Shear Stress Induction of the Endothelial Nitric Oxide Synthase Gene Is Calcium-Dependent But Not Calcium-Activated

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Arterial levels of shear stress (25 dynes/cm^2) can elevate constitutive endothelial nitric oxide synthase (eNOS) gene expression in cultured endothelial cells (Ranjan et al., 1995). By PhosphorImaging of Northern blots, we report that the eNOS/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) ratio in bovine aortic endothelial cells (BAEC) increased by 4.8- and 7.95-fold after 6-hr shear stress exposure of 4 and 25 dynes/cm^2, respectively. Incubation of BAEC with dexamethasone (1 μM) had no effect on shear stress induction of eNOS mRNA. Buffering of intracellular calcium in BAEC with bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′, *N*′-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM) reduced shear stress induction of eNOS mRNA by 70%. Yet, stimulation of BAEC with ionomycin (0.1–1.0 μM) for 6–24 hr to elevate intracellular calcium had no effect on eNOS mRNA. These studies indicated that the shear stress induction of eNOS mRNA was a calcium-dependent, but not calcium-activated, process. Shear stress was a very potent and rapid inducer of the eNOS mRNA, which could not be mimicked with phorbol myristrate acetate or endotoxin. Inhibition of tyrosine kinases with genistein (10 μM) or tyrphostin B46 (10 μM) or inhibition of G-protein signaling with guanosine 5′-*O*-(2-thiodiphosphate) (GDP-βS) (600 μM, 6-hr preincubation) did not block the shear stress elevation of eNOS mRNA. **J. Cell. Physiol. 171:205–211,1997.** © 1997 Wiley-Liss, Inc.

The induction of the constitutive endothelial nitric oxide synthase (eNOS, also cNOS or NOSIII) gene expression by shear stress has been observed by several laboratories using cultured human and bovine endothelial cells (Nishida et al., 1992; Ranjan et al., 1995; Uematsu et al., 1995). Actinomycin D can prevent the elevation of eNOS messenger RNA (mRNA) by shear stress, indicating that new transcription is required (Uematsu et al., 1995). Cyclic strain of cultured endothelial cells can also enhance eNOS expression levels (Awolesi et al., 1995). The chronic exercise of dogs increases the coronary vascular NO production and endothelial NOS gene expression (Sessa et al., 1994) possibly through a hemodynamic mechanism.

The eNOS gene can be induced in endothelial cells by treatment with basic fibroblast growth factor (Kostyk et al., 1995), oxidized low density lipoprotein and lysophosphatidylcholine (Hirata et al., 1995), and protein kinase C (PKC) inhibitors (Ohara et al., 1995). The human eNOS promoter has a CCAAT box, is TATA-less (not uncommon for constitutive genes), and contains two AP-1 sites, five SP-1 (GC rich) sites, a CRE site, and a shear stress response element (SSRE) and NF1 site (Marsden et al., 1993). The SP-1 sites in the NOS promoter have been shown to be functional by gel shift assays and SP-1 binding to the site between −104/−90 in the promoter is critical for over 90% of basal eNOS expression (Wariishi et al., 1995; Teng et al., 1995; Zhang et al., 1995).

From in vitro studies, various DNA promoter sites, DNA binding proteins, and signaling events have been shown to mediate the shear stress induction of endothelial genes in a promoter-specific manner. The SSRE site and nuclear factor kB (NFkB) binding are involved in platelet-derived growth factor (PDGF) B chain induction by shear stress (Khachigian et al., 1995). However, only one of two AP-1 sites, and not the SSRE site, are involved in monocyte chemoattractant protein-1 (MCP-1) promoter activation by shear stress (Shyy et al., 1995). Also, SP-1 sites (with concomitant SP-1 tyrosine phosphorylation) are involved in the transient induction of the tissue factor gene in shear stressed endothelium (Lin et al., 1996). Because NFkB can bind SSRE and the eNOS promoter has an SSRE, we explored the role of dexamethasone in the induction of eNOS. We used PKC activators as a test of the role of the AP-1 site in eNOS expression. We also explored the requirements for intracellular calcium, tyrosine kinase, and G-protein signaling on the induction of eNOS mRNA by shear stress.

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MATERIALS AND METHODS

Cell culture and shear stress exposures

Bovine aortic endothelial cells (BAEC) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated newborn calf serum, 0.30 mg/ml glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (Gibco-BRL Laboratories, Gaithersburg, MD) (10% complete DMEM) as described previously (Ranjan et al., 1995). In one experiment where noted, the glass slides were incubated with 2 mg/cm^2 of human plasma fibronectin (Gibco-BRL Laboratories), rinsed, and dried before seeding the cells. In some experiments, monolayers were incubated with the membrane permeable bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM; 5 μM; Molecular Probes, Eugene, OR) for 30 min to load the cells and allow hydrolysis to the membrane impermeant form of the active calcium chelator. In some experiments, monolayers were preincubated for 6 hr with 600 μM guanosine 5'-*O*-(2-thiodiphosphate) (GDP-β S; Sigma, St. Louis, MO). Ionomycin and the tyrosine kinase inhibitors, genistein and tyrphostin B46, were obtained from CalBiochem (LaJolla, CA).

