areas of the vasculature exposed to high shear are protected from the development of atherosclerosis, whereas areas exposed to low shear are prone to atherosclerotic lesion formation.[2] It is thought that increases in eNOS caused by shear may contribute to this phenomenon because of the many antiatherogenic properties of nitric oxide ($NO^{\bullet}$). In vivo, exercise training increases eNOS expression, probably by increasing shear stress.[3]

To date, the mechanisms linking mechanical forces like laminar shear to gene expression have remained poorly defined. Shear stress is known to activate numerous intracellular signaling molecules, including tyrosine kinases (in particular c-Src), G-proteins, PI-3 kinase, the mitogen activated protein kinases (MAPK) extracellular-related kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and protein kinase C.[4,5] How these interact and relate to increases in eNOS expression remains undefined.

There is also evidence that shear stress may increase activity of the eNOS promoter through transcriptional regulation of the eNOS gene, but this has only been demonstrated using studies of promoter-luciferase constructs.[6] To date, there has been no evidence of posttranscriptional regulation of eNOS mRNA by laminar shear stress, although many other stimuli seem to modulate eNOS expression by changing its mRNA stability.[7,8]

The present study was performed to examine specific mechanisms responsible for increased eNOS expression in response to shear stress. Given the documented effect of shear on activation of c-Src, we began by examining a potential role of this tyrosine kinase. Subsequently, we found that eNOS expression is modulated by a pathway involving c-Src activation of Ras/Raf and ERK1/2 to increase the transcription rate and a pathway dependent on c-Src but independent of Ras/Raf/ERK to increase mRNA half-life.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAECs; Cell Systems, Kirkland, Wash) were cultured in Media 199 (M199; Gibco Laboratories) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah) as described earlier.[9] Postconfluent BAECs between passages 4 and 9 were used for experiments.

Original received December 4, 2000; resubmission received July 19, 2001; revised resubmission received October 16, 2001; accepted October 16, 2001.

From the Division of Cardiology (M.E.D., H.C., G.R.D., D.G.H.) and the Molecular and Systems Pharmacology Program (M.E.D., D.G.H.), Emory University, Atlanta, Ga; and the Atlanta Veterans Hospital Medical Center (M.E.D., H.C., G.R.D., D.G.H.), Atlanta, Ga.

Correspondence to David G. Harrison, Division of Cardiology, Emory University, 1639 Pierce Dr, WMB 319, Atlanta, GA 30322. E-mail dharr02@emory.edu

Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/hh2301.100806**

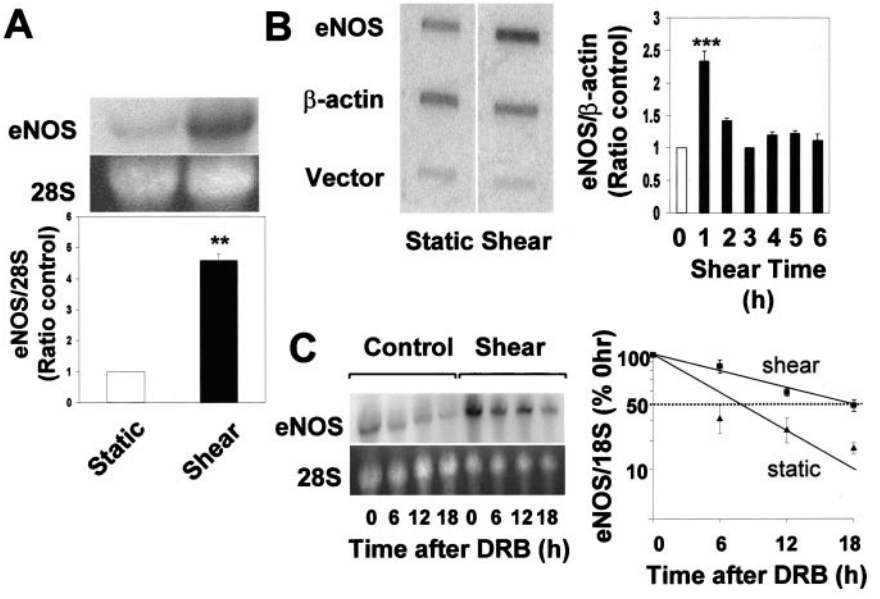

Figure 1. A, Northern analysis illustrating the effect of 6 hours of laminar shear stress on eNOS mRNA expression in BAECs. Cells were exposed to either no shear or 15 dynes/cm^2 of shear stress for 6 hours. Top, Representative blot for eNOS mRNA; middle, corresponding 28S band; bottom, grouped densitometric data (mean±SEM) from 4 separate experiments. Asterisks indicate significant difference from static (**$P<0.01$, t test). B, Nuclear run-on analysis demonstrating an increase in eNOS gene transcription after 1 hour of laminar shear stress. Nuclei harvested from BAECs after 1 to 6 hours of 15 dynes/cm^2 of laminar shear stress and 1 to 6 hours of static conditions were incubated with ^{32}P-UTP and unlabelled ATP, CTP, and GTP in reaction buffer for 30 minutes at 30°C. Transcripts containing equal amounts of radiolabel from each group were hybridized to membranes previously slot-blotted with cDNAs for eNOS, β-actin, and vector DNA (2 μg each). Hybridization was allowed to proceed for 36 hours at 68°C. Left, Representative blot of the significant time point; right, grouped densitometric data (mean±SEM) for all time points from 3 separate experiments. Asterisks indicate significant difference from unsheared (***$P<0.001$, Dunnett's test after 1-way ANOVA). C, DRB-chase experiments to examine changes in eNOS mRNA half-life in response to shear. BAECs were exposed to static or exposed to 15 dynes/cm^2 of laminar shear stress for 6 hours before addition of DRB (60 μmol/L). The cells were then harvested at the time points indicated and Northern analyses performed to determine the rate of eNOS mRNA decay in each group. Top left, Representative blot for eNOS mRNA; bottom left, corresponding 18S bands; right, grouped densitometric data (mean±SEM) from 4 separate experiments.

