Shear Stress Causes Nuclear Localization of Endothelial Glucocorticoid Receptor and Expression From the GRE Promoter

Julie Y. Ji, Huiyan Jing, Scott L. Diamond

Abstract—We tested the hypothesis that steady laminar shear stress activates the glucocorticoid receptor (GR) and its transcriptional signaling pathway in an effort to investigate the potential involvement of GR in shear stress–induced atherothrombosis actions in the vasculature. In both bovine aortic endothelial cells (BAECs) and NIH3T3 cells expressing GFP-GR chimeric protein, wall shear stress of 10 or 25 dynes/cm² caused a marked nuclear localization of GFP-GR within 1 hour to an extent comparable to induction with 25 μmol/L dexamethasone. The shear mediated nuclear localization of GFP-GR was significantly reduced by 25 μmol/L of the MEK1 inhibitor (PD098059) or the PI 3-kinase inhibitor (LY294002). Also, Western blots demonstrated translocation of endogenous GR into nucleus of sheared BAECs. Promoter construct studies using glucocorticoid response element (GRE)–driven expression of secreted alkaline phosphatase (SEAP) indicated that BAECs exposed to shear stress of 10 and 25 dynes/cm² for 8 hours produced 9-fold more SEAP (n = 6; P < 0.005) than control cells, a level comparable to that observed with dexamethasone. Shear stress enhanced SEAP expression at 6 hours was reduced 50% (n = 5; P < 0.005) by MEK1/2 or PI 3-kinase inhibitors, but not by the NO inhibitor, L-NAME. Finally, in human internal mammary artery, endothelial GR is found to be highly nuclear localized. We report a new shear responsive transcriptional element, GRE. The finding that hemodynamic forces can be as potent as high dose glucocorticoid steroid in activating GR and GRE-regulated expression correlates with the atheroprotective responses of endothelial cells to unidirectional arterial shear stress. (Circ Res. 2003;92:279-285.)

Key Words: endothelium • shear stress • hemodynamics • glucocorticoid receptor

The vascular endothelium plays a central role in vessel tone regulation while endothelial dysfunction is implicated in various wall-thickening pathologies. Atherosclerosis is preferentially initiated at bifurcation sidewalls with reversing or vortexing flows, or regions of low mean wall shear stress.1 In cultured endothelium, the onset of flow causes the transient induction of proatherogenic genes such as platelet-derived growth factor A and B chain, macrophage chemoattractant protein 1 (MCP-1), and endothelin-1 (ET-1), whereas arterial shear stress over many hours downregulates these genes.2–3 Sustained shear is also associated with long-term elevated expression of tissue plasminogen activator (tPA), endothelial nitric oxide synthase (eNOS), and C-type natriuretic peptide, genes typically associated with an atheroprotective phenotype.6,7

A variety of kinases and signaling proteins are involved in shear-modulated responses. It is thought that shear stress activates phosphatidylinositol 3-kinase (PI3-kinase), leading to phosphorylation of Akt (protein kinase B) and serine phosphorylation of eNOS at RIRTQ51179FSLQ8–10. A recent study also shows, however, that shear stress phosphorylates eNOS at Ser1179 through an Akt-independent mechanism that involves protein kinase A.11 The subsequent eNOS activation results in elevated production of NO, another potent endothelial signaling molecule. Garcia-Cardena also demonstrated the association of heat shock protein 90 (hsp90) with eNOS in human umbilical vein endothelial cells (HUVECs) after 15 to 30 minutes of shear,12 whereas NO production occurs within seconds after flow onset. Mitogen-activated protein kinase (MAPK) activation in shear-stressed endothelium is also well documented; extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK), big MAPK (BMK1), and to a lesser extent, p38 are all activated by shear stress.13–16 Proximal to gene expression changes in steady flow, kinases activated during mechanotransduction can alter the activity of transcription factors such as nuclear factor (NF)-κB, AP-1, and erg-1.17–19 Thus in the endothelium, cellular responses to shear stress occur through diverse signaling pathways involving, among others, NO, heat shock proteins, and protein kinases such as PI 3-kinase and MAPK.

