Nitric Oxide Synthase Expression in Endothelial Cells Exposed to Mechanical Forces

Thierry Ziegler, Paolo Silacci, Vanessa J. Harrison, Daniel Hayoz

Abstract—Nitric oxide (NO) has been demonstrated to play a central role in vascular biology and pathobiology. The expression of endothelial NO synthase (eNOS) is regulated in part by blood flow–induced mechanical factors. The purpose of this study was to evaluate how the expression of eNOS mRNA correlates with the activation of its promoter in both arterial and venous endothelial cells (ECs) exposed to mechanical forces, ie, shear stress and cyclic circumferential stretch. Bovine aortic ECs (BAECs) and EA hy.926, a cell line derived from human umbilical vein ECs, were grown on the inside of elastic tubes and subjected to combinations of pressure, pulsatile shear stress, and cyclic circumferential stretch for 24 hours. Two patterns of shear stress were used: unidirectional (mean of 6, ranging from 3 to 9 dyne/cm²) and oscillatory (mean of 0.3, ranging from −3 to +3 dyne/cm²). The expression of eNOS mRNA was quantified by Northern blot analysis. Activation of the promoter was assessed by luciferase activity after the cells were transiently transfected before the flow experiments with a plasmid construct containing the fully functional eNOS promoter coupled to a luciferase reporter gene. Expression of eNOS mRNA was increased and promoter activity was enhanced by unidirectional shear stress compared with static control. Oscillatory shear slightly upregulated eNOS mRNA in BAECs, whereas it downregulated eNOS mRNA in EA hy.926. In both BAECs and EA hy.926, there was a good correlation between the increase in eNOS mRNA expression and promoter activation by unidirectional shear stress. In contrast, in both BAECs and EA hy.926 cells exposed to shear stress, cyclic stretch did not change eNOS mRNA expression, but the activation of eNOS promoter was significantly lower. Moreover, when ECs were exposed to oscillatory shear stress, there was a dramatic activation of the eNOS promoter. These results demonstrate that unidirectional shear stress increases eNOS mRNA expression via a transcriptional mechanism. However, oscillatory shear stress and cyclic stretch appear to control eNOS expression through posttranscriptional regulatory events.

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Key Words: atherosclerosis • endothelium • stress, mechanical • nitric oxide synthase • promoters

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Nitric oxide is the end product of the conversion of L-arginine to L-citrulline by an enzyme called NO synthase. Vascular ECs are known to constitutively express 1 of the 3 isoforms of NO synthase called eNOS. NO has been demonstrated to interfere with key events involved in atherogenesis. Not only is NO the most potent vasodilator, it also inhibits platelet aggregation and leukocyte adhesion to ECs and suppresses vascular smooth muscle cell proliferation and migration. The production of NO, as well as the expression of eNOS by ECs, has been shown to be dependent on mechanical factors. Shear stress and cyclic circumferential stretch have been demonstrated to increase NO release as well as upregulate eNOS mRNA and protein. More recently, using an in vitro tube model in which pressure, shear stress, and cyclic circumferential stretch can be combined, we have shown that shear stress represents the major mechanical factor inducing an increase in eNOS expression in BAECs. Pressure and cyclic stretch, however, did not significantly alter changes in eNOS expression in cells exposed to shear stress.

A functional sequence of the human eNOS that confers the basal transcription of eNOS in BAECs has already been cloned and studied. The promoter fragment contains 1600 bp with many putative sites for binding of transcriptional factors. By using deletion-promoter constructs, it was observed that eNOS basal transcription was positively modulated by sequences ranging from −1033 to −779 and from −494 to −166 bp. The role of the latter sequence in eNOS basal transcription was further characterized by mutation analysis and was found to contain the primordial consensus site for Sp1.

Several risk factors for atherosclerosis, such as hypercholesterolemia, diabetes, hypertension, and smoking, have been associated with impaired arterial vasodilatation, caused by reduced NO bioavailability. Endothelial dysfunction commonly observed during atherosclerosis may be due in part to either a decreased NO production or an increased scavenging of extracellular NO by free oxygen species. These mechanisms and the correlation between atherogenesis and perturbed mechanical environment, ie, oscillatory shear stress,
are not fully understood. We have recently shown that unlike unidirectional shear stress, oscillatory shear stress did not induce any change in eNOS expression compared with static culture. 4

The purpose of this study was to assess activation of the above-mentioned eNOS promoter in 2 types of endothelial cell: BAECs and EA hy.926, a hybridoma cell line created by fusing primary HUVECs with the human carcinoma cell line, A549. This hybridoma cell line has been shown to preserve many features of HUVECs. 5,6 These 2 cell types were exposed to 2 different mechanical environments, unidirectional and oscillatory, for 24 hours.

Methods

Cell Culture

BAECs were isolated from aorta obtained from the local slaughterhouse and grown in DMEM (Gibco) supplemented with 10% FBS (Seromed), 100 μg/mL penicillin-streptomycin (Gibco), and 2 mmol/L glutamine (Gibco). EA hy.926 was obtained from Dr Edgell (University of North Carolina) and cultured in DMEM with 4.5 g/L glucose, 10% FBS, penicillin-streptomycin, glutamine, and 50 mmol/L HEPES.