Shear Apparatus

A cone-in-plate viscometer with a 1° angle was used to shear cells.[10] All shear studies used a shear rate of 15 dynes/cm^2 and were performed in an incubator at 37°C in 5% CO_2. The culture medium was changed to 5% FCS the night before the experiments. Cells were pretreated with the indicated agent in media containing 5% FCS for 1 hour prior to shear.

Assessment of eNOS Expression

Northern analyses and nuclear run-ons were performed as previously described.[11]

Assessment of MAP Kinase Activation

Western analysis for ERK1/2 was performed using a 1:1000 dilution of a polyclonal antibody against either total or phosphorylated ERK1/2 (New England Biolabs) as well as a 1:5000 dilution of a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, Calif). Western analysis for Raf was performed using a 1:1000 dilution of either a polyclonal antibody against phosphorylated Raf (New England Biolabs) or a monoclonal antibody against total Raf (Transduction Laboratories) as well as a 1:5000 dilution of a secondary goat anti-rabbit antibody or a goat anti-mouse antibody (Bio-Rad) conjugated to horseradish peroxidase.

Materials

PD98059, UO126, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and PP2 were obtained from Calbiochem. PP1 was obtained from Biomol. All drugs were dissolved in DMSO and the resulting stock solutions 0.2 μmol/L filtered before use. Adenoviruses containing either the β-galactosidase cDNA or a cDNA encoding a kinase inactive c-Src were a generous gift of Dr Bradford C. Berk (University of Rochester, NY) and were prepared as described previously.[12] The adenovirus containing dominant negative Ras (RasN17) was a generous gift of Dr Craig D. Logsdon (University of Michigan, Ann Arbor, Mich) and was amplified in the same manner as c-Src$_{KI}$. Estimates of viral titer were determined using absorbance at 260 nm as described previously.[13]

Results

Laminar Shear Stress Causes an Early Increase in eNOS mRNA Transcription and a Sustained Stabilization of eNOS mRNA

Six hours of laminar shear stress resulted in a 4- to 5-fold increase in eNOS mRNA (Figure 1A). Steady state levels of mRNA are dependent on both the rate of mRNA transcription and decay. To determine if endogenous eNOS gene transcription was increased by shear, cells were sheared for 0 to 6 hours and transcriptional rate was measured using nuclear run-on analyses. At only 1 hour of shear was an increase in eNOS mRNA transcription rate appreciable (Figure 1B); after this, eNOS transcription rate was similar between sheared and nonsheared cells (Figure 1B).

To examine the effect of shear on eNOS mRNA stability, cells were either exposed to shear or static conditions for 6 hours. After this, the RNA polymerase inhibitor DRB was added to the media and cells harvested for Northern analysis at the times indicated. In unsheared cells, the eNOS mRNA half-life was approximately 5 hours. This value is similar to that previously reported.[11] In contrast, laminar shear stress increases the eNOS mRNA half-life by more than 3-fold (Figure 1C).

Role of c-Src in Induction of eNOS mRNA by Shear

Shear stress has been shown to activate the tyrosine kinase c-Src within seconds in a calcium-dependent fashion.[12] Because of its role in signaling and mechanical force transduc-

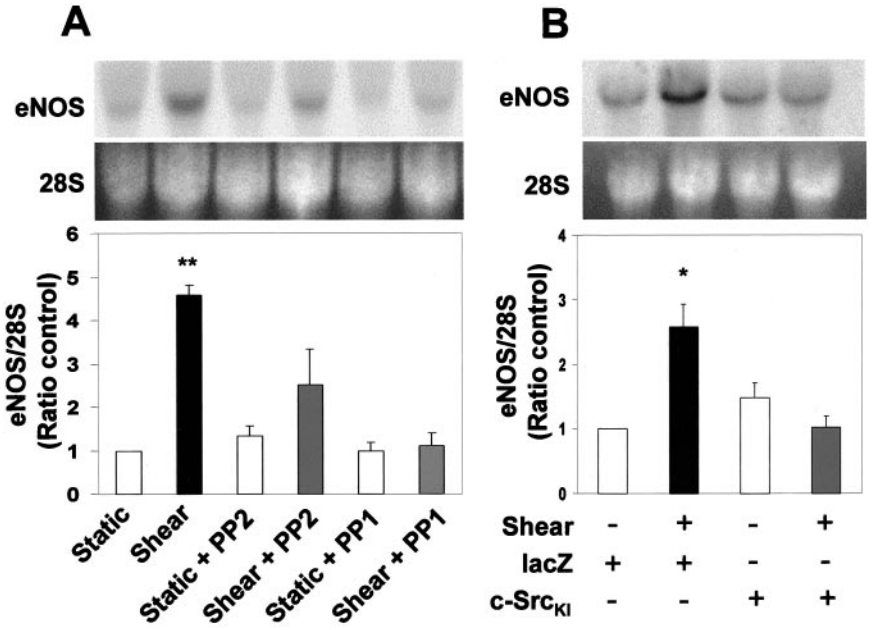

Figure 2. Northern analysis showing the effect of c-Src inhibition on shear-induced eNOS mRNA expression in BAECs. Cells were pretreated with media alone, PP2 (10 μmol/L), and PP1 (10 μmol/L) for 1 hour (A) or pretreated with adenoviruses encoding either lacZ or c-Src$_{KI}$ (50 PFU/cell for 48 hours) to inhibit endogenous c-Src (B). Cells were then exposed to 15 dynes/cm^2 of laminar shear stress for 6 hours. Top panels, Representative blots for eNOS mRNA; middle panels, corresponding 28S bands; bottom panels, grouped densitometric data (mean±SEM) from 4 separate experiments. Asterisks indicate significant difference from shear alone (*$P<0.05$, **$P<0.01$, Dunnett's test after 1-way ANOVA).