The antiinflammatory effects of glucocorticoids on the endothelium suggest a possible role for the glucocorticoid...
receptor (GR) in mediating atheroprotective actions of shear stress in the vasculature. Cytokine induction of inducible NOS in endothelium is inhibited by pretreatment with dexamethasone, a potent glucocorticoid, whereas induction of eNOS expression by shear stress can occur in the presence of dexamethasone. Recent studies have linked corticosteroid-mediated eNOS activation and NO production to its acute cardiovascular protective effects, and vascular inflammation processes are becoming increasingly implicated in atherosclerosis pathology.

The glucocorticoid receptor is present in endothelial cells and smooth muscle cells. In its inactive state, the GR resides mainly in the cytoplasm in complexes with chaperone proteins such as hsp90, hsp70, and immunophilins (FKBP59, cyp40). On steroid binding, chaperone proteins dissociate from GR, allowing for nuclear import. GR becomes activated as its transcriptional regulatory region is phosphorylated on threonine 171 and serine 246 residues by MAPK, and on serine 224 and 232 residues by cyclin-dependent kinases (CDK) complexes. Liganded GR form dimers that bind to specific glucocorticoid response element (GRE) to regulate transcription of glucocorticoid-responsive genes.

Steroid-activated GR can suppress immunological and inflammatory responses and regulate gene expression through either classical (GR binding to GRE) or nonclassical (NF-κB downregulation) mechanisms. In this study, we examined the effect of steady shear stress on GR activation and transcriptional regulation as compared with dexamethasone, particularly in the context of various shear-activated kinase activities and NO production that may also regulate GR function. Such regulation may have relevance to the atheroprotective role of unidirectional shear stress on endothelial function.

**Materials and Methods**

**Materials**

Dexamethasone, 1,4-pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione (Steraloids) was dissolved in DMSO and stored at −20°C. PD098059 (MEK inhibitor), U0126 (MEK1/2 inhibitor), and LY294002 (PI 3-kinase inhibitor) were all obtained from Cell Signaling, dissolved in DMSO, and stored at −20°C. Nitric oxide synthase inhibitor, N-nitro-l-arginine methyl ester hydrochloride (L-NAME) from Sigma, was dissolved in water and stored at −70°C. The pGFP-GR plasmid was provided by Dr Mario Galigniana (University of Michigan Medical School, Ann Arbor, Mich). In this vector, the mouse GR cDNA N-terminus was fused in frame to the C-terminus of green fluorescence protein (GFP) on the pEGFP-C3 plasmid (Clontech). The pGRE-SEAP plasmid (Clontech) utilizes a secreteable form of human placental alkaline phosphatase (SEAP) driven by 3 tandem copies of the GRE sequence, GTTACA(N)7TGTCTC, fused to a weak TATA-like (P100) region of the Herpes simplex virus thymidine kinase (HSV-TK) promoter.

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were cultured (passage <12) in Dulbecco’s modified Eagle’s medium (DMEM) (BioWhitaker) supplemented with 10% heat-inactivated newborn calf serum, 1% L-glutamine (200 mmol/L), and 2% penicillin streptomycin (Invitrogen). Cells were seeded on 38×75-mm glass slide treated with 0.1% gelatin at a density of 1 to 2×10⁶ cells per slide and cultured to confluence. BAECs on slides were transfected for 2 hours with pGFP-GR or pGRE-SEAP using Lipofectamine (Invitrogen) as previously described, and allowed to recover overnight. The G418-selected cell line 3676 is a derivative of 3T3 fibroblasts that stably express a GFP-rat GR chimeric protein (gift from Dr Gordon Hager, National Institutes of Health, Bethesda, Md). The GFP-GR protein is expressed in these cells through removal of tetracycline from the culture medium (tetr-off induction). 3T3 or transfected BAECs were maintained in phenol-free DMEM with 2.5% charcoal/dextran-treated fetal bovine serum (HyClone) for minimal background growth factor and steroid concentrations.

