

Hemodynamic Regulation of Inflammation at the Endothelial–Neutrophil Interface

JULIE Y. JI,¹ HUIYAN JING,² and SCOTT L. DIAMOND^{1,2}

¹Department of Bioengineering, Institute for Medicine and Engineering, University of Pennsylvania, 1024 Vagelos Research Laboratory, 3340 Smith Walk, Philadelphia, PA 19104, USA; and ²Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, 1024 Vagelos Research Laboratory, 3340 Smith Walk, Philadelphia, PA 19104, USA

(Received 20 July 2007; accepted 7 February 2008; published online 26 February 2008)

Abstract—Arterial shear stress can regulate endothelial phenotype. The potential for anti-inflammatory effects of shear stress on TNF α -activated endothelium was tested in assays of cytokine expression and neutrophil adhesion. In cultured human aortic endothelial cells (HAEC), arterial shear stress of 10 dyne/cm² blocked by >80% the induction by 5 ng/mL TNF α of interleukin-8 (IL-8) and IL-6 secretion (50 and 90% reduction, respectively, in the presence of nitric oxide synthase antagonism with 200 μ M nitro-L-arginine methylester, L-NAME). Exposure of TNF α -stimulated HAEC to arterial shear stress for 5 h also reduced by 60% ($p < 0.001$) the conversion of neutrophil rolling to firm arrest in a venous flow assay conducted at 1 dyne/cm². Also, neutrophil rolling lengths at 1 dyne/cm² were longer when TNF α -stimulated HAEC were presheared for 5 h at arterial stresses. In experiments with a synthetic promoter that provides luciferase induction to detect *cis* interactions of glucocorticoid receptor (GR) and NF κ B, shear stress caused a marked 40-fold induction of luciferase in TNF α -treated cells, suggesting a role for GR pathways in the anti-inflammatory actions of fluid shear stress. Hemodynamic force exerts anti-inflammatory effects on cytokine-activated endothelium by attenuation of cytokine expression and neutrophil firm arrest.

Keywords—Shear stress, Inflammation, Glucocorticoid, Neutrophils.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that is often hemodynamically localized at sites of low and reversing shear stress.^{18,33} Inflammatory markers are distinct indicators in the development and the

progression of atherosclerotic lesions, which are heavily infiltrated with macrophages, T-lymphocytes and other cellular components of inflammation.⁴⁰ During inflammation, the initial rolling interaction of neutrophils on activated endothelium is mediated by selectins.²³ Firm adhesion occurs when β 2 integrins on neutrophils are up regulated by chemokines such as IL-8 to mediate binding to adhesion molecules (ICAM-1) on the activated endothelium.³⁸ Proinflammatory cytokines such as TNF α or IL-1 induce the expression of several cytokines and cell adhesion molecules by endothelial cells.⁴⁰

With respect to steroidal anti-inflammatory drugs, there are two main mechanisms by which glucocorticoids are thought to exert their therapeutic actions in suppressing inflammatory and immune responses. Glucocorticoids diffuse into the cytoplasm and bind to glucocorticoid receptor (GR), which then translocate into the nucleus and bind as dimers to the glucocorticoid response elements (GRE) present in various promoters.² Examples of genes regulated through GRE in their promoters include IL-8 and IL-2 receptor α .²⁴ Activated GR can also suppress inflammation by directly interacting with activated transcription factors, such as nuclear factor-kappa B (NF κ B) and activator protein-1 (AP-1), thus altering NF κ B or AP-1 participation in inflammatory gene expression.^{43,45} In most cases, the active form of NF κ B is a heterodimer of RelA (p65) and NF κ B1 (p50) released from its inhibitor I κ B. Steroid-liganded GR can directly interact with the p65 subunit of NF κ B²⁹ as well as interfering with transcriptional cofactors CREB binding protein (CBP) and steroid receptor coactivator-1 (SRC-1).³⁵ In the endothelium, TNF α inducible genes that are down regulated by the presence of dexamethasone include: interferon β , platelet-derived growth factor (PDGF) B subunit, transforming growth factor (TGF) β 2, vascular endothelial growth factor receptor 3 (VEGFR3),

Address correspondence to Scott L. Diamond, Department of Chemical and Biomolecular Engineering, Institute for Medicine and Engineering, University of Pennsylvania, 1024 Vagelos Research Laboratory, 3340 Smith Walk, Philadelphia, PA 19104, USA. Electronic mail: sld@seas.upenn.edu

IL-1, 2, 7, and 8.¹ Glucocorticoids also suppress the expression of inflammatory markers such as adhesion molecule expression (ICAM-1, E-selectin, VCAM-1)^{3,6,37} and various interleukins. GR suppresses NF κ B induction of the IL-6 gene in vascular endothelial cells,⁴⁷ indicating that the precise mechanisms of NF κ B down-regulation by nuclear steroid receptors can be gene-specific since IL-6 lacks any apparent GRE in its promoter.

The endothelium also acts as a dynamic interface between biochemical triggers and mechanical factors (hemodynamics) and inflammatory cell adhesion. Various kinases activated during mechanotransduction can alter the activity of transcription factors such as NF κ B, AP-1, erg-1, and GR.^{15,16,21,34} A recent study by Chiu et al. showed that shear stress on endothelial cells alters TNF α -stimulated expression of ICAM-1, VCAM-1 and E-selectin while decreasing TNF α -stimulated NF κ B-DNA binding activity in mobility shift assay,⁵ although no tests of cytokine secretion or endothelial adhesiveness were conducted in that study. Glucocorticoid receptors are present in endothelium and smooth muscle cells.^{10,14} In our prior study, we demonstrated that shear stress caused endothelial GR nuclear localization and activated transcription from a GRE promoter through pathways sensitive to inhibitors of the shear-activated kinases, MEK1/2 kinases and PI-3 kinase.¹⁵ This finding suggests certain parallels between the atheroprotective role of unidirectional shear stress and the anti-inflammatory actions of the GR. We now test the hypothesis that shear stress is anti-inflammatory, specifically in suppressing TNF α -induced endothelial activation with respect to the expression of secreted cytokines and neutrophil adhesion.