tion, we examined the importance of c-Src in induction of eNOS mRNA expression by shear stress. Pretreatment of BAECs for 1 hour with the Src family inhibitors PP1 (10 μmol/L) and PP2 (10 μmol/L) had no effect on baseline eNOS mRNA levels. In contrast, PP2 significantly attenuated and PP1 completely abolished the increase in eNOS mRNA caused by shear (Figure 2A).

To further address the role of c-Src in modulation of eNOS expression in response to shear, adenoviruses were used to overexpress either β-galactosidase (β-gal) or a kinase inactive c-Src (c-Src$_{KI}$). For these studies, the virus was diluted (50 plaque-forming unit (PFU)/cell) in serum-free medium and exposed to cells for 2 hours. Serum was then added to achieve a final concentration of 5% and experiments performed 46 hours later. BAECs treated with β-gal responded to shear by increasing eNOS mRNA by 2.6-fold. In contrast, cells infected with c-Src$_{KI}$ had no increase in eNOS mRNA in response to shear (Figure 2B). Lower concentrations of the c-Src$_{KI}$ also inhibited eNOS expression caused by shear, albeit to a lesser extent (data not shown).

Role of c-Src Activation of Raf

It has been shown that c-Src can activate the Raf cascade in other systems.[14] We therefore considered the possibility that Raf may be activated by c-Src in response to shear in endothelial cells. Cells were pretreated with either PP1 (10 μmol/L) or media alone for 1 hour and sheared for 15 minutes. Pretreatment with PP1 had no effect on the basal phosphorylation of Raf but completely prevented the shear-induced phosphorylation of Raf to below baseline levels (Figure 3A).

Role of Ras in eNOS mRNA Induction by Shear

In many cases, Raf and Ras are both required to activate downstream components such as MEK and ERK1/2.[15] Because c-Src inhibition prevented Raf phosphorylation, we hypothesized a role for Ras in eNOS mRNA induction by shear. To test this hypothesis, cells were treated with adenoviruses containing either β-gal or dominant negative Ras (RasN17) in a similar fashion as the c-Src$_{KI}$ experiments. BAECs treated with the β-gal adenovirus responded to 6 hours of laminar shear by increasing eNOS mRNA levels about 3.2-fold. Treatment of cells with RasN17, however, completely prevented the increase in eNOS mRNA in response to shear at this early time point (Figure 3B).

Role of ERK1/2 Activation in Regulation of eNOS mRNA Expression

As reported previously,[16] we found in preliminary experiments that shear stress resulted in a rapid phosphorylation of ERK1/2. This occurred as early as 1 minute after onset of shear and peaked at 15 minutes (data not shown). To determine if ERK1/2 activation by shear was dependent on c-Src, cells were pretreated for 1 hour with PP1 (10 μmol/L) and then exposed to shear for 15 minutes. Treatment with PP1 abolished the shear-induced phosphorylation of ERK1/2 but had no effect on basal phosphorylation of ERK1/2 or total levels of ERK1/2 (Figure 4A). Further, pretreatment of BAECs for 1 hour with the RasN17 adenovirus resulted in a complete inhibition of ERK1/2 phosphorylation whereas treatment with the lacZ virus did not (Figure 4B).

Because our data indicate a role of c-Src and Ras/Raf in both eNOS mRNA upregulation and ERK1/2 phosphorylation by shear stress, we examined the role of ERK1/2 activation in regulation of eNOS mRNA. BAECs were pretreated with either PD98059 or UO126 for 1 hour and were then exposed to shear stress for 6 hours. At this early time point, both PD98059 and UO126 completely abolished the upregulation of eNOS mRNA in response to shear stress without affecting the basal levels (Figure 4C).

Numerous other downstream targets of c-Src have been described that could potentially mediate the effect of laminar shear on eNOS expression. These include p38 MAPK,[17] various isoforms of protein kinase C, and PI-3 kinase.[18] In