**Shear Stress Exposure and Fluorescence Assays**

Cells were exposed to laminar shear stress in parallel plate flow chambers attached to flow loops for media recirculation (15 mL in a 37°C incubator as previously described. Wall shear stress was calculated from solution of the Navier-Stokes equation for Newtonian fluid between parallel plates: \( \tau_w = 6 \mu Q/bh^2 \) for viscosity, \( \mu = 0.01 \) dynes-sec/cm²; \( Q \), volumetric flow rate (cm³/s); \( b \), flow chamber width (2.5 cm); and \( h \), the total plate separation (0.025 cm). After experiments involving GFP-GR localization, cells were digitally imaged at 40× using a Leica DM IRBE fluorescence microscope (FITC cube) and a cooled CCD camera (Hamamatsu, C4742-95) driven by OpenLab software (Improvision). For experiments involving SEAP expression, 200-μL media samples were taken without disruption of flow. SEAP was quantified using a SEAP Fluorescence Detection Kit (Clontech). Heating samples at 65°C and incubating them in assay buffer containing l- homoarginine, a SEAP inhibitor, eliminated endogenous alkaline phosphatase activity. Total SEAP activity was measured using the fluorescent substrate 4-methylumbelliferyl phosphate (MUP) in black 96-well plates read by the Fluoroscan Ascent FL microplate fluorometer (EX 360 nm; EM 449 nm).

**Western Blotting**

After experiments, BAECs were washed once in PBS, removed from slides using 0.05% trypsin and harvested by centrifugation. Cells were then lysed and separated into cytoplasmic and nuclear proteins using a nuclear extraction kit (Active Motif). Protein concentration was determined using the Bradford protein assay (BioRad). Cytoplasmic and nuclear proteins (200 μg/lane) were separated by SDS-PAGE on 7.5% Tris-HCl Ready Gel (BioRad), transferred onto nitrocellulose membrane by electroblotting, and blocked overnight at 4°C in 5% nonfat dry milk and 0.1% TBS. Incubation with the primary antibody, rabbit polyclonal anti-GRα IgG (Santa Cruz Biotechnology) at 1:400 dilution, was done at room temperature for 2 hours. Subsequently, the blot was washed 5 times (10 minutes each) in 0.5% TBS, incubated for 1.5 hours at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) diluted 1:400, before being developed in ECL Plus Western Blotting Detection Reagents (Amersham Biosciences). Recombinant human GR (PanVera) was used as positive control. Bands were quantified by scanning densitometry and analyzed using ImageQuant software (Molecular Dynamics).

**Histology and Immunohistochemistry**

Human internal mammary artery segments were fixed overnight in 10% formalin, dehydrated, and embedded in paraffin before being cut into 5-μm thick sections and mounted onto glass slides. For immunohistochemical staining, slides were deparaffinized and labeled with rabbit polyclonal IgG against human GR C-terminal (Santa Cruz Biotechnology) at 1:10 or 1:100 dilutions. Alternatively, sections were stained for endothelial cells using mouse anti-human CD31 antibody diluted 1:10. Detection was done using the anti-rabbit and mouse biotinylated secondary antibody as part of the Vectastain Elite Kit with DAB peroxidase substrate kit (Vector). Simultaneous nuclear staining on GR labeled sections was done using either VectaShield mounting medium with DAPI (Vector) or Hematoxylin stain (Fisher).

**Results**

**Laminar Shear Stress Causes GR Nuclear Localization**

BAECs transiently transfected with pGFP-GR displayed predominantly cytoplasmic localization of GFP-GR when cul-
tured overnight under stationary conditions. BAECs exposed to laminar shear stress of 10 or 25 dynes/cm² displayed prominent nuclear localization of GFP-GR over the course of a 2-hour shear stress exposure (Figure 1). Within 15 minutes after the onset of flow, the cell nuclei became distinguished with increased fluorescence. Nuclear import of the GFP-GR was further enhanced at 30 minutes as fluorescence increased in the cell nuclei and decreased in the cytoplasm. After 60 to 120 minutes of applied shear stress, a majority of the fluorescent GR was present in the nuclei. In separate experiments, sustained nuclear localization of GFP-GR in BAECs was consistently observed after 8 and 12 hours of shear stress exposure at 25 dynes/cm² (data not shown).