MATERIALS AND METHODS

Cell Culture and Reagents

Human aortic endothelial cells (HAEC) were maintained in EGM-2 endothelial media system (Clonetics). Glass slides were coated with type I collagen (BD Biosciences). For flow chamber experiments, cells were seeded on collagen coated 38 $\times$ 75 mm glass slides at a density of 1 to 2 $\times$ 10⁶ cells per slide and cultured to confluency. TNF α was obtained from Sigma. Incubated cell culture media from HAEC was collected and measured for cytokine content using human IL-8 and IL-6 ELISA immunoassays (R&D Systems) according to the manufacturer's instructions. IL-8 and IL-6 concentrations were used to calculate the total amount of cytokine produced after accounting for volume changes, and normalized with respect to the total number of cells in each experimental group.

Neutrophil Isolation

Human blood was collected from healthy adult donors by venipuncture and anticoagulated with Na-citrate (9 parts blood to 1 part Na-citrate) and neutrophils were isolated over neutrophil isolation medium (Robbins Scientific) as previously described.¹³ After isolation, neutrophils were resuspended in Hank's balanced salt solution (HBSS, Gibco Laboratories) supplemented with 2% HBS, counted, and diluted to a final concentration of 0.75 $\times$ 10⁶ cells/mL.

Shear Stress Exposure and Neutrophil Adhesion Studies

Cells were exposed to laminar shear stress in parallel plate flow chambers attached to flow loops for media recirculation (15 mL) in a 37 $^{\circ}$ C incubator as previously described.¹⁵ Wall shear stress was calculated as: $\tau_{\text{wall}} = 6 \mu Q / bh^2$ for viscosity, $\mu = 0.01$ dynes-s/cm²; Q , volumetric flow rate (cm³/s); b , flow chamber width (2.5 cm); h , the total plate separation (0.025 cm). For neutrophil adhesion studies, following 5 h arterial shear stress exposure at 10 dyne/cm² (± 5 ng/mL TNF α), the flow chambers were reconnected to a Harvard syringe pump for infusion of a neutrophil suspension at wall shear stress of 1 dyne/cm². During the neutrophil adhesion studies at venous flow conditions, flow chambers were imaged by phase contrast microscopy (Zeiss Axiovert 135, 20X Plan Apochromat) and recorded on videotape for subsequent digital image analysis. Neutrophils were perfused over HAEC for 5 min before the start of image acquisition. Each field of view (FOV; 0.1 mm²) of neutrophils flowing over HAEC was recorded in 10-s video segments from which total and firmly adherent neutrophil counts were determined. "Firm adhesion" refers to neutrophils that remained stationary during 10 s, and "total" refers to average number of neutrophils that interacted with the endothelial monolayer in the FOV over the 10-s interval. Rolling distance was generated using the multi-tracking function of ImageJ (NIH).

Promoter Constructs

The pGRED was kindly provided by Dr. Alexander Whitehead (U. Penn.).³⁹ The pGRED contains the SAA2 promoter with a deletion of a 9-basepair interruption of the GRE consensus sequence, thus providing an active GRE and an active NF κ B site in the promoter upstream of firefly luciferase. *Renilla* luciferase transfection control plasmid was from Promega. For dual luciferase assays, endothelial cultures were washed in PBS and lysed in Passive Lysis Buffer (Promega). Lysates were assayed for luciferase and *Renilla* activity using the LAR II and Stop and Glo Reagents (Promega) in a dual-injection luminometer.

RESULTS

Shear Stress Attenuates TNF α -induced IL-8 and IL-6 Secretion

Addition of 5 ng/mL TNF α under static conditions induced a marked 45-fold increase ($p < 0.001$, $n = 3$) in the total amount of IL-8 secreted by HAEC in 8 h compared to static control (Fig. 1a). However, shearing the cells during TNF α exposure blocked this IL-8 secretion by 80% ($p < 0.001$, $n = 3$). Shear stress alone caused a small increase relative to static culture of IL-8 secretion from 1 to 3 ng/10⁶ cells at 8 h. This small up-regulation of IL-8 by flow alone was not seen in the presence of LNAME (Fig. 2a) since

LNAME-treated cells maintained in static culture produced about 4 ng of IL-8 per 10⁶ cells at 8 h.

With respect to IL-6 secretion, adding TNF α to cell culture media induced a striking 33-fold increase ($p < 0.01$, $n = 3$) over static control that was reduced significantly by 90% ($p < 0.01$, $n = 3$) by flow (Fig. 1b). Shear stress alone caused an increase of IL-6 secretion at 8 h compared to static control from 0.05 to 0.25 ng/10⁶ cells that was not seen in the presence of LNAME (Fig. 2b). These data demonstrated that shear stressed endothelium, when compared to stationary cultures, were considerably less responsive to TNF α with respect to IL-8 and IL-6 secretion.

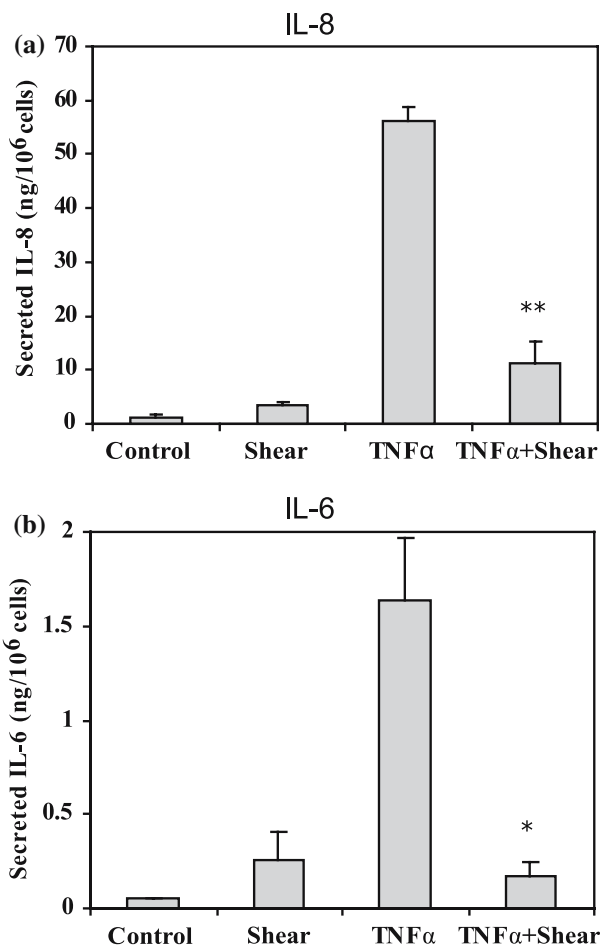


FIGURE 1. Effect of shear stress on TNF α activated expression of cytokines IL-8 (a) and IL-6 (b) in HAEC. Cells were cultured and maintained under static condition (Cont) or treated with TNF α , 5 ng/mL, for 8 h (TNF α). HAEC were pre-treated with shear stress at 10 dynes/cm² for 1 h before TNF α was injected into flow media without interruption. Cells were sheared in either the presence or absence of TNF α media for 8 h. Data are presented as mean $\pm$ SE ($n = 3$). * $p < 0.01$ and ** $p < 0.001$ refer to significant difference compared to static TNF α alone.