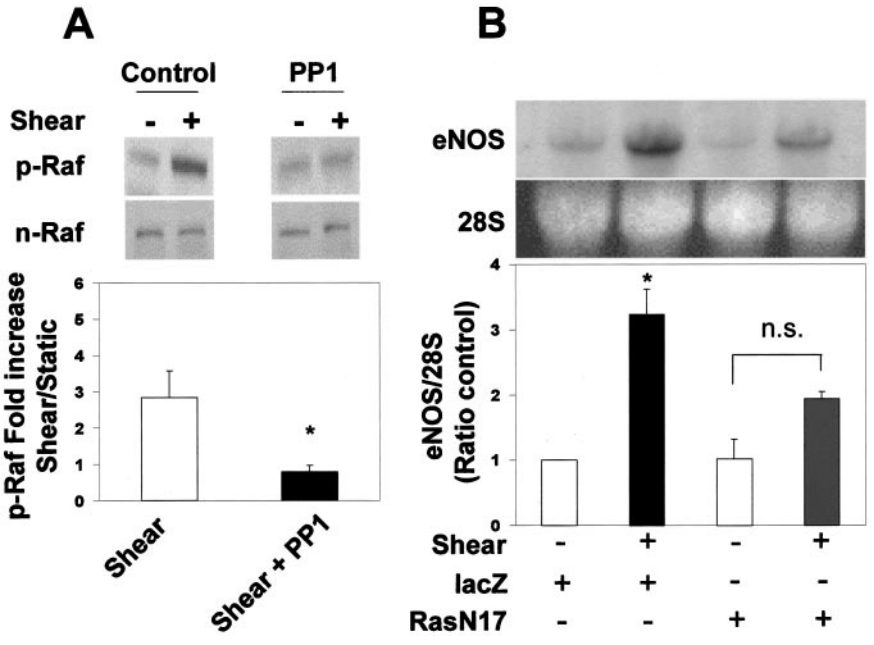

Figure 3. Western and Northern analysis showing the effect on Raf phosphorylation and eNOS mRNA expression by shear stress. A, Cells were pretreated with media alone or PP1 (10 μmol/L) for 1 hour and either held static or exposed to 15 dynes/cm^2 of laminar shear for 15 minutes. Cells were harvested in 1× sample buffer and ≈15 μg were loaded in a 12.5% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies for phospho-specific Raf (A, top) or total Raf (A, middle). Bottom, Grouped densitometric (mean±SEM) data from 4 separate experiments. Asterisks indicate significant differences from shear alone (*$P<0.05$, Dunnett's test after 1-way ANOVA). B, Cells were pretreated with lacZ or RasN17 (50 PFU/cell for 48 hours) to inhibit endogenous Ras and then exposed to 15 dynes/cm^2 of laminar shear stress for 6 hours. Top, Representative blots for eNOS mRNA; middle, corresponding 28S bands; bottom, grouped densitometric data (mean±SEM) from 4 separate experiments. Asterisks indicate significant difference from shear alone (*$P<0.05$, Dunnett's test after 1-way ANOVA).

preliminary experiments, we found that specific inhibition of these pathways with Wortmanin (100 nmol/L, to inhibit PI-3 kinase), SB202190 (10 μmol/L, to inhibit p38 MAP kinase), and GF109203X (1 μmol/L, to inhibit protein kinase C) had no effect on either the phosphorylation status of ERK1/2 or eNOS mRNA expression (data not shown).

Role of c-Src/Ras/ERK Pathway in Regulation of eNOS Transcription

Our data have shown that eNOS mRNA upregulation by laminar shear stress is in part due to a transient increase in transcription rate. Activation of ERK may result in activation of several transcription factors including SP-1[19] and AP-1,[20] for which numerous binding sites are found in the eNOS promoter.[21] To determine if c-Src, Ras, and ERK1/2 regulate eNOS transcription, nuclear run-on analysis was used. Pretreatment of BAECs with the c-Src inhibitor PP1 for 1 hour completely prevented the increase in transcription rate caused by 1 hour of laminar shear stress (Figure 5A). In contrast, pretreatment the lacZ virus did not inhibit the increase in eNOS transcription rate (2.5-fold). In addition, exposure of cells to RasN17 adenovirus completely inhibited the tran-