We conducted tests to determine if GR nuclear localization during fluid shear stress exposure was an endothelial specific response. In either BAECs or 3T3 fibroblast cells, shear stress of 10 dynes/cm² for 1 hour caused GFP-GR to nuclear localize at a rate and extent similar to treatment with 25 μmol/L dexamethasone (Figure 2). Both transiently transfected BAECs and 3T3 fibroblasts stably expressing GFP-GR showed uniform cellular fluorescence under control conditions. Induction with 25 μmol/L dexamethasone quickly activated the GR with a dramatic concentration of green fluorescence to the nucleus that began within 10 minutes. This experiment demonstrated that shear stress induced GR nuclear localization in two different cell types, BAECs and 3T3 fibroblasts, and the level of receptor translocation under shear was comparable to that achieved with dexamethasone.

**Kinase Inhibitors Prevents GR Nuclear Localization in BAECs Exposed to Flow**

We used kinase inhibitors to investigate the potential effects of phosphorylation on GR nuclear localization by shear stress. LY294002 is an inhibitor of PI3-kinase (Kᵢ = 1.4 μmol/L) and PD98059 is an inhibitor of MEK1 activation and MAP kinase (Kᵢ = 2 to 7 μmol/L). Preincubation of BAECs for 30 minutes with LY294002 or PD98059 (25 μmol/L) prevented shear stress–induced nuclear localization of the GFP-GR (Figure 3). Without inhibitor pretreatment, BAECs displayed distinct nuclear localization of GFP-GR with shear stress. In cells with inhibitor pretreatment, GFP-GR remained in the cytoplasm even after 1 hour of shear stress exposure (10 dynes/cm²).

**Glucocorticoid Response Element (GRE) Is Shear Stress Sensitive**

To study if endogenous GR can mediate transcriptional activation in BAECs exposed to shear stress, we used the

![Figure 1](https://example.com/figure1.png)

*Figure 1.* Time course pictures of fluorescent GR (GFP-GR) nuclear localization in BAECs exposed to steady laminar shear stress of 10 or 25 dynes/cm² for up to 2 hours. BAECs are cultured on glass slides and transfected with GFP-GR plasmid one day before subjecting cells to shear stress in parallel plate flow chambers. Imaging was conducted on live cells at each time point.

![Figure 2](https://example.com/figure2.png)

*Figure 2.* Nuclear translocation of GFP-GR with dexamethasone or shear stress in BAECs and 3T3 fibroblasts. Complete nuclear translocation of GFP-GR was observed after 1 hour induction with 25 μmol/L dexamethasone as well as with laminar shear stress of 10 dynes/cm² in both cell types.

![Figure 3](https://example.com/figure3.png)

*Figure 3.* Effect of kinase inhibitors on shear-induced nuclear localization of GFP-GR. BAECs expressing GFP-GR were preincubated with either 25 μmol/L of PD98059, an inhibitor of MEK1 activation and the MAP kinase cascade, or LY294002, a PI3-kinase inhibitor, for 30 minutes before exposure to laminar shear stress of 10 dynes/cm² for up to 1 hour.
GRE-SEAP promoter construct. Shearing BAECs at 10 and 25 dynes/cm² activated production of SEAP to a level similar to that obtained with 25 μmol/L dexamethasone (Figure 4). Enhanced SEAP production was detectable within 3 hours in response to shear level of 10 dynes/cm², slightly sooner than dexamethasone induction, but not statistically significant. With shear stress or steroid induction, SEAP levels in cell media were detectable as early as 2 hours. By the end of 4 hours of induction, there was a highly significant presence of the SEAP in the culture media from 10 dynes/cm² shear as compared with control. SEAP production in response to 25 dynes/cm² shear became significantly different from control as late as 7 hours after flow onset. For all conditions tested, total amount of SEAP produced gradually increased with time. By 8 hours after induction, dexamethasone or shear stress, SEAP production in response to all three stimuli was over 9-fold greater than the static control case ($P<0.005$, $n=6$).