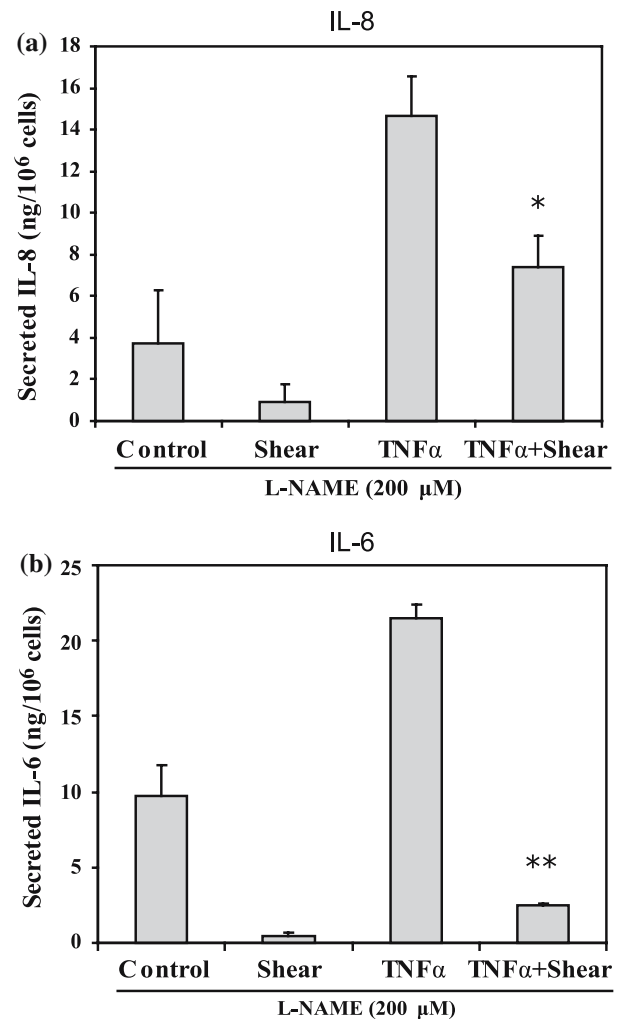


FIGURE 2. Effect of L-NAME on shear attenuation of TNF α induced IL-8 (a) and IL-6 (b) secretion in HAEC. Cells were pretreated with the eNOS inhibitor L-NAME (200 μ M) for 1 h. Static cells were maintained in media containing L-NAME and treated with TNF α (5 ng/mL) for 8 h. Sheared cells were further pre-conditioned at 10 dynes/cm² for 1 h in L-NAME media before TNF α was injected and sheared for another 8 h. Data are mean $\pm$ SE ($n = 3$ in each group). * $p < 0.005$, ** $p < 0.001$, refers to significant difference compared to static TNF α alone.

Either TNF α receptor mediated signaling proximal of NF κ B activation was disrupted in sheared cells and/or shear stress triggered factors, e.g. nitric oxide (NO), that antagonized NF κ B function. To test the role of shear induced NO production¹⁹ on TNF α stimulation of HAEC, we used an eNOS inhibitor nitro-L-arginine methylester (L-NAME) in conjunction with TNF α and shear stress (Fig. 2). HAEC were pretreated with the L-NAME (200 μ M, 1 h), a concentration known to block shear induced NO release.⁴⁶ HAEC preconditioned in static culture or shear condition (10 dynes/cm², 1 h) in the presence of L-NAME, were then maintained further in the presence or absence of TNF α (5 ng/mL). In comparing the static control groups from Figs. 1 and 2, there was a small increase of basal IL-8 secretion and a marked increase in IL-6 secretion by HAEC cells pretreated with L-NAME, indicating that the basal production of NO by static cells limited IL-8 and IL-6 expression. In static HAEC cultures, pretreatment with L-NAME did not prevent the marked increase of IL-8 and IL-6 secretion by TNF α , and again shearing in media with TNF α yielded a significant reduction of cytokine expression (50% for IL-8 and 90% for IL-6). With L-NAME present, shear stress reduced IL-6 production in TNF α -stimulated cells to levels below the matched static control cultures.

These data indicate that shear stress interfered with TNF α -induced increase of IL-8 and IL-6 without a strict requirement for flow-induced NO.

Shear Stress Attenuates Neutrophil Firm Arrest on TNF α -Activated HAEC

HAEC monolayers were treated with 5 ng/mL TNF α in the presence or absence of arterial shear stress for 5 h before a neutrophil adhesion assay at 1 dyne/cm². Time averaged images (Fig. 3) allowed detection of rolling and arrested neutrophils. On control cultures without TNF α , neutrophils passed over the endothelial surfaces with essentially no rolling or arrest. HAEC exposed to shear stress for 5 h alone (no TNF α) did not promote neutrophil adhesion, indicating that shear stress alone was not pro-adhesive. HAEC cells treated with TNF α , on the other hand, were strongly activated with about $38.61 \pm 6.92\%$ ($n = 15$ FOV) of interacting neutrophils becoming firmly arrested (Fig. 4a). Cells maintained under arterial shear stress for 5 h during the TNF α induction, however, had 60% ($p < 0.001$) fewer neutrophils converting to firm arrest ($11.7 \pm 4.59\%$ of all interacting neutrophils, $n = 15$ FOV). The total number of neutrophils (rolling and arrested) that came to interact