Confluent monolayers of BAEC (Passage 2–14) were then exposed to a steady laminar shear stress of 4 or 25 dynes/cm^2 in individual, sterile, parallel-plate flow chamber systems with recirculating medium (20 ml of 10% complete DMEM) or maintained in a CO_2 incubator as stationary controls as described previously (Ranjan et al., 1995). The pH and aeration of the perfusion media was controlled using a sterile 5% CO_2-air mixture. Experiments using the various inhibitors and activators were conducted in replicate.

RNA isolation and mRNA analysis

Total RNA was extracted from control and shear stress stimulated endothelial cells using the method of Chomczynski and Sacchi (1987). Briefly, endothelial cell monolayers (15 cm^2) were rinsed with sterile phosphate-buffered saline (PBS), lysed in 1.0 ml of denaturing buffer (4.0 M guanidium isothiocyanate, 0.5 % sarkosyl, 25 mM sodium citrate, and 0.1 M mercaptoethanol), followed by addition of 0.1 ml of 2.0 M sodium acetate (pH 4.0). The mixture was extracted twice with phenol and chloroform/isoamyl alcohol (49:1). RNA was precipitated by addition of an equal volume of isopropyl alcohol and overnight incubation at −20°C followed by centrifugation at 12,000g for 30 min. The RNA pellet was rinsed with ethanol, dried, and redissolved in diethylpyrocarbonate-treated water.

A 296-bp segment of bovine eNOS complimentary DNA (cDNA) corresponding to position 2730–3025 (Sessa et al., 1992) was amplified using a coupled reverse transcription-polymerase chain reaction, subcloned into T-tailed pBluescript II SK(+), and verified by sequencing (Ranjan et al., 1995). The cDNA probe was synthesized by the random priming hexanucleotide method using [α-^{32}P]deoxyadenosine triphosphate ([α-^{32}P]dATP). An identical quantity of each total RNA sample was run on each lane of a 1.0% agarose/formaldehyde gel. The gel was blotted overnight to Millipore Immobilon N membrane, and then membranes were probed using 10^6 cpm/ml of probe in hybridization buffer followed by high stringency washes. Autoradiography of membranes was performed at −75°C for 16 hr followed by scanning densitometry. In some experiments, a 425S PhosphorImaging system (Molecular Dynamics, Sunnyvale, CA) was used to scan plates that had been exposed for up to 48 hr at room temperature. The imaging plates were scanned with a 10-mW HeNe laser at a speed of 15 μsec/176 μm pixel and analyzed using ImageQuant software. Each lane was normalized by the PhosphorImaging signal for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Although PhosphorImaging has superior sensitivity and dynamic range when compared with autoradiography/scanning densitometry, the image printouts generated with the available equipment in this study were limited to binary images of moderate spatial resolution. The difference between the eNOS/GAPDH ratio obtained by scanning densitometry and PhosphorImaging was primarily the result of the enhanced dynamic range of PhosphorImaging and the fact that the scanning densitometry relied on line scans whereas the PhosphorImaging used area scans. Also, the lower background, increased sensitivity, and higher linearity of phosphorImaging can cause a difference in the fold-induction observed with PhosphorImaging and autoradiography. The precise value of the eNOS/GAPDH ratio in each experiment was dependent also on the ^{32}P incorporation yield of each labeling reaction as well as the age of each probe. The lower background, increased sensitivity, and higher linearity of PhosphorImaging are the primary cause for the difference in fold-induction observed with PhosphorImaging and autoradiography. All comparisons were made for RNA samples that had been isolated simultaneously from replicate monolayers, electrophoresed, blotted, probed, and then imaged.