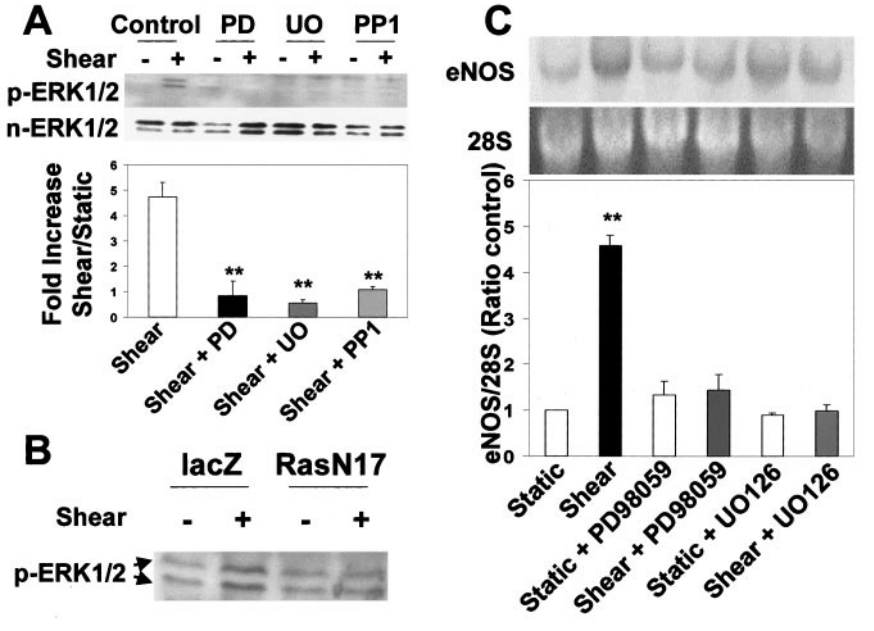

Figure 4. Western and Northern analysis illustrating the effect of ERK1/2 phosphorylation inhibition on shear-induced eNOS mRNA expression in BAECs. A, Cells were pretreated with adenoviruses encoding either lacZ or RasN17 (50 PFU/cell for 48 hours) and exposed to 15 dynes/cm^2 for 15 minutes. Western analysis was performed using a phospho-ERK1/2 specific antibody and the representative blot from 4 separate experiments is shown. B, Cells were pretreated with media alone, PD98059 (PD, 50 μmol/L), UO126 (UO, 50 μmol/L), or PP1 (10 μmol/L) for 1 hour and either held static or exposed to 15 dynes/cm^2 of laminar shear for 15 minutes. Western analysis was performed using antibodies for phospho-specific ERK1/2 (B, top) and total ERK1/2 (B, middle). Bottom, Grouped densitometric (mean±SEM) data from 4 separate experiments. Asterisks indicate significant differences from shear alone (**$P<0.01$, Dunnett's test after 1-way ANOVA). C, Cells were pretreated with media alone, PD98059 (50 μmol/L), or UO126 (50 μmol/L) for 1 hour and then exposed to 15 dynes/cm^2 of laminar shear stress for 6 hours. Top, Representative Northern blot for eNOS mRNA; middle, corresponding 28S bands; bottom, grouped densitometric data (mean±SEM) from 4 separate experiments. Asterisks indicate significant differences from shear alone (**$P<0.01$, Dunnett's test after 1-way ANOVA).

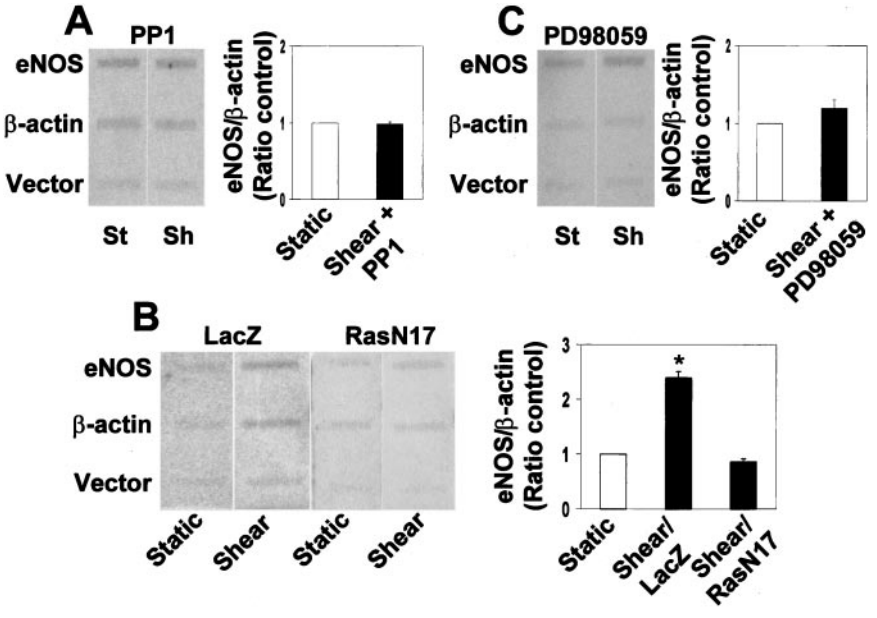

Figure 5. Role of c-Src, Ras, and ERK1/2 in regulation of eNOS transcription by shear. Cells were pretreated with either PP1 (10 μmol/L for 1 hour, A), adenoviruses encoding either lacZ or RasN17 (50 PFU/cell for 48 hours, B), or PD98059 (50 μmol/L for 1 hour, C) and exposed to 15 dynes/cm^2 for 1 hour. Left panels, Representative nuclear run-on experiments; right panels, grouped densitometric (mean±SEM) data from 4 separate experiments. Asterisks indicate significant differences compared with shear alone (*$P<0.05$, Dunnett's test after 1-way ANOVA)

scriptional increase caused by 1 hour of shear (Figure 5B). Finally, pretreatment of BAECs with PD98059, the MEK1/2 inhibitor completely inhibited eNOS transcription in response to shear stress (Figure 5C). These data indicate that the c-Src/Ras/ERK pathway modulates eNOS mRNA by increasing the rate of transcription of the gene.

Role of c-Src/Ras/ERK Pathway in Regulation of eNOS mRNA Stability

Our data suggest that a major component of the steady state upregulation of eNOS mRNA by shear stress is the result of prolonged mRNA stabilization. To determine if the pathway that modulates eNOS transcription in response to shear also affects mRNA stability, additional DRB-chase studies were performed. Pretreatment of endothelial cells with the c-Src inhibitor PP1 significantly attenuated the response of shear on eNOS mRNA half-life reducing it from >18 hours to under 10 hours, compared with 6 to 7 hours in static cells (Figure 6A). In contrast, pretreatment of BAECs with either the MEK1/2 inhibitor PD98059 or the RasN17 adenovirus had no effect on eNOS mRNA stabilization by shear stress (Figures 6B and 6C).

Effect of Long-Term Shear on eNOS Expression During c-Src and MEK1/2 Inhibition

The pervious data suggest that shear activates eNOS transcription transiently, and that this involves a pathway involving Raf/Ras/MEK/ERK but this pathway is not involved in stabilization of the eNOS mRNA. We hypothesized that prolonged shear, in excess of the normal eNOS half-life, would increase steady state levels of eNOS even in the presence of MEK1/2 inhibition. As shown in Figure 7, eNOS mRNA levels were increased 2.6- and 3.8-fold in cells