Neutrophil adhesion at 1 dyne/cm²

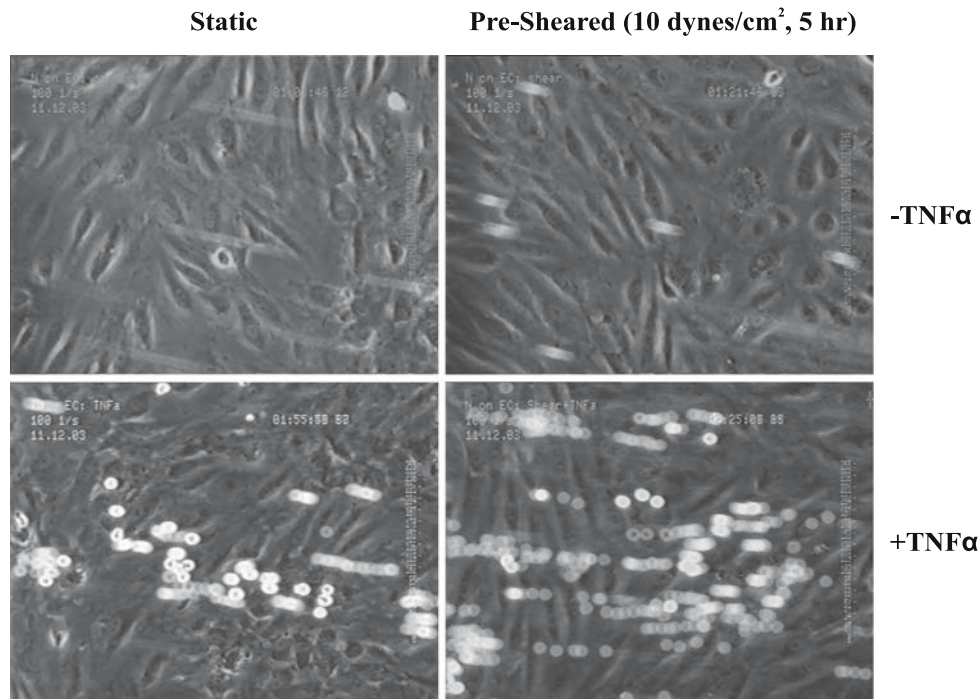


FIGURE 3. Neutrophil adhesion studies on HAEC monolayers. Static HAEC cultured on glass slides are treated with either media or TNF α (5 ng/mL) for 5 h before neutrophil assays. Sheared HAEC on glass slides are exposed to shear stress at 10 dynes/cm² for 5 h in media with or without TNF α before assaying with neutrophils. One-second image sequences were captured from 10-s video segments, and processed to generate the time-sequence images.

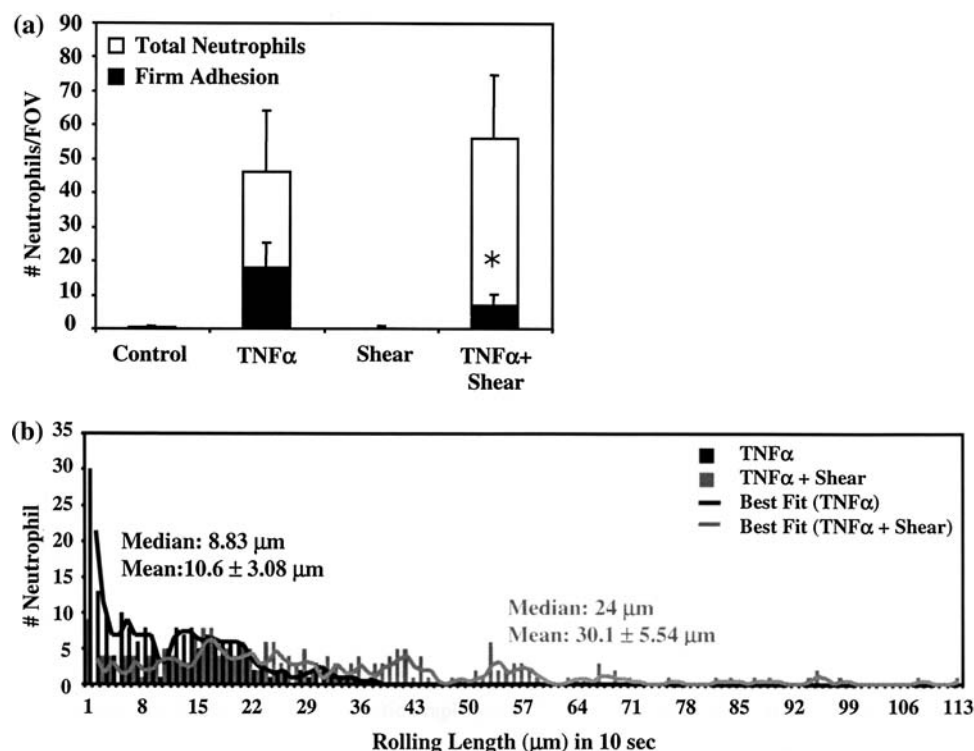


FIGURE 4. Total and firmly adhered neutrophil over TNF α treated sheared and static endothelial cells are quantified in (a) for 5 different images in each treatment. Data are presented as mean $\pm$ SE ($n = 5$). * $p < 0.001$, refers to significant difference in firm adhesion between TNF α treatment of HAEC under static or shear stress conditions. Finally, rolling length for 188 and 212 neutrophils over static and sheared HAEC, respectively, are generated and presented in a histogram (b). In each case, neutrophils are collected from 5 different FOVs.

with shear stressed HAEC plus TNF α vs. static HAEC treated with TNF α was not significantly different (56.1 ± 18.8 vs. 46.3 ± 17.9 per FOV).

To further quantify the neutrophil interactions with endothelial surfaces at 1 dyne/cm², the rolling length in microns over a 10-s interval was determined. The results are presented in Fig. 4b, in the form of a histogram, generated on the tracked movement of 188 and 212 neutrophils over static and sheared HAEC, respectively. On TNF α -stimulated HAEC in the absence of arterial shear stress exposure, most neutrophils had short rolling lengths (90% < 21 μ m) with the longest being 37 μ m and with a median length of 8.83 μ m. On sheared HAEC treated with TNF α , however, the rolling length increased up to 113 μ m with the median length of 24 μ m. The mean rolling length for both cases was also significantly different: 10.6 μ m (no shear) vs. 30.1 μ m (10 dyne/cm²) ($p < 0.001$).