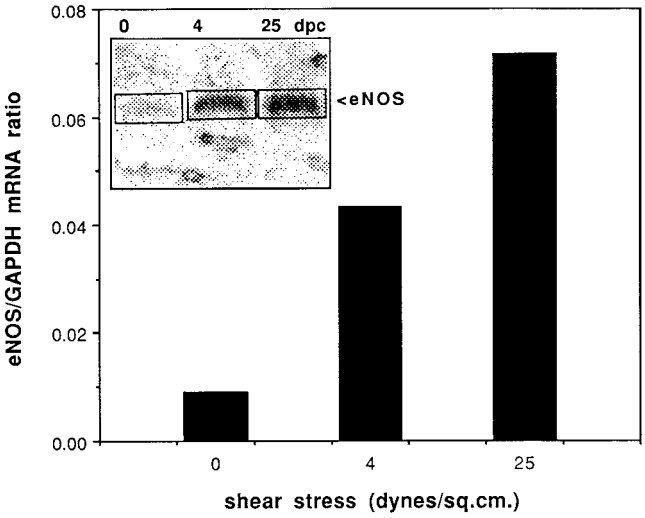

Fig. 1. Laminar shear stress induces BAEC eNOS mRNA levels. PhosphorImaging of eNOS mRNA levels (insert) analyzed by Northern blotting of total RNA from BAEC that were maintained in stationary culture (0) or exposed to laminar shear stress of 4 or 25 dynes/cm^2 (dpc) for 6 hr. The eNOS mRNA signal for each lane was normalized to its GAPDH mRNA signal, which had a lane-to-lane SD of ±15.5%.

RESULTS

With the use of the eNOS/GAPDH mRNA ratio as an internal metric of eNOS gene expression, we found

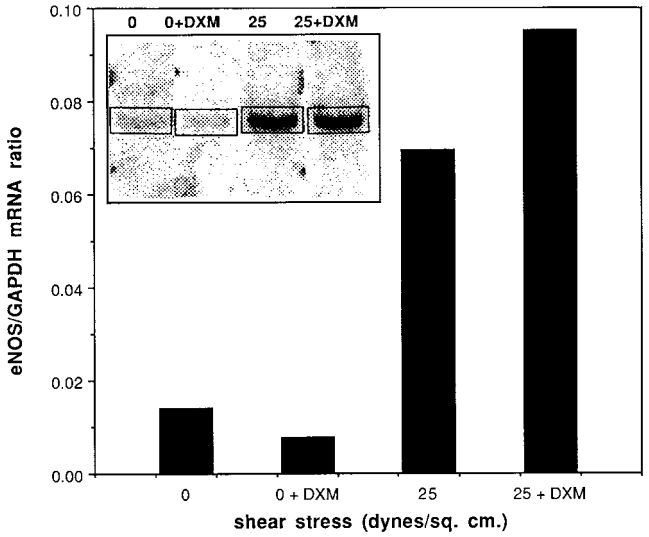

Fig. 2. Laminar shear stress induces BAEC eNOS mRNA levels in the presence or absence of 1 μM dexamethasone (DXM). PhosphorImaging of eNOS mRNA levels (insert) analyzed by Northern blotting of total RNA from BAEC that were maintained in stationary culture (0) or exposed to laminar shear stress 25 dynes/cm^2 for 6 hr in the presence or absence of dexamethasone. The eNOS mRNA signal for each lane was normalized to its GAPDH mRNA signal, which had a lane-to-lane SD of ±15.2%.

that 6-hr exposure to laminar shear stress of 4 and 25 dynes/cm^2 induced BAEC eNOS mRNA levels by 4.8- and 7.95-fold, respectively, compared with stationary control (Fig. 1). The sensitivity, linearity, and dynamic range of PhosphorImaging provided resolution of the dose-response of eNOS induction by shear stress that was not fully apparent in our earlier studies using autoradiography/scanning densitometry of the eNOS mRNA band (Ranjan et al., 1995). Also, BAEC eNOS mRNA levels were elevated by shear stress exposure either in the absence or presence of 1 μM dexamethasone (Fig. 2) confirming that NFkB-dependent pathways were not involved in the induction.

To test whether a calcium-dependent step was required for the elevation of the eNOS mRNA levels in cells exposed to shear stress, BAEC were preincubated for 30 min with the cell-permeable intracellular calcium chelator, BAPTA/AM (5.0 μM). A 6-hr exposure to shear stress increased the eNOS/GAPDH mRNA ratio by 2.5-fold relative to stationary endothelial control levels as measured in the autoradiograph. BAPTA caused over a 70% reduction in the shear stress stimulation of eNOS mRNA levels (Fig. 3A). In shear stressed cells maintained in BAPTA, the eNOS mRNA level was only 43% greater than control levels as indicated by scanning densitometry of the autoradiograph in Fig. 3A. This experiment was repeated with an independent passage of BAEC using PhosphorImaging as an alternative method of quantitation (Fig. 3B). We found that BAPTA reduced the shear stress induction of eNOS mRNA by 68% as indicated by the eNOS/GAPDH mRNA phosphoImaging ratio (Fig. 3C). The GAPDH mRNA signal had a lane-to-lane standard deviation of ± 25.6%. Chelation of intracellular calcium substantially attenuated the elevation of the eNOS mRNA levels by shear stress.