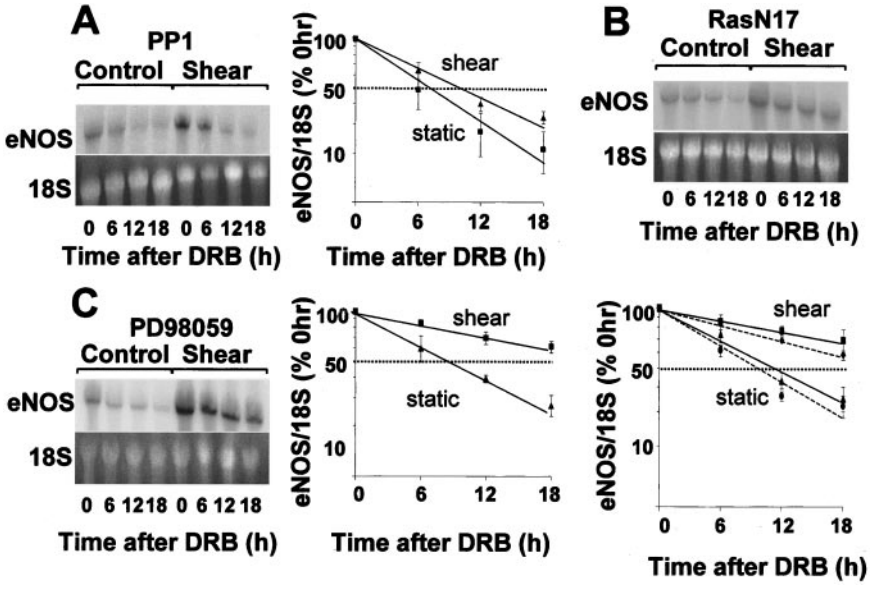

Figure 6. DRB-chase experiments examining the role of c-Src, Ras, and ERK1/2 on eNOS mRNA half-life in response to shear. BAECs were pretreated with either PP1 (10 μmol/L, 1hour, A), PD98059 (50 μmol/L, 1 hour, B), or adenoviruses encoding lacZ or RasN17 (50 PFU/cell, 48 hours, C). The cells were then exposed to no shear or sheared at 15 dynes/cm^2 for 6 hours before the addition on DRB (60 μmol/L). Cells were then harvested at the time points indicated and Northern analyses performed to determine the rate of eNOS mRNA decay in each group. Shown are representative blots for eNOS mRNA, the corresponding 18S bands, and grouped densitometric data (mean±SEM) from 4 separate experiments. The dotted lines (C) indicate lacZ mRNA levels.

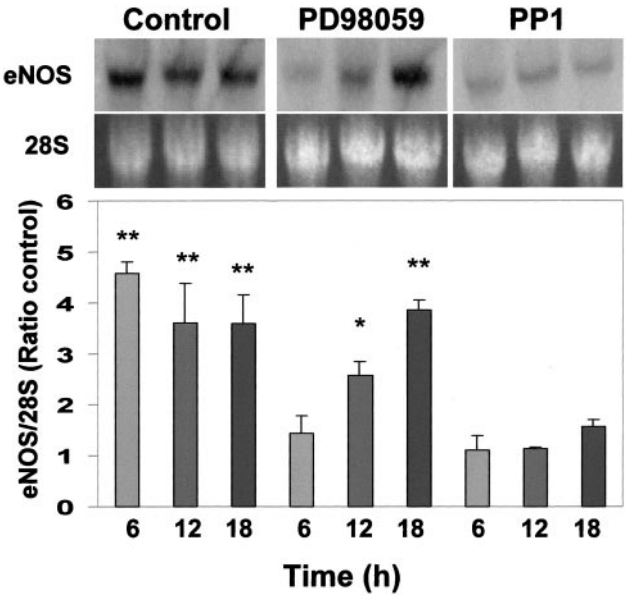

Figure 7. Northern analysis illustrating the differences between short- and long-term shear on eNOS mRNA expression. BAECs were pretreated with media alone, PP1 (10 μmol/L), or PD98059 (50 μmol/L) for 1 hour and exposed to 15 dynes/cm^2 for 6, 12, or 18 hours. Top, Representative Northern blots for eNOS; middle, corresponding 28S bands; bottom, grouped densitometric data (mean±SEM) from 3 separate experiments. Asterisks indicate significant differences from static control (*$P<0.05$, **$P<0.01$, Dunnett's test after 1-way ANOVA).

exposed to shear stress for 12 or 18 hours, respectively, even in the presence of PD98059. In contrast, cells exposed to 12 or 18 hours of shear in the presence of PP1 had no change in eNOS mRNA levels.

Discussion

Although it well established that shear stress upregulates eNOS mRNA and protein expression, the signaling pathways that mediate this response have for the most part remained unidentified. In this study, we identified two pathways involving c-Src that lead to a transient increase in eNOS mRNA transcription and prolongation of the eNOS mRNA half-life. The increase in mRNA transcription is dependent on a pathway involving Raf, Ras, and ERK1/2, whereas the stabilization of eNOS mRNA is independent of these. Thus, c-Src leads to increased eNOS expression in response to shear via 2 divergent pathways, one leading to a transient increased eNOS expression and a second leading to a prolonged stabilization of eNOS message.

Shear stress has been shown to increase transcription of several genes. Many of these genes contain a shear stress response element (SSRE), with the core sequence GAGACC.[22] Several such elements are found in the bovine and human eNOS promoter. Of note, as we found in the case of eNOS, the increase in transcription of several other genes in response to shear is very transient.[23,24] The prolongation of the eNOS mRNA stability in response to shear therefore seems critical in allowing sustained expression of eNOS in response to shear stress. In many ways, the combination of an early increase in transcription and a prolongation of mRNA stability seems analogous to a loading dose, followed by a maintenance dose of a medication to obtain rapid and sustained levels of a drug. Increased levels of a drug can ultimately be achieved without a loading dose if treatment is sustained for a sufficient length of time. Indeed, in the case of eNOS, we found that prolonged shear stress (18 hours) increased its mRNA levels even when the pathway leading to increased transcription was blocked. On first inspection, these two pathways may seem redundant, but one should not discount the importance of the early increase in transcription. For example, brief episodes of exercise may predominantly increase eNOS expression via the rapid, transcription-mediated pathway.