Interactions Between Glucocorticoid Receptor and NF κ B in Sheared Endothelium

While disturbed hemodynamics may enhance endothelium susceptibility to atherosclerosis, we have

detected the anti-inflammatory effects of unidirectional arterial shear stress in attenuating TNF α -activated endothelial cytokine production (Figs. 1 and 2) and neutrophil adhesion (Figs. 3 and 4). The net effect of shear stress, however, encompasses a variety of transcriptional factors such as AP-1, SP-1, and GR^{15,16,21,34} to potentially regulate NF- κ B function in a promoter specific manner. We sought to investigate possible interactions between shear stress activated GR functions, independent of dexamethasone,¹⁵ on NF κ B function. To detect *cis* interactions between GR and NF κ B on a promoter, we employed an artificial promoter construct that is induced when GR and NF κ B bind the promoter. The wildtype SAA2 promoter contains binding sites for NF κ B, AP-1, and NF-IL6, along with a disrupted GRE site.²⁵ The inactive GRE sequence is interrupted in the middle by a function-blocking 9-bp insertion. Removal of this 9-bp insertion renders the promoter responsive to dexamethasone potentiation (Fig. 5a) in the presence of cytokine stimulation.³⁹ This artificial promoter construct based on the SAA2 deletion (pGRED) is unique in that, when induced by a cytokine, its transcriptional activity is *enhanced*, not repressed, by dexamethasone. This allows “light-up”

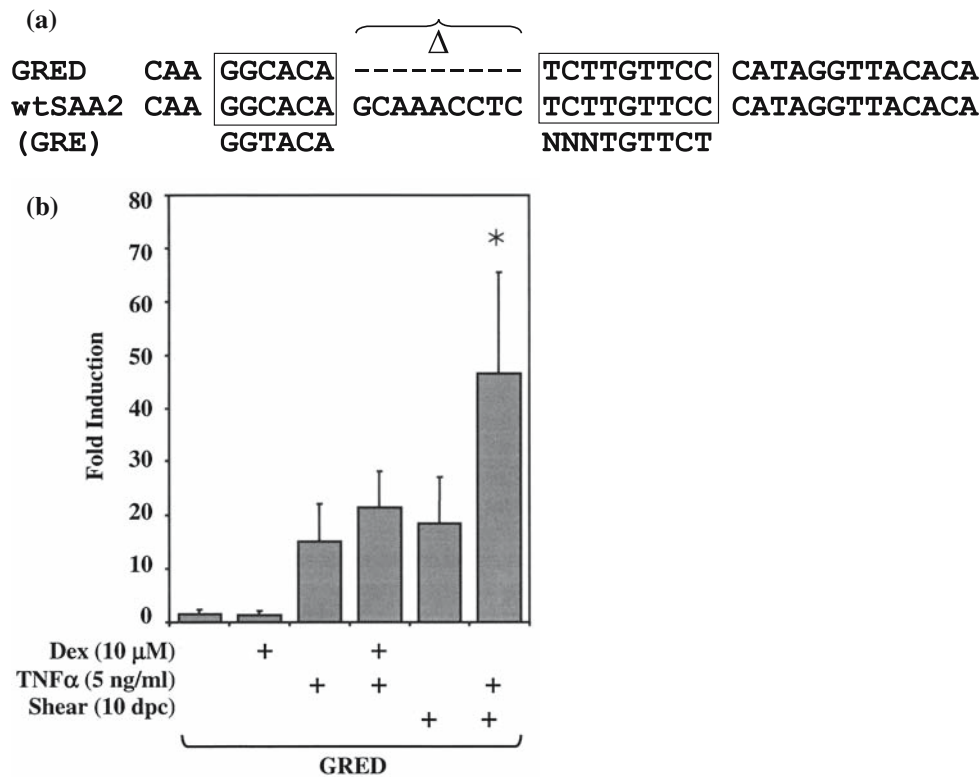


FIGURE 5. *cis* Interactions of GRE and NFκB in sheared endothelium. (a) Alignment of the region of SAA2 and GRED promoters encompassing the GRE sequence, as compared to a consensus GRE sequence. GRED carries an intact GRE sequence following a 9-basepair deletion (Δ) from the SAA2 promoter. (b) BAEC transfected with GRED plasmid with the Renilla control plasmid are maintained in medium only, 10 μM dexamethasone, 5 ng/mL TNFα, dexamethasone with TNFα, shear stress alone (10 dynes/cm², dpc) or shear stress and TNFα. Cells were harvested after 5 h treatment and relative luciferase values were quantified. Data are presented as mean ± SE (n = 3). *p = 0.05, **p < 0.005, refers to significant difference between treatments with shear stress alone and shear stress with TNFα.

detection of NFκB-GR cross talk. While dexamethasone typically down regulates NFκB function, this may occur via GR binding to NFκB, either on or off the promoter. Because GRED involves dexamethasone potentiation of NFκB, the GRED construct allows detection of GR modulation of activated NFκB on the promoter.

TNFα (5 ng/mL) caused a 15-fold induction of GRED which was further enhanced by dexamethasone (10 pM) (Fig. 5b). The GRED promoter was not responsive to dexamethasone alone, consistent with the enhancer function of GR when in the presence of NFκB on the promoter. Applying shear stress alone activated GRED, thus detecting activation of both GR and NFκB by shear stress. The combination of TNFα with shear stress caused a striking 40-fold elevation of transcriptional activity from GRED. Taken together, these data suggest that having the intact GRE sequence present renders the GRED promoter highly responsive to shear stress, particularly in the presence of strong NFκB functionality in TNFα-stimulated endothelium.

DISCUSSION

Shear stress activation of GR receptor and GRE transcriptional regulation¹⁵ provides a mechanism for potential cross talk between mechanotransduction and anti-inflammatory actions. In this study, we demonstrated that shear stress at 10 dynes/cm² attenuated TNFα-stimulated IL-6 and IL-8 expression in cultured human endothelial cells (Figs. 1 and 2). Shear induced inhibition of TNFα-stimulated IL-6 and IL-8 expression did not strictly require the presence of NO (Fig. 2), which suggests that the anti-inflammatory actions of shear stress is independent of its vasodilatory effects through stimulated NO release. We have previously shown that pretreating endothelial cells with L-NAME had no effect on shear induced GRE-SEAP promoter construct activation at 6 h, as a metric of shear activation of endogenous GR function. The anti-inflammatory effect of shear stress was also apparent in neutrophil-endothelial interactions. Endothelial monolayers exposed to arterial shear and static endothelium respond differently to TNFα

stimulation, as evidenced by more sustained neutrophil rolling (longer rolling length) and less conversion to firm arrest on cells pre-exposed to 10 dyne/cm² (Figs. 3 and 4). A likely explanation for the increased rolling length could be the decreased expression of IL-8 and/or reduced presentation of ICAM-1 or VCAM-1 by sheared endothelial cells. The GRED promoter construct was designed to provide a “light-up” signal to detect GR-NF κ B interactions at the level of a promoter. Shear stress proved to be a particularly strong inducer of the GRED promoter in TNF α -stimulated endothelium.