To evaluate whether elevation of intracellular calcium caused the induction of eNOS by fluid shear stress, we conducted experiments with ionomycin. Elevation of BAEC intracellular calcium with ionomycin (0.1–1.0 μM) for 6–22 hr did not markedly induce eNOS mRNA (Fig. 4). PhosphorImaging of eNOS and GAPDH mRNA levels (Table 1) demonstrated that ionomycin was not a significant inducer of eNOS mRNA. To evaluate if other potent endothelial agonists could induce eNOS mRNA, we used endotoxin and a phorbol ester. We have observed repeatedly ($n > 20$ monolayers tested) that laminar shear stress can elevate BAEC eNOS mRNA levels within 6 hr . Exposure of BAEC for 6 hr to lipopolysaccharide endotoxin (LPS; 1.0 μg/ml) or phorbol myristrate acetate (PMA; 0.5 μg/ml) had little effect on eNOS mRNA levels relative to stationary controls (Fig. 5).

The preincubation of cells with the G-protein inhibitor, GDP-β S (600 μM) had little effect on the induction of eNOS mRNA (Fig. 6) in BAEC exposed to laminar shear stress of 25 dynes/cm^2 for 6 hr. By scanning densitometry analysis of the autoradiograph, we found that the shear stress elevated the eNOS/GAPDH mRNA ratio to a level nearly 3-fold greater than that of stationary controls regardless of the use of GDP-β S. In a replicate experiment with an independent passage of BAEC, we used PhosphorImaging to monitor the induction of eNOS mRNA in the presence of GDP-β S. The 5.9-fold induction of eNOS mRNA by shear stress in the presence of GDP-β S was substantial (Fig. 7A), and was reduced only by 30% compared with sheared monolayers without the inhibitor (Fig. 7B).

The tyrosine phosphorylation of paxillin in focal adhesion plaques of shear stressed endothelium (Davies, 1995) demonstrates that tyrosine kinases are activated during shear stress exposures. To study the potential role of tyrosine kinases in the shear stress induction of the eNOS gene, we used the tyrosine kinase inhibitors genistein and tyrphostin B46. Genistein did not block the shear stress induction of eNOS (Fig. 7A,B). Similarly, the presence of 10 μM tyrphostin B46 did not cause any significant change in the level of induction of eNOS in tyrphostin B46–treated BAEC as compared with untreated BAEC exposed to arterial levels of shear stress (Fig. 8). Tyrphostin B46 caused some elevation of GAPDH mRNA levels in BAEC stationary controls because uniform loading of RNA in the different lanes of Fig. 8 was confirmed by ethidium bromide staining. In light of the role of tyrosine phosphorylation during integrin-mediated adhesion and remodeling (Burridge et al., 1992; Romer et al., 1994), we grew BAEC on fibronectin-coated slides to modulate focal adhesion function and cytoskeletal function during eNOS induction by shear stress. BAEC grown on both uncoated glass slides and glass slides coated with 2 mg/cm^2 of human plasma fibronectin were subjected simultaneously to shear stress of 25 dynes/cm^2. Northern blot analysis of RNA isolated from both types of slides showed that the presence of fibronectin had no effect on the shear stress induction of eNOS gene, which was 2- to 3-fold higher (by autoradiography/scanning densitometry of the eNOS/GAPDH ratio) in both cases when compared with stationary controls (not shown).