One of the earliest signaling events in response to laminar shear in the endothelium is activation of tyrosine kinases.[25] The tyrosine kinase c-Src is activated within seconds after onset of shear and in turn causes phosphorylation of the tyrosine kinase Flk-1.[26] Our data strongly implicate c-Src as a mediator of eNOS expression in response to shear stress. Both c-Src inhibitors PP1 and PP2 markedly attenuated the increase in eNOS mRNA in response to shear. Further, treatment with an adenovirus encoding kinase inactive c-Src completely prevented the effect of shear on eNOS expression, without affecting basal levels of eNOS mRNA. Our data do not exclude a role for inhibition of c-Src dephosphorylation. Indeed, shear stress has been shown to alter the endothelial cell redox state and several lines of evidence suggest that redox stress can inhibit tyrosine phosphatases,[27] which could in turn augment the effect of c-Src activation. In prior studies, we could not prevent the increase in eNOS expression in response to shear using antioxidants.[9]

The current experiments strongly suggest a role for Raf and ERK1/2 as being downstream of c-Src in modulation of eNOS mRNA transcription by shear stress. This is similar to many previously described pathways where c-Src has been shown to activate the Raf/MEK/ERK cascade.[25,28,29] Inhibition of MEK1/2, the upstream activator of ERK1/2, with PD98059[30] and UO126[31] completely blocked the shear-induced eNOS mRNA upregulation at 6 hours. We also demonstrated that Raf phosphorylation, a critical step in this process, was prevented by c-Src inhibition with PP1. This pathway, and ERK1/2 in particular, may represent the final component activated by laminar shear stress leading to an increase in eNOS transcription.

Of note, it has been shown that PD98059 and UO126 may also inhibit MEK5 activation in response to epidermal growth factor.[32] Further, it has been shown that shear stress can activate MEK5/ERK5 in endothelial cells.[33] In order to exclude a role of MEK5/ERK5 in shear induction of eNOS mRNA, cells were treated with a dominant negative MEK5 adenovirus (DNMEK5A, a generous gift of Dr J.D. Lee). Of note, there was a slight increase in basal phosphorylation of ERK5 in the DNMEK5A treated cells. This increase in basal phosphorylation of ERK5 was not associated with an increase in basal eNOS message. Further, the DNMEK5A completely blocked phosphorylation of ERK5 in response to shear. In fact, shear caused a slight decrease in ERK5 phosphorylation in these cells. In spite of this, shear still produced a 2.5-fold increase in eNOS mRNA in DNMEK5A-treated cells that

was identical to that observed in control cells infected with only a lacZ virus (data not shown). In addition, shear stress–induced increase in ERK5 phosphorylation has been shown to be insensitive to c-Src inhibition using both PP1 and $c\text{-}Src_{KI}$.[33] Given these results, it is very unlikely that MEK5/ERK5 play a role in regulation of eNOS transcription in response to shear stress.

From our studies, it also appears likely that c-Src activates the MAPK pathway at the Ras/Raf level, and not via cross talk from other pathways. In preliminary studies, we found that inhibitors of p38 MAPK, JNK, and PI-3 kinase had no effect on phosphorylation status of ERK1/2 or eNOS mRNA expression (data not shown). Interestingly, PI-3 kinase has been shown to be important in acute second-to-second regulation of eNOS activity by shear in a phosphorylation-dependent manner.[34] Our data, however, suggest that this pathway does not modulate the long-term expression of eNOS mRNA in response to shear.

The mechanisms whereby shear and c-Src modulates eNOS mRNA transcription and stability will require substantial additional study. In preliminary studies, we have found that shear stress increases expression of a chimeric eNOS promoter/chloramphenicol acetyltransferase reporter construct transfected into BAECs, and that this is completely inhibited by PP1, PD98059, and UO126. This confirms our nuclear run-on analyses and directly demonstrates that shear activates the eNOS promoter in a c-Src- and MEK-dependent fashion. Regulation of mRNA stability is very complex and often involves mRNA/protein interactions. Recently, we have identified 2 cytosolic proteins that bind to the eNOS mRNA 3′untraslated region that act as destabilizing proteins.[8,35] In preliminary studies, it does not appear that binding of either of these is regulated by shear stress. Previous reports have described a role of the SH3 domain of c-Src in recruiting mRNA-binding proteins.[36–38] However, it is also conceivable that phosphorylation of an mRNA binding protein by c-Src or an upstream effector might alter its activity. Furthermore, it is also possible that this c-Src might modulate expression of relevant RNA binding proteins.

In summary, these studies have identified a crucial role of c-Src in regulation of eNOS expression in response to shear stress. Activation of c-Src seems to lay upstream of 2 divergent pathways, one leading to a transient increase in eNOS transcription and a second leading to prolonged stabilization of eNOS mRNA. These pathways seem complimentary in that they allow for both short- and long-term changes in eNOS mRNA expression. Finally, these data stress the importance of the tyrosine kinase c-Src as a modulator of unidirectional laminar shear stress in the endothelium.

Acknowledgments

The study was supported by NIH grants HL390006 and HL59248, NIH Program Project Grant HL58000, and a Department of Veterans Affairs Merit Grant.