Previous research has shown that IL-8 in solution rapidly induces rolling neutrophils to arrest. Also, increasing immobilized IL-8 decreases neutrophil rolling distance and promotes firm adhesion.⁸ IL-8 in the fluid phase or bound to endothelium glycoaminoglycan may increase β 2-integrin avidity, leading to neutrophil firm arrest through ICAM-1/ β 2 integrin interactions.³⁸ Sheared endothelial cells express less IL-8 upon cytokine stimulation (Fig. 1a), which may lead to less neutrophil firm adhesion and longer rolling lengths. Chiu *et al.* showed decreased DNA binding activity of NF κ B in cells that were exposed to shear stress in addition to TNF α .⁵ This finding of NF κ B down regulation is quite consistent with the reduced IL-6 and IL-8 expression in sheared HAEC stimulated with TNF α that we demonstrated. In fact, this decreased IL-6 and IL-8 presentation may contribute significantly to altered interactions between neutrophils and sheared endothelium since its mechanism is independent of NO inhibitor. On the other hand, NO inhibitor has been shown to abolish the attenuating effect of shearing on elevated endothelial VCAM-1 expression induced by TNF α and lipopolysaccharide.⁴¹ Finally, we also saw that shearing endothelial cells alone, in the absence of cytokine stimulation, does not promote neutrophil adhesiveness, possible because shear stress alone does not substantially induce expression of VCAM-1 or ICAM-1 on HAEC.⁴²

While increased P-selectin or E-selectin facilitates neutrophil rolling and ICAM-1 or VCAM-1 expression aids in neutrophil firm adhesion, our findings in Fig. 4a suggest that shear stress influenced only the conversion to firm arrest since total interacting neutrophils (rolling and arrested) were the same regardless of preshearing. Prior studies have investigated various aspects of endothelial response to stimulation by TNF α in the presence of shear stress, although none have previously measured endothelial adhesiveness to human neutrophils. Yamawaki *et al.*⁴⁸ showed in an *ex vivo* model of rabbit aorta that shear stress inhibited TNF α stimulated VCAM-1 expression. Chiu *et al.*⁵ also demonstrated in human umbilical endothelial cells that that stress decreases TNF α -induced VCAM-1 and

E-selectin expression, while enhancing TNF α -induced ICAM-1 mRNA and protein expression. It is difficult to predict the net effect on neutrophil adhesion from these two prior studies since E-selectins, ICAM-1 and VCAM-1 are being altered in differing ways. Additionally, the membrane-cytoskeletal structure function is likely altered in endothelial cells during shear stress exposure and this may have subsequent effect on bond life, independent of receptor number due to changes in membrane extension and tethering. The VCAM-1 data from these studies correlates well with the reduced neutrophil interactions that we saw. No change in net rolling, a selectin-mediated process, was observed in our measurements due to preshearing of the TNF α -stimulated endothelium. On the other hand, ICAM-1 levels under shear has been consistently observed to be different from VCAM-1 or E-selectin,^{5,7,17,32} and these studies suggested that both NF κ B transcriptional activation and oxidative stress (reactive oxygen species) differentially influence TNF α induced secretion of cytokines and adhesion molecules. The ICAM-1 promoter region contains binding sites for AP-1, SP-1 and NF κ B, rendering its expression sensitive to regulation by a number of transcriptional factors under both shear stress and cytokine stimulations.³¹ The effect of arterial shear stress on TNF α activation of endothelium has been addressed before in previous studies.⁴⁸ However, this is the first functional assay of direct neutrophil-endothelial interactions under both TNF α and shear stress stimulation. Though previous studies have presented data on expression levels of adhesion molecules, there were no direct measurements of altered endothelial adhesiveness toward neutrophils. This is the first study to measured alteration of neutrophil rolling on TNF α -stimulated endothelium due to pre-exposure to arterial shear stress.

In an experiment of this type, LNAME may have regulatory effects on baseline properties of endothelium, effects on TNF α -stimulated properties, and effects on mechanobiological responses via NO in the presence or absence of TNF α . For example, LNAME is known to have unexpected additional actions on endothelium beyond the inhibition of NO production²² since NO is active as an autocrine agent. We note that LNAME reduced IL-8 production by TNF α -stimulated endothelium under no-flow conditions. In contrast, LNAME on its own (without TNF α (or flow) enhanced IL-6 production indicating an additional role of LNAME on IL-6 regulation not seen for IL-8. Complex autocrine loops regulating the IL-6 and IL-8 genes may become unmasked with the use of chemical inhibitors such as LNAME and this is seen with IL-6 which was up-regulated by the use of LNAME on its own. Still, shear stress markedly reduced TNF α -stimulated IL-8 and IL-6 production as seen in Figs. 1 and 2.

The anti-inflammatory effect of shear stress on TNF α activation is further supported by microarray studies of endothelial gene expression.⁴ As microarray studies of gene expression became more sophisticated, a recent study analyzed differential changes in endothelial transcription profiles of disturbed vs. undisturbed laminar flow regions of the same pig aorta.²⁸ Proinflammatory adhesion molecules such as VCAM-1, ICAM-1, E-selectin, P-selectin were not differentially expressed in these regions, while IL-6 and IL-8 receptor β are up-regulated in disturbed regions and IL-8 is slightly down-regulated in disturbed regions. These data are in good agreement with our data on the attenuating effect of elevated shear stress on IL-6 and IL-8 expression. Taken together, these data suggest that interleukins (IL-6, IL-8) display increased sensitivity toward varying flow conditions, and changes in their expression may be a precursor to altered presentation of inflammatory adhesion molecules.