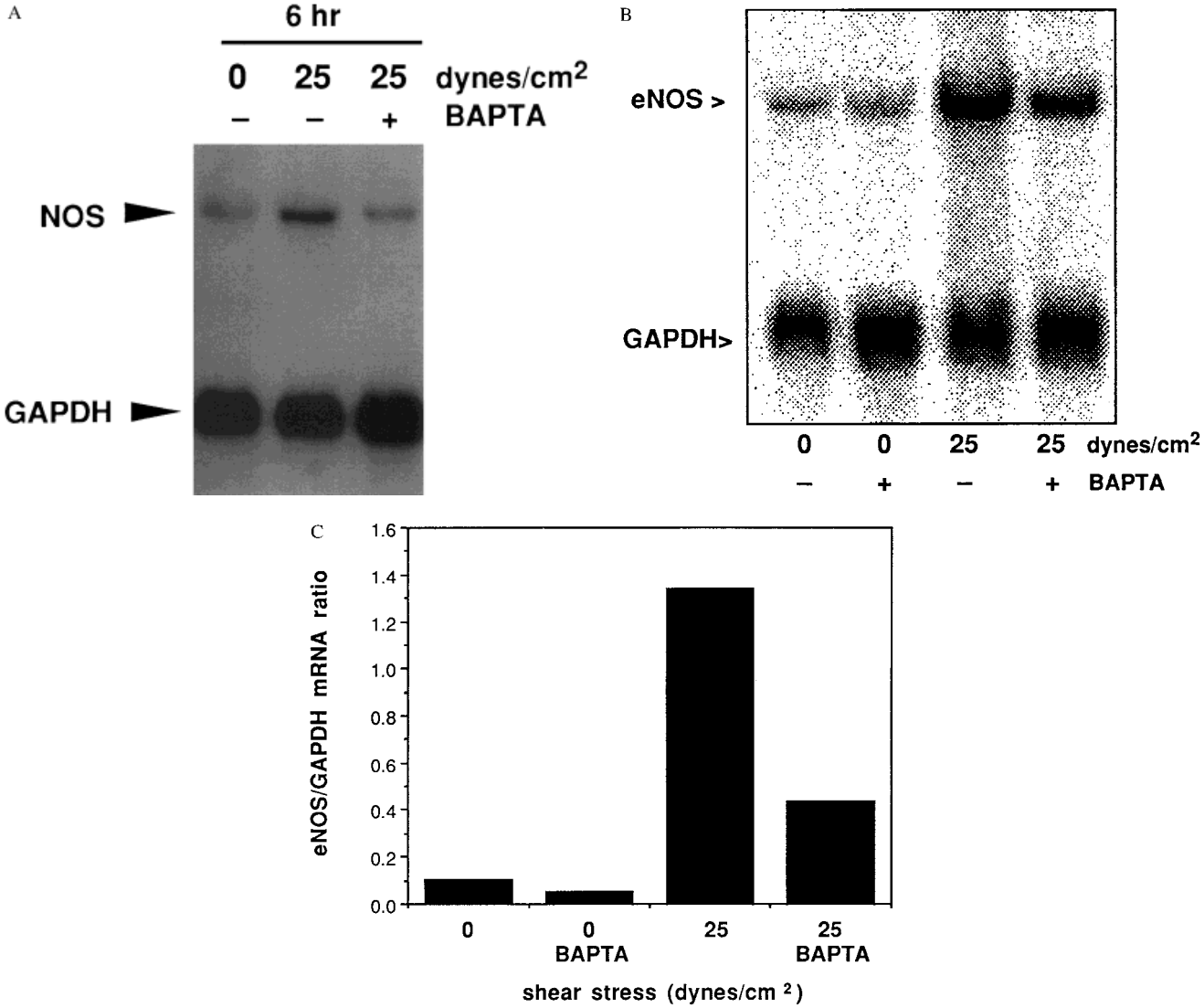

Fig. 3. The induction of BAEC eNOS mRNA levels by shear stress is attenuated by chelation of intracellular calcium by BAPTA. Some monolayers were incubated in 5 μM BAPTA/AM for 30 min. Replicate experiments with independent passages of BAEC were conducted by quantification of Northern blotting by autoradiography/scanning densitometry (**A**) or PhosphorImaging (**B**). In the first experiment (A), BAPTA reduced the shear stress induction of eNOS mRNA by 70% as indicated by the eNOS/GAPDH mRNA density ratio. A total of 5 μg RNA was loaded in each electrophoresis lane. In the second experiment (B), BAPTA reduced the shear stress induction of eNOS mRNA by 68% as indicated by the eNOS/GAPDH mRNA PhosphoImaging ratio (**C**). In B and C, the GAPDH mRNA signal had a lane-to-lane SD of ±25.6%.

DISCUSSION

We investigated signal transduction pathways involved in the elevation of eNOS mRNA levels by fluid shear stress. The induction of eNOS mRNA levels by shear stress was inhibited by 70% by chelation of intracellular calcium with BAPTA. However, ionomycin did not enhance eNOS mRNA levels, suggesting that elevated levels of intracellular calcium were not sufficient for the induction of this gene. Chelation of intracellular calcium may interfere with the transport of proteins from the cytoplasm into the nucleus by depletion of calcium from the endoplasmic reticulum (Greber and Gerace, 1995) and this may be the mechanism by which BAPTA prevented the induction of the eNOS gene. Also, the induction of eNOS mRNA did not require the activation of G-proteins or tyrosine kinases by shear stress. The rapid and strong induction of eNOS mRNA by shear stress is somewhat unique in that it is not easily mimicked by treating cells with ionomycin, endotoxin, or phorbol ester.