**Effect of Kinase Inhibitors on GRE-Dependent Expression**

Pretreating GRE-SEAP transfected BAECs with the kinase inhibitors LY294002, PD98059, or U0126 reduced the production of SEAP after 6 hours of shear stress exposure (Figure 5A). LY294002 is an inhibitor of PI3-kinase. U0126 ($K_i=0.53$ μmol/L) is a potent inhibitor of both MEK1 and MEK2, whereas PD98059 is a stronger inhibitor of MEK1. Cells were preincubated with 25 μmol/L of the inhibitor for up to 2 hours before shear stress experiments at 10 dynes/cm². Consistent with attenuation of nuclear localization of GFP-GR (Figure 3), the inhibitors also reduced expression from the GRE transcription pathway in response to shear stress. Kinase inhibitors caused an average of 53.25% reduction from shear alone. The inhibitory effect was statistically significant when compared with matched BAECs sheared without inhibitor ($P<0.005$, $n=5$). In agreement with Figure 4, induction with dexamethasone (25 μmol/L) or shear stress (10 dynes/cm²) strongly activated the GRE transcription pathway. Determination of GR nuclear localization or GRE-SEAP induction under conditions of simultaneous MEK1 and PI 3-kinase inhibition (25 μmol/L PD98059 and LY294002) was attempted. However, BAECs were not adhesive under flow conditions in the presence of both inhibitors.

**Effect of NOS Inhibitor L-NAME on Shear-Induced GR Activation**

To further assess the role that NO might play in shear activation of GR, cells were incubated with L-NAME (200 μmol/L), a well-characterized NO synthase inhibitor, for 30 minutes before subjecting them to shear (10 dynes/cm²) in circulating media containing 200 μmol/L L-NAME. Pretreating GRE-SEAP transfected BAECs with the NOS inhibitors L-NAME (200 μmol/L) for 30 minutes did not significantly reduce SEAP production after extended shear in circulating media also containing L-NAME (Figure 5B). L-NAME failed to interfere with shear induced activation of the GRE promoter after 6 hours of shear at 10 dynes/cm². Again, induction with dexamethasone (25 μmol/L) or shear stress (10 dynes/cm²) caused no significant reduction in transcriptional activation of GRE-SEAP plasmid after 6 hours as compared with dexamethasone or shear induction. Data are presented as mean±SE ($n=3$).
The increase in nuclear localized endogenous GR was highly significant for cells induced with shear stress or dexamethasone (Dex) or shear-treated cells caused an increase of nuclear GR and a decrease of cytoplasmic GR. GR antibody revealed nuclear localization is comparable with induction by 25\mu mol/L dexamethasone in both BAECs and 3T3 fibroblasts, and using Western blots, we have confirmed increased nuclear presence of endogenous GR in BAECs. PI3-kinase, along with MAPK members such as ERK1/2, JNK, and BMK1, plays major roles in shear stress activated endothelial signaling pathways. PI3-kinase also phosphorylates the closely related estrogen receptor (ER) and regulates the vasoprotective effect in endothelium, partly by regulating production of vasoactive factors such as NO, prostacyclin, endothelin-1, MCP-1, and vascular epidermal growth factor (VEGF). In endothelial cells, glucocorticoids exert an antiinflammatory action via inhibition of adhesion molecule expression (ICAM-1, E-selectin, VCAM-1) and attenuation of MCP-1 promoter activity, possibly through attenuation of AP-1 or NF-κB pathways. Similarly, it has been shown that other nuclear receptors such as estrogen, progesterin, and retinoic acid receptors can serve potential antiinflammatory roles by inhibiting adhesion molecule expression.

**Discussion**

Arterial levels of shear stress may exert an antiatherosclerosis effect in endothelium, partly by regulating production of vasoactive factors such as NO, prostacyclin, endothelin-1, MCP-1, and vascular epidermal growth factor (VEGF). In endothelial cells, glucocorticoids exert an antiinflammatory action via inhibition of adhesion molecule expression (ICAM-1, E-selectin, VCAM-1) and attenuation of MCP-1 promoter activity, possibly through attenuation of AP-1 or NF-κB pathways. Similarly, it has been shown that other nuclear receptors such as estrogen, progesterin, and retinoic acid receptors can serve potential antiinflammatory roles by inhibiting adhesion molecule expression. This study provides the first evidence of shear activation of GR and the role of GRE in mechanotransduction.