As an initial step toward studying shear activated GR and NF κ B function, we studied the interaction between shear activated GR and NF κ B at their corresponding promoter sites, utilizing a modified SAA2 promoter constructs that presents binding sites for AP-1, SP-1, and a functional GRE sequence resulted in overall increased activation of reporter gene. It should also be noted that binding of shear induces and activates fos/jun (the AP-1 complex), which can also antagonize NF κ B function. Shear stress activated GR pathway, independent of dexamethasone, may interfere with cytokine enhanced NF κ B functions in inflammation. However, the overall effect of shear stress encompasses a variety of transcriptional factors, including AP-1, SP-1, and NF- κ B that may interfere with GR transcriptional functions as well.

TNF α is a strong activator of NF κ B,^{27,30} a key transcription factor in the up-regulated expression of inflammatory markers including IL-6 and IL-8.^{20,44} Previous studies have shown that NF κ B mediated interleukin expression can be repressed by ligand activated GR,^{26,45} suggesting that shear stress may also exert its inhibitory effects against TNF α through the activation of GR and GRE pathway. The atheroprotective effects of shear stress on the endothelium may be attributed to various anti-inflammatory processes, along with its ability to modulate the release of vasoactive factors such as NO, prostacyclin, endothelin-1, MCP-1, and vascular epidermal growth factor (VEGF).^{9,11,12,36} Suppressing inflammation could be a key mechanism by which shear stress exerts its atheroprotective functions in the endothelium. Recent analysis of gene expression profile in normal pig aorta revealed that the endothelium in disturbed flow region is primed for inflammation²⁸ where genes for several

general pro-inflammatory cytokines and receptors such as interleukin 1 α , IL-1 receptor 1, IL-6, IL-8 receptor β , and monocyte chemotactic protein 1 (MCP-1) are up regulated compared to laminar flow area. However, the NF κ B system is primarily inactivated, consistent with the unaffected expression of inflammatory cells adhesion molecules between two flow regions. Thus, while varying hemodynamics may alter endothelium liability to atherosclerosis, we presented data supporting the anti-inflammatory effects of shear stress in inhibiting TNF α activated endothelial activation and neutrophil interactions. The net effect of shear stress encompasses a variety of transcriptional factors such as AP-1, SP-1, and GR to regulate NF κ B function in a promoter specific manner.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant #HL64388 and #HL56621. J.Y.J. is a National Science Foundation Graduate Fellow and NIH Bioengineering Training Grant in Cardiovascular Physiology Recipient (5T32HL07954).

REFERENCES

- ¹Asgeirsdottir, S. A., R. J. Kok, M. Everts, D. K. Meijer, and G. Molema. Delivery of pharmacologically active dexamethasone into activated endothelial cells by dexamethasone-anti-E-selectin immunoconjugate. *Biochem. Pharmacol.* 65(10):1729–1739, 2003.
- ²Barnes, P. J. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. (Lond.)* 94(6):557–572, 1998.
- ³Brostjan, C., J. Anrather, V. Csizmadia, G. Natarajan, and H. Winkler. Glucocorticoids inhibit E-selectin expression by targeting NF-kappaB and not ATF/c-Jun. *J. Immunol.* 158(8):3836–3844, 1997.
- ⁴Chen, B. P., Y. S. Li, Y. Zhao, *et al.* DNA microarray analysis of gene expression in endothelial cells in response to 24-h shear stress. *Physiol. Genomics* 7(1):55–63, 2001.
- ⁵Chiu, J. J., P. L. Lee, C. N. Chen, *et al.* Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor-[alpha] in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 24(1):73–79, 2004.
- ⁶Cronstein, B. N., S. C. Kimmel, R. I. Levin, F. Martiniuk, and G. Weissmann. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 89(21):9991–9995, 1992.
- ⁷De Caterina, R., P. Libby, H. B. Peng, *et al.* Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion

- molecules and proinflammatory cytokines. *J. Clin. Invest.* 96(1):60–68, 1995.
- ⁸DiVietro, J. A., M. J. Smith, B. R. Smith, L. Petruzzelli, R. S. Larson, and M. B. Lawrence. Immobilized IL-8 triggers progressive activation of neutrophils rolling *in vitro* on P-selectin and intercellular adhesion molecule-1. *J. Immunol.* 167(7):4017–4025, 2001.
 - ⁹Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A. M. Zeiher. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399(6736):601–605, 1999.
 - ¹⁰Eickelberg, O., M. Roth, R. Lox, et al. Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. *J. Biol. Chem.* 274(2):1005–1010, 1999.
 - ¹¹Frangos, J. A., S. G. Eskin, L. V. McIntire, and C. L. Ives. Flow effects on prostacyclin production by cultured human endothelial cells. *Science* 227(4693):1477–1479, 1985.
 - ¹²Gan, L., M. Miocic, R. Doroudi, L. Selin-Sjogren, and S. Jern. Distinct regulation of vascular endothelial growth factor in intact human conduit vessels exposed to laminar fluid shear stress and pressure. *Biochem. Biophys. Res. Commun.* 272(2):490–496, 2000.
 - ¹³Goel, M. S., and S. L. Diamond. Neutrophil enhancement of fibrin deposition under flow through platelet-dependent and -independent mechanisms. *Arterioscler. Thromb. Vasc. Biol.* 21(12):2093–2098, 2001.
 - ¹⁴Inoue, H., K. Umehono, T. Nishimori, Y. Hirata, and T. Tanabe. Glucocorticoid-mediated suppression of the promoter activity of the cyclooxygenase-2 gene is modulated by expression of its receptor in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 254(2):292–298, 1999.
 - ¹⁵Ji, J. Y., H. Jing, and S. L. Diamond. Shear stress causes nuclear localization of endothelial glucocorticoid receptor and expression from the GRE promoter. *Circ. Res.* 92(3):279–285, 2003.
 - ¹⁶Khachigian, L. M., N. Resnick, M. A. Gimbrone, Jr., and T. Collins. Nuclear factor-kappa B interacts functionally with the platelet-derived growth factor B-chain shear-stress response element in vascular endothelial cells exposed to fluid shear stress. *J. Clin. Invest.* 96(2):1169–1175, 1995.
 - ¹⁷Khan, B. V., D. G. Harrison, M. T. Olbrych, R. W. Alexander, and R. M. Medford. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 93(17):9114–9119, 1996.
 - ¹⁸Ku, D. N., D. P. Giddens, C. K. Zarins, and S. Glagov. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis* 5(3):293–302, 1985.
 - ¹⁹Kuchan, M. J., and J. A. Frangos. Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am. J. Physiol.* 266(3 Pt 1):C628–636, 1994.
 - ²⁰Lakshminarayanan, V., E. A. Drab-Weiss, and K. A. Roebuck. H₂O₂ and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells. *J. Biol. Chem.* 273(49):32670–32678, 1998.
 - ²¹Lan, Q., K. O. Mercurius, and P. F. Davies. Stimulation of transcription factors NF kappa B and AP1 in endothelial cells subjected to shear stress. *Biochem. Biophys. Res. Commun.* 201(2):950–956, 1994.
 - ²²Lefer, D. J., R. Scalia, B. Campbell, et al. Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia-reperfusion injury in rats. *J. Clin. Invest.* 99(4):684–691, 1997.
 - ²³McEver, R. P., K. L. Moore, and R. D. Cummings. Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J. Biol. Chem.* 270(19):11025–11028, 1995.
 - ²⁴McKay, L. I., and J. A. Cidlowski. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* 20(4):435–459, 1999.
 - ²⁵Meek, R. L., S. Urieli-Shoval, and E. P. Benditt. Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: implications for serum amyloid A function. *Proc. Natl. Acad. Sci. U.S.A.* 91(8):3186–3190, 1994.
 - ²⁶Nyhlen, K., M. Linden, R. Andersson, and S. Uppugunduri. Corticosteroids and interferons inhibit cytokine-induced production of IL-8 by human endothelial cells. *Cytokine* 12(4):355–360, 2000.
 - ²⁷Osborn, L., S. Kunkel, and G. J. Nabel. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. U.S.A.* 86(7):2336–2340, 1989.
 - ²⁸Passerini, A. G., D. C. Polacek, C. Shi, et al. Coexisting proinflammatory and antioxidative endothelial transcription profiles in a disturbed flow region of the adult porcine aorta. *Proc. Natl. Acad. Sci. U.S.A.* 101(8):2482–2487, 2004.
 - ²⁹Ray, A., and K. E. Prefontaine. Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* 91(2):752–756, 1994.
 - ³⁰Read, M. A., M. Z. Whitley, A. J. Williams, and T. Collins. NF-kappa B and I kappa B alpha: an inducible regulatory system in endothelial activation. *J. Exp. Med.* 179(2):503–512, 1994.
 - ³¹Roebuck, K. A., and A. Finnegan. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J. Leukoc. Biol.* 66(6):876–888, 1999.
 - ³²Roebuck, K. A., A. Rahman, V. Lakshminarayanan, K. Janakidevi, and A. B. Malik. H₂O₂ and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. *J. Biol. Chem.* 270(32):18966–18974, 1995.
 - ³³Ross, R. Atherosclerosis-an inflammatory disease. *N. Engl. J. Med.* 340(2):115–126, 1999.
 - ³⁴Schwachtgen, J. L., P. Houston, C. Campbell, V. Sukhatme, and M. Braddock. Fluid shear stress activation of egr-1 transcription in cultured human endothelial and epithelial cells is mediated via the extracellular signal-related kinase 1/2 mitogen-activated protein kinase pathway. *J. Clin. Invest.* 101(11):2540–2549, 1998.
 - ³⁵Sheppard, K. A., K. M. Phelps, A. J. Williams, et al. Nuclear integration of glucocorticoid receptor and nuclear factor-kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. *J. Biol. Chem.* 273(45):29291–29294, 1998.
 - ³⁶Shyy, Y. J., H. J. Hsieh, S. Usami, and S. Chien. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular

- endothelium. *Proc. Natl. Acad. Sci. U.S.A.* 91(11):4678–4682, 1994.
- ³⁷Simoncini, T., S. Maffei, G. Basta, *et al.* Estrogens and glucocorticoids inhibit endothelial vascular cell adhesion molecule-1 expression by different transcriptional mechanisms. *Circ. Res.* 87(1):19–25, 2000.
- ³⁸Smith, C. W. Leukocyte-endothelial cell interactions. *Semin. Hematol.* 30(4 Suppl. 4):45–53, 1993: discussion 54–45.
- ³⁹Thorn, C. F., and A. S. Whitehead. Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. *J. Immunol.* 169(1):399–406, 2002.
- ⁴⁰Tousoulis, D., G. Davies, C. Stefanadis, P. Toutouzas, and J. A. Ambrose. Inflammatory and thrombotic mechanisms in coronary atherosclerosis. *Heart* 89(9):993–997, 2003.
- ⁴¹Tsao, P. S., R. Buitrago, J. R. Chan, and J. P. Cooke. Fluid flow inhibits endothelial adhesiveness. Nitric oxide and transcriptional regulation of VCAM-1. *Circulation* 94(7):1682–1689, 1996.
- ⁴²Tsao, P. S., N. P. Lewis, S. Alpert, and J. P. Cooke. Exposure to shear stress alters endothelial adhesiveness. Role of nitric oxide. *Circulation* 92(12):3513–3519, 1995.
- ⁴³Tuckermann, J. P., H. M. Reichardt, R. Arribas, K. H. Richter, G. Schutz, and P. Angel. The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin. *J. Cell. Biol.* 147(7):1365–1370, 1999.
- ⁴⁴Vanden Berghe, W., K. De Bosscher, E. Boone, S. Plaisance, and G. Haegeman. The nuclear factor-kappaB engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *J. Biol. Chem.* 274(45):32091–32098, 1999.
- ⁴⁵Vanden Berghe, W., E. Francesconi, K. De Bosscher, M. Resche-Rigon, and G. Haegeman. Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-kappaB-dependent mechanism. *Mol. Pharmacol.* 56(4):797–806, 1999.
- ⁴⁶Wang, W., and S. L. Diamond. Does elevated nitric oxide production enhance the release of prostacyclin from shear stressed aortic endothelial cells? *Biochem. Biophys. Res. Commun.* 233(3):748–751, 1997.
- ⁴⁷Xu, X., M. Otsuki, H. Saito, *et al.* PPARalpha and GR differentially down-regulate the expression of nuclear factor-kappaB-responsive genes in vascular endothelial cells. *Endocrinology* 142(8):3332–3339, 2001.
- ⁴⁸Yamawaki, H., S. Lehoux, and B. C. Berk. Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused *ex vivo*. *Circulation* 108(13):1619–1625, 2003.