Our observations of shear induction of eNOS mRNA in the presence of BAPTA were similar to earlier studies of shear stress induction of PDGF-B chain mRNA in which BAPTA provided a marked inhibition of the induction (Hsieh et al., 1992). In contrast, BAPTA has no effect on the activation of mitogen-activated protein (MAP) kinase by shear stress (Tseng et al., 1995). We found that 600 μM GDP-β S does not block eNOS

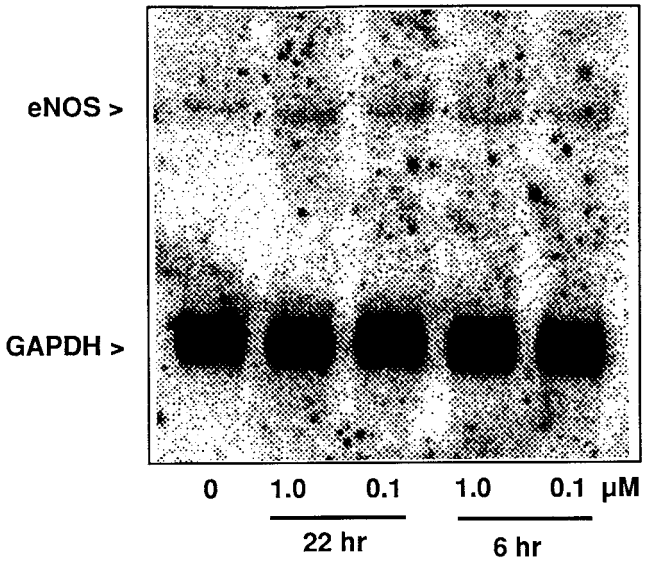

Fig. 4. Elevation of BAEC intracellular calcium with ionomycin (0.1–1.0 μM) for 6–22 hr does not induce eNOS mRNA. PhosphorImaging values are given in Table 1.

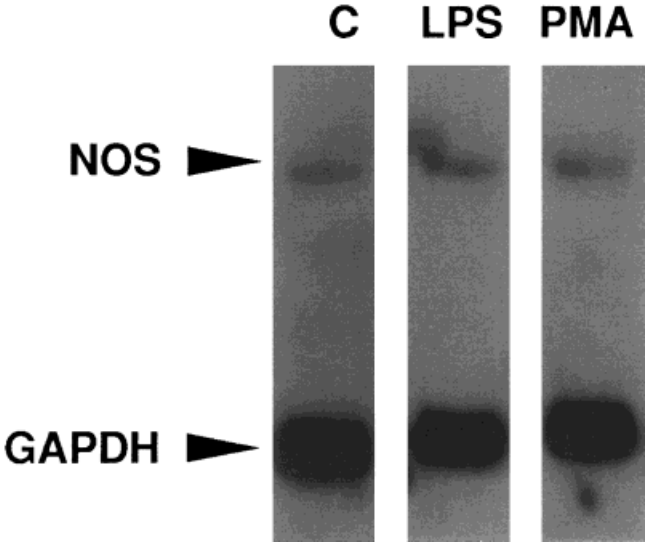

Fig. 5. Failure to elevate eNOS mRNA levels in BAEC after 6 hr of stimulation with 1.0 μg/ml of LPS or 0.5 μg/ml PMA as compared with control cells (C). A total of 5 μg RNA was loaded in each electrophoresis lane.

TABLE 1. Ionomycin does not induce BAEC eNOS mRNA levels.[1]

	eNOS/GAPDH
Control	0.0277
Ionomycin 0.1 μM for 6 hr	0.0317
Ionomycin 1.0 μM for 6 hr	0.0270
Ionomycin 0.1 μM for 22 hr	0.0286
Ionomycin 1.0 μM for 22 hr	0.0345

[1]Replicate monolayers of BAEC were exposed to 0.1 or 1.0 μM ionomycin for 6–22 hr. PhosphorImaging counts for eNOS mRNA and GAPDH mRNA analyzed by Northern blotting of 9 μg of total BAEC RNA. The lane-to-lane variation of the eNOS/GAPDH mRNA ratio had an SD of ±9.4%, indicating the absence of significant induction of eNOS mRNA by treatment of BAEC with ionomycin.

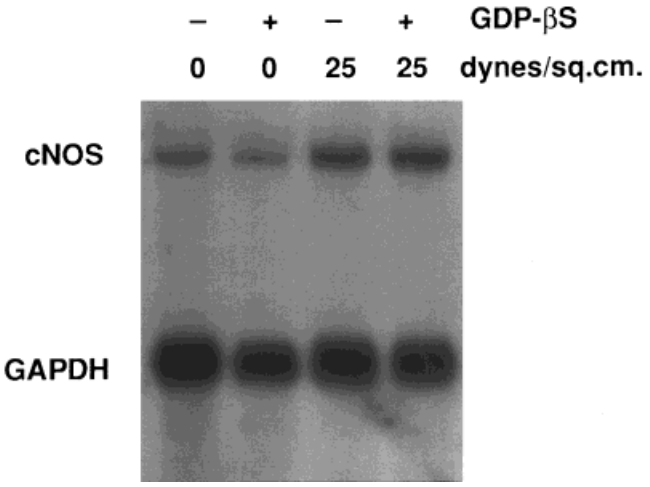

Fig. 6. Induction of eNOS mRNA by shear stress does not require G-protein signaling. The GAPDH and NOS mRNA levels are shown in BAECs that were maintained in stationary culture or exposed for 6 hr to arterial levels of laminar shear stress (25 dynes/cm^2) in the presence (+) or absence (–) of 6-hr preincubation with 600 μM of GDP-β S, an inhibitor of G-protein function. A total of 5 μg RNA was loaded in each electrophoresis lane.