We have shown that after 1 hour of shear, GR nuclear localization is comparable with induction by 25\mu mol/L dexamethasone in both BAECs and 3T3 fibroblasts, and using Western blots, we have confirmed increased nuclear presence of endogenous GR in BAECs. PI3-kinase, along with MAPK members such as ERK1/2, JNK, and BMK1, play major roles in shear stress activated endothelial signaling pathways. PI3-kinase also phosphorylates the closely related estrogen receptor (ER) and regulates the vasoprotective effect in endothelium, partly by regulating production of vasoactive factors such as NO, prostacyclin, endothelin-1, MCP-1, and vascular epidermal growth factor (VEGF). In endothelial cells, glucocorticoids exert an antiinflammatory action via inhibition of adhesion molecule expression (ICAM-1, E-selectin, VCAM-1) and attenuation of MCP-1 promoter activity, possibly through attenuation of AP-1 or NF-κB pathways. Similarly, it has been shown that other nuclear receptors such as estrogen, progesterin, and retinoic acid receptors can serve potential antiinflammatory roles by inhibiting adhesion molecule expression. This study provides the first evidence of shear activation of GR and the role of GRE in mechanotransduction.

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activities that estrogen exerts on the endothelium. Our data indicate that pretreating the cells with MAPK and PI3-kinase inhibitors significantly reduced shear stress–induced GR nuclear localization at 1 hour, suggesting that GR fails to move into the nucleus without phosphorylation downstream of either kinase. It is unknown, however, which step of the GR activation cascade is interrupted by the lack of kinase activity.

With the observation that shear stress can cause GR to nuclear localize, we investigated next if the GR transported activity. We have presented in vivo data indicating that under physiological conditions, GR is nuclear localized in the endothelium of a functional blood vessel under continuous shear. Thus, the present study here offers strong evidence that, through induction of the GRE sequence, shear stress is a potent activator of the GR transcriptional regulatory pathway. The observation that GR is activated by shear stress is quite consistent with the most prominent observations of recent mRNA expression profiling of sheared HUVECs using DNA microarrays, where glucocorticoid-induced leucine zipper protein (GILZ) and P450A1A were some of the most highly induced genes, whereas ET-1 and MCP-1 were some of the most significantly repressed genes. Dexamethasone is a known inducer/potentiator of the antiinflammatory GILZ and P450A1A and a known suppressor of ET-1 and MCP-1 expression under stimulatory conditions. In fact, the biphasic induction of MCP-1 by shear stress, where MCP-1 eventually is suppressed by 5 hours, may be a result of activation of endogenous GR by shear stress because it is known that dexamethasone pretreatment blocks shear-induced MCP-1 expression. Interestingly, dexamethasone can suppress COX2 induction by lipopolysaccharide (LPS) if the GR is sufficiently expressed in BAECs. Some labs have reported transient induction of COX2 in shear-stressed HUVECs, whereas others observed sustained induction at 24 hours. This variability may be due to differences in endogenous GR levels in endothelial cultures and the precise level of shear stress exposure or flow changes used experimentally. Also, activation of GR pathways by shear stress may help physiologically to limit or moderate the transient 2- to 3-fold induction of adhesion molecules such as ICAM-1 and E-selectin in sheared endothelial cells via mechanisms similar to dexamethasone suppression of cytokine induction of these adhesion molecules.

In summary, this study demonstrated that steady arterial levels of unidirectional shear stress cause nuclear localization of endogenous GR through processes that were sensitive to MAPK and PI 3-kinase inhibition. Thus, arterial hemodynamics may prove as potent as glucocorticoids to elevate a constitutive nuclear localization of GR activity through a steroid-independent mechanism. This finding is significant in further delineating the interplay between shear stress, inflammation, and atherogenic pathology.

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