BAECs and EA hy.926 were grown in 6-well plates to 60% confluence. At this point, they were transfected with plasmid constructs containing the functional promoter for eNOS provided by Dr W.C. Sessa (Yale University). The construction of this plasmid is described elsewhere. 4 Briefly, this plasmid was made by inserting the promoter fragment into a vector containing the luciferase reporter gene (pGL2, Promega). The cells were rinsed twice with Optimem-I (Invitrogen) and grown in DMEM with 5% CO2/95% air to keep a constant pH of 7.2.

Northern Blot Analysis

For Northern blot analysis, the cells were left overnight to recover. The cells from the two 6-well plates were trypsinized and seeded at a density of 90 000 cells/cm2. The cell suspension obtained was centrifuged for 10 minutes at 800 g. The pellets were resuspended in 3 mL of a trypsin-EDTA solution (Gibco). After 3 minutes, the cell suspension was washed once with PBS and resuspended in 5 mL of a trypsin-EDTA solution. The final cell suspension was used to prepare nuclei for Northern blot analysis. At the end of the experiment, the fittings were quickly dismounted. The tubes were then rinsed once with PBS and filled with 3 mL of a trypsin-EDTA solution (Gibco). After 3 minutes, the cells were completely detached by gentle tapping of the tubes, and the cell suspension obtained was centrifuged for 10 minutes at 800 g at 4 °C. The cell pellet was lysed in a buffer containing guanidinium isothiocyanate, and total RNA was isolated using a kit (RNeasy, Qiagen). Total RNA (5 μg) was loaded into wells and separated according to its size in a 1% agarose/6% formaldehyde gel. RNA was transferred overnight by capillarity to a nylon membrane (Hybond-N, Amersham). The membranes were prehybridized at 42 °C for a minimum of 1 hour in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll, 0.05 mol/L Tris (pH 7.5), 1.0 mol/L NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA (Boehringer Mannheim). Hybridization was carried out in the same solution containing 32P-random prime-labeled cDNA (Boehringer Mannheim) specific for human eNOS (a gift of Dr Quetermous, Harvard Medical School), bovine eNOS (a gift of Dr D.G. Harrison, Emory University), and human GADPH (Clonetech). After overnight incubation, the membranes were washed in 2× SSC/0.1% SDS at room temperature for 30 minutes and in 0.1× SSC/0.1% or 0.5% SSC/0.1% SDS (GAPDH) at 60 °C for 1 hour and then exposed to x-ray film (Kodak X-O Mat) at 80 °C. Transcript levels were quantified using an electronic autoradiography apparatus (Instant Image, Packard) or by scanning the x-ray film (Apple OneScanner) followed by densitometric analysis (NIH Image, NIH).

Results

Northern Blot Analysis

EA hy.926 and BAECs were subjected to unidirectional and oscillatory flow for 24 hours. Total RNA was analyzed for the expression of eNOS (Figures 1 and 2). Both BAECs and EA hy.926 showed upregulated eNOS mRNA when subjected to increased levels of unidirectional shear stress. Expression was dose-dependent on the level of shear stress and was significantly higher at 6 dyne/cm2 than at 0.3 dyne/cm2 and at no shear (static control). It is interesting to note that at the same shear stress level, the response of BAECs was greater than that of EA hy.926. When a 4% cyclic stretch was combined with the different levels of shear stress, a slight increase in...
eNOS mRNA expression was observed for both cell types. However, this increase was not statistically significant for either cell type. Oscillatory shear stress slightly upregulated eNOS mRNA expression in BAECs but downregulated expression in EA hy.926 compared with the static control. These effects, however, were not statistically significant. When compared with unidirectional shear stress level having the same mean value, oscillatory shear stress significantly downregulated eNOS expression in both BAECs and EA hy.926.

**eNOS Promoter Analysis**

The activation of the eNOS promoter was assessed by measuring luciferase activity in BAECs and EA hy.926 transiently transfected with a sequence coding for the functional human eNOS promoter coupled to a luciferase reporter gene and subsequently exposed to unidirectional and oscillatory flow for 24 hours. The trend of activation of the eNOS promoter by mechanical factors was similar in BAECs and EA hy.926. Unidirectional shear stress induced an increase in luciferase activity that was dependent on the level of shear stress (Figures 3 and 4). EA hy.926 seem to be more sensitive to lower shear stress levels than BAECs, but the activation by a shear stress of 6 dyne/cm² was similar in both cell types. Oscillatory shear stress induced a significant 7- to 10-fold higher promoter activation compared with the static control.
and a unidirectional shear stress of 0.3 dyne/cm². A 4% cyclic stretch decreased the expression induced by either low, high, or oscillatory shear stress. This decrease was statistically significant for all conditions in EA hy.926 and for the higher level of unidirectional and oscillatory shear stress in BAECs.