mRNA induction by shear stress. In contrast, Hsieh et al. (1993) showed that 300 μM GDP-β S can reduce the elevation of c-fos mRNA by pulsatile flow by about 90% (mean shear stress of 16 dynes/cm^2). If GDP-β S blocks the induction of c-fos (Hsieh et al., 1993) but does not inhibit shear stress induction of eNOS mRNA, then the induction of eNOS mRNA by shear stress would appear to be AP-1 independent. This is consistent with our observations that phorbol ester does not elevate eNOS mRNA and PKC inhibition with H7 (Ranjan et al., 1995) does not prevent shear stress induction of eNOS. Uematsu et al. (1995) also found that the PKC inhibitor calphostin C (100 nM) does not block the induction of eNOS by shear stress. These findings with GDP-β S and PKC inhibitors, in concert with the present observations with ionomycin (Table 1) and PMA, suggest that one of the well-studied signaling pathways activated during endothelial response to shear stress, namely, the activation cascade of G-protein (Hsieh et al., 1992) → phospholipase C/Ca^{2+} → PKC (Tseng et al., 1995) → c-fos/c-jun (Hsieh et al., 1993; Ranjan and Diamond, 1993) → AP-1 site-dependent transcription (Lan et al., 1994; Shyy et al., 1995) is not operative in the elevation of eNOS mRNA by shear stress. In fact, a recent study indicates that PKC activity may even down-regulate eNOS expression (Ohara et al., 1995). PKC activation may serve as an amplifier (but not the trigger) of the shear stress induction, but that interpretation is not consistent with the study of Ohara et al. (1995).

Mechanical deformation of endothelial cells also can induce eNOS (Awolesi et al., 1995). Although calcium mobilization is a hallmark during deformation of cells (Sigurdson et al., 1993; Naruse and Sokabe, 1993) and less so for sheared endothelium (Davies, 1995), it would

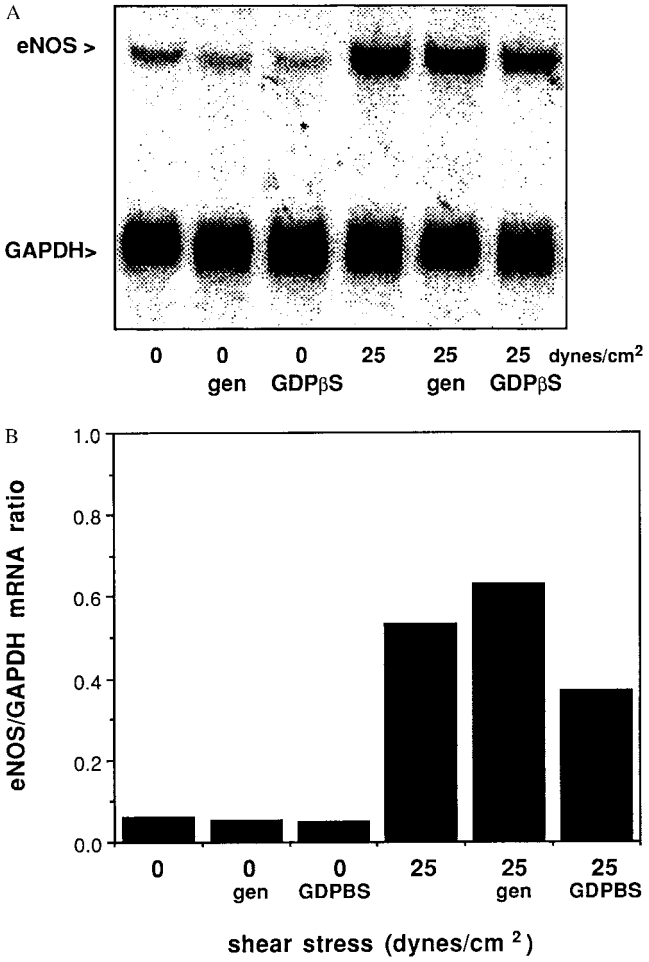

Fig. 7. Induction of eNOS mRNA by shear stress does not require G-protein signaling or tyrosine kinase activity. The GAPDH and eNOS mRNA levels are shown in BAECs that were maintained in stationary culture or exposed for 6 hr to shear stress (25 dynes/cm^2) in the presence or absence of the tyrosine kinase inhibitor, genistein (gen, 10 μM) or with preincubation with the G-protein inhibitor, GDP-β S (**A**). In this experiment, genistein had no effect on the induction of eNOS, whereas GDP-β S caused only a slight decrease in the large induction of eNOS by shear stress, as indicated by the eNOS/GAPDH mRNA phosphoImaging ratio (**B**). The GAPDH mRNA signal had a lane-to-lane SD of ±17.9%.