References

1. Harrison DG, Venema RC, Arnal JF, Inoue N, Ohara Y, Sayegh H, Murphy TJ. The endothelial cell nitric oxide synthase: is it really constitutively expressed? *Agents Actions Suppl*. 1995;45:107–117.
2. Ku DN, Giddens DP, Zarins CK, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis*. 1985; 5:293–302.
3. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res*. 1994;74: 349–353.
4. Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol*. 1998;18:677–685.
5. Ishida T, Takahashi M, Corson MA, Berk BC. Fluid shear stress-mediated signal transduction: how do endothelial cells transduce mechanical force into biological responses? *Ann N Y Acad Sci*. 1997;811: 12–23; discussion 23–24.
6. Malek AM, Jiang L, Lee I, Sessa WC, Izumo S, Alper SL. Induction of nitric oxide synthase mRNA by shear stress requires intracellular calcium and G-protein signals and is modulated by PI 3 kinase. *Biochem Biophys Res Commun*. 1999;254:231–242.
7. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation*. 1998;97: 1129–1135.
8. Searles CD, Miwa Y, Harrison DG, Ramasamy S. Posttranscriptional regulation of endothelial nitric oxide synthase during cell growth. *Circ Res*. 1999;85:588–595.
9. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000;86:347–354.
10. Dewey CF, Jr, Bussolari SR, Gimbrone MA, Jr, Davies PF. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng*. 1981;103:177–185.
11. Ramasamy S, Parthasarathy S, Harrison DG. Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J Lipid Res*. 1998;39:268–276.
12. Okuda M, Takahashi M, Suero J, Murry CE, Traub O, Kawakatsu H, Berk BC. Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent c-Src activation. *J Biol Chem*. 1999;274: 26803–26809.
13. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A*. 1998;95:2509–2514.
14. Morrison DK. Mechanisms regulating Raf-1 activity in signal transduction pathways. *Mol Reprod Dev*. 1995;42:507–514.
15. Moodie SA, Willumsen BM, Weber MJ, Wolfman A. Complexes of Ras. GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science*. 1993;260:1658–1661.
16. Tseng H, Peterson TE, Berk BC. Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ Res*. 1995;77:869–878.
17. Nagao M, Yamauchi J, Kaziro Y, Itoh H. Involvement of protein kinase C and Src family tyrosine kinase in Galphaq/11-induced activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. *J Biol Chem*. 1998;273:22892–22898.
18. Daulhac L, Kowalski-Chauvel A, Pradayrol L, Vaysse N, Seva C. Gastrin stimulates the formation of a p60Src/p125FAK complex upstream of the phosphatidylinositol 3-kinase signaling pathway. *FEBS Lett*. 1999;445: 251–255.
19. Merchant JL, Du M, Todisco A. Sp1 phosphorylation by Erk 2 stimulates DNA binding. *Biochem Biophys Res Commun*. 1999;254:454–461.
20. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med*. 1996;74:589–607.
21. Robinson LJ, Weremowicz S, Morton CC, Michel T. Isolation and chromosomal localization of the human endothelial nitric oxide synthase (NOS3) gene. *Genomics*. 1994;19:350–357.
22. Resnick N, Yahav H, Khachigian LM, Collins T, Anderson KR, Dewey FC, Gimbrone MA Jr. Endothelial gene regulation by laminar shear stress. *Adv Exp Med Biol*. 1997;430:155–164.
23. Hsieh HJ, Li NQ, Frangos JA. Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am J Physiol*. 1991;260: H642–H646.
24. Hsieh HJ, Li NQ, Frangos JA. Pulsatile and steady flow induces c-fos expression in human endothelial cells. *J Cell Physiol*. 1993;154:143–151.
25. Takahashi M, Ishida T, Traub O, Corson MA, Berk BC. Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res*. 1997;34:212–219.

26. Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, Shyy JY. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J Biol Chem*. 1999;274:18393–18400.
27. Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol*. 2000;20:1430–1442.
28. Takahashi M, Berk BC. Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells: essential role for a herbimycin-sensitive kinase. *J Clin Invest*. 1996;98:2623–2631.
29. Traub O, Monia BP, Dean NM, Berk BC. PKC-epsilon is required for mechano-sensitive activation of ERK1/2 in endothelial cells. *J Biol Chem*. 1997;272:31251–31257.
30. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem*. 1995;270:27489–27494.
31. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem*. 1998;273:18623–18632.
32. Kamakura S, Moriguchi T, Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases: identification and characterization of a signaling pathway to the nucleus. *J Biol Chem*. 1999;274:26563–26571.
33. Yan C, Takahashi M, Okuda M, Lee JD, Berk BC. Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. *J Biol Chem*. 1999;274:143–150.
34. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.
35. Miwa Y, Kanazawa K, Harrison DG. Post-transcriptional regulation of the endothelial nitric oxide synthase by the RNA binding protein monsder II: role of the cytoskeleton. *Circulation*. 2000;102:II-110. Abstract.
36. Weng Z, Thomas SM, Rickles RJ, Taylor JA, Brauer AW, Seidel-Dugan C, Michael WM, Dreyfuss G, Brugge JS. Identification of Src, Fyn, and Lyn SH3-binding proteins: implications for a function of SH3 domains. *Mol Cell Biol*. 1994;14:4509–4521.
37. Taylor SJ, Shalloway D. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. *Nature*. 1994;368:867–871.
38. Fumagalli S, Totty NF, Hsuan JJ, Courtneidge SA. A target for Src in mitosis. *Nature*. 1994;368:871–874.