**Discussion**

It has been demonstrated that mechanical forces, i.e., shear stress and cyclic stretch, can regulate the expression of eNOS mRNA and protein. However, little is known about the exact mechanisms of this induction in both arterial and venous ECs. Both shear stress and cyclic stretch were shown to upregulate eNOS in BAECs and HUVECs. Recently, we have shown in our elastic tube model that eNOS mRNA and protein expression in BAECs was not significantly increased by the combination of shear stress, pressure, and cyclic stretch compared with pressure and shear stress. It appeared that shear stress was the major mechanical factor influencing eNOS and that both pressure and cyclic stretch were only secondary when combined to shear stress.

The main findings of this study are that (1) eNOS induction in response to mechanical factors is similar in the cell line EA hy.926 compared with in BAECs; (2) the increase in eNOS mRNA by unidirectional shear stress is associated with an activation of eNOS promoter; (3) cyclic stretch downregulates eNOS promoter activation by shear stress even though it does not result in a decrease in eNOS mRNA; and (4) oscillatory shear stress activates eNOS promoter but does not affect eNOS mRNA.

The cell line EA hy.926 is derived from primary HUVECs and a human carcinoma cell line A549. This cell line has been shown to preserve many cell characteristics of HUVECs (e.g., release of endothelin-1, vascular cell adhesion molecules, and von Willebrand factor), but to our knowledge it has never been assessed for eNOS expression. The results shown here imply that EA hy.926 can be safely used as a model of eNOS expression in ECs, especially under mechanical forces.

The activation of the eNOS promoter by shear stress using a reporter gene assay has never been reported to date. Transcription inhibition studies have already been performed to show that gene transcription is necessary for the shear stress–induced eNOS mRNA increase. Our findings of promoter activation can be explained by the presence of several shear stress responsive sites in this promoter region. It contains one shear stress–responsive element (SSRE), several AP-1, and one nuclear factor-κB sequence. SSRE has been shown to be responsible for platelet-derived growth factor-β activation by shear stress via binding of nuclear factor-κB. Moreover, genes coding for intracellular adhesion molecule, transforming growth factor-β, and tissue plasminogen activator, which were shown to be altered by shear stress, have one or several SSRE sequences in their promoter. AP-1 was shown to mediate shear-induced monocyte chemotactic protein-1 expression. Further characterization of the role of these sites in the shear-induced eNOS expression is underway in our laboratory.

The intriguing results here are the contrasting modulation of the eNOS promoter and mRNA by oscillatory shear stress and cyclic stretch. These opposing results could be explained by the fact that other regulatory elements, which are not present in the plasmid constructions, can regulate transcription of eNOS gene by cyclic stretch and oscillatory shear stress. Elongation may also be differently regulated in the eNOS gene, through the altered binding of a factor to the DNA during transcription. Finally, this could also be due to a posttranscriptional mechanism altering mRNA half-life.

The growth state of ECs has been shown to regulate eNOS mRNA by modulating mRNA half-life. Confluent ECs produced less mRNA than subconfluent cells even though transcription activation was similar. It may be that ECs exposed to cyclic stretch have an increased eNOS mRNA half-life because of an increase in their growth rate. Cyclic stretch was shown to increase EC growth rate. Similarly for ECs exposed to oscillatory shear stress, some posttranscriptional mechanism may induce a degradation of eNOS mRNA, which would counterbalance the increased production. Recently, 2 domains were found within the distal portion of untranslated eNOS mRNA containing sequences that are determinants of mRNA stability. Deletions of these regions resulted in a >18-fold increase in mRNA stability. Moreover, a 53-kDa protein binding to these domains was isolated from BAECs. The binding affinity of this protein was lower in proliferating ECs.

BAECs and EA hy.926 cells were subjected to different mechanical environments to assess eNOS promoter activation and mRNA expression. Unidirectional shear stress was found to activate eNOS promoter and upregulate mRNA. Oscillatory shear stress, found in regions prone to the development of atherosclerosis, activated eNOS promoter but did not upregulate eNOS mRNA. Cyclic circumferential stretch inhibited shear-induced promoter activation but did not downregulate mRNA expression. These data imply that regulation of eNOS expression by mechanical factors occurs by both transcriptional and posttranscriptional mechanisms that still need to be determined.

Booth in hypertension and atherosclerotic plaque formation, the balance between NO and reactive oxygens is affected, resulting in a deleterious increase in oxidative stress. Several observations suggest that this is one of the triggering events in vascular remodeling. Using this new perfusion system, we were able to demonstrate that oscillatory flow decreases eNOS mRNA expression in ECs in the absence of other factors. This decrease in eNOS expression may result in decreased availability of NO and increased oxidative stress. Interestingly, oscillatory flow occurs frequently at the site where atherosclerotic plaques develop. Thus, investigations to determine the mechanisms of eNOS regulation by mechanical forces can be expected to contribute to the understanding of the plaque formation process.

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