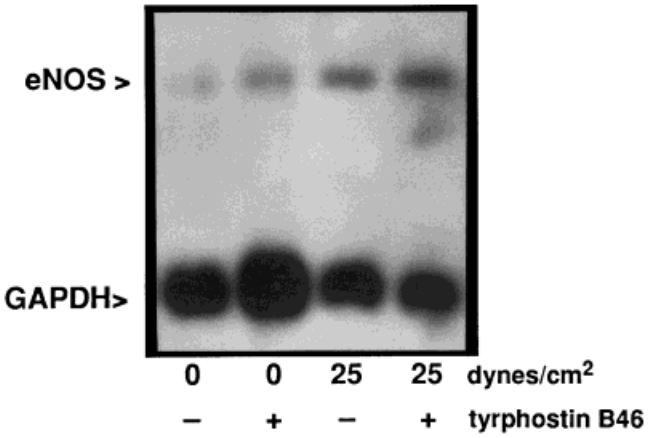

Fig. 8. The tyrosine kinase inhibitor tyrphostin B46 does not inhibit shear stress induction of BAEC eNOS mRNA. Cells were incubated in 10 μM tyrphostin B46. A total of 5 μg RNA was loaded in each electrophoresis lane. The GAPDH mRNA level in the second lane was elevated because a variation in gel loading was not apparent by ethidium bromide staining of total RNA.

appear that calcium is not the central player in induction of eNOS by shear stress or substrate deformation because ionomycin stimulation of BAEC does not induce eNOS. Although NO can stimulate the production of cyclooxygenase products in endothelium (Davidge et al., 1995) and astrocytes (Molina-Holgado et al., 1995), it does not appear that altered arachidonic acid metabolism by shear stress is responsible for the induction of eNOS because ionomycin, which would elevate both NO production and prostacyclin (PGI_2) production, did not induce eNOS mRNA. Also, we have shown previously that the elevation eNOS protein levels by shear stress can occur in the presence of N^G-nitro-L-arginine methyl ester to block shear stress-induced NO production (Ranjan et al., 1995).

Cytoplasmic proteins such as $p125^{FAK}$, paxillin, and tensin can be tyrosine phosphorylated during cell adhesion to extracellular matrix proteins and the inhibition of tyrosine kinase activity prevents the formation of focal adhesions and stress fibers (Burridge et al., 1992). There is evidence that expression of MCP-1, a shear stress-sensitive gene in endothelial cells (Shyy et al., 1995), in human monocytes is regulated by cell density through PKC and tyrosine kinase. Also, the eNOS mRNA, protein, and NO production levels decrease as the monolayer reaches confluency (Arnal et al., 1994). Focal adhesions have been studied extensively as the possible cytoskeleton-linked mechanotransduction sites to which cell tension is transmitted by flow (Davies, 1995). However, we found that when the glass substrate was coated with fibronectin, it had no effect on the shear induction of the eNOS gene. Also, inhibition of tyrosine kinase activity does attenuate the induction of eNOS by shear stress.

Considerable evidence suggests that the chronic exposure of endothelial cells to physiological levels of arterial shear stress promotes a beneficial phenotype. Examples include the elevation of eNOS gene expression with potential vasodilatory enhancement and smooth muscle cell (SMC) growth antagonism, the elevated expression of endothelial Cu/Zn superoxide dismutase with potentially reduced NO consumption by superoxide anion (Inoue et al., 1996), the shut down of endothelin expression (Sharefkin et al. 1991; Malek et al., 1993) with reduced constrictor and SMC mitogenic activity, the elevation of PGI_2 and NO production with impedance of platelet and neutrophil interactions with the vessel wall, the transient induction and then suppression at longer times of PDGF and MCP-1 expression, and the large enhancement of C-type natriuretic peptide mRNA (Okahara et al., 1995) with potential SMC growth inhibition and vasodilation. The loss of NO production by human endothelial cells with cell division in culture (Sato et al., 1993) may represent the loss of this unique in vivo phenotype due to the absence of shear stress.

ACKNOWLEDGMENTS

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