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Relative Reduction of Endothelial Nitric-Oxide Synthase Expression and Transcription in Atherosclerosis-Prone Regions of the Mouse Aorta and in an *in Vitro* Model of Disturbed Flow

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Atherosclerosis develops in distinct regions of the arterial tree. Defining patterns and mechanisms of endothelial cell gene expression in different regions of normal arteries is key to understanding the initial molecular events in atherogenesis. In this study, we demonstrated that the expression of endothelial nitric-oxide synthase (eNOS), an atheroprotective gene, and its phosphorylation on Ser¹¹⁷⁷, a marker of activity, were lower in regions of the normal mouse aorta that are predisposed to atherosclerosis. The same expression pattern was observed in mouse strains that are both susceptible and resistant to atherosclerosis, and the topography of eNOS expression was inverse to p65, the main nuclear factor- κ B subunit. Modeling of disturbed and uniform laminar flow *in vitro* reproduced the expression patterns of eNOS and p65 that were found *in vivo*. Heterogeneous nuclear RNA expression and RNA polymerase II chromosome immunoprecipitation studies demonstrated that regulation of transcription contributed to increased eNOS expression in response to shear stress. *In vivo*, the transcription of eNOS was reduced in regions of the mouse aorta predisposed to atherosclerosis, as defined by reporter gene expression in eNOS promoter- β -galactosidase reporter transgenic mice. These data suggest that disturbed hemodynamic pat-

terns found at arterial branches and curvatures uniquely modulate endothelial cell gene expression by regulating transcription, potentially explaining why these regions preferentially develop atherosclerosis when risk factors such as hypercholesterolemia are introduced. (Am J Pathol 2007, 171:1691–1704; DOI: 10.2353/ajpath.2007.060860)

Endothelial cells (ECs) line the inner wall of arteries and play a central role in atherogenesis. In normal arteries, ECs at sites predisposed to atherosclerosis exhibit unique gene expression patterns,^{1,2} and during hypercholesterolemia, they regulate the recruitment of circulating monocytes to the arterial intima, a process that begins during the earliest stages of atherogenesis and is critical to the initiation and progression of lesions.³ Thus, elucidating mechanisms of EC gene expression in specific regions of the arterial tree may provide insights into disease pathogenesis.

Most risk factors for atherosclerosis, such as hyperlipidemia, hypertension, diabetes mellitus, and smoking, are systemic, and the endothelium of the entire arterial tree is exposed to them. However, atherosclerotic lesions form preferentially at or near branch points, bifurcations, and curvatures.⁴ This distribution pattern suggests that local factors, such as hemodynamic forces (shear stress), influence the initiation of atherosclerosis. Blood flow in these regions is complex or disturbed laminar, in

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contrast to straight segments of the arterial tree, where the blood flow is uniformly laminar.⁵ ECs sense hemodynamic forces and respond to temporal or spatial gradients by modulating the expression of genes. Introduction of shear stress to endothelium cultured under static conditions activates various signal transduction pathways and modulates the expression of adhesion molecules,^{6,7} and mechanosensory complexes have been identified.^{8,9} Different shear stress profiles can induce distinct repertoires of endothelial gene expression.¹⁰ *In vitro* modeling has revealed that uniform laminar flow induces a sustained expression of genes that may be "atheroprotective," such as endothelial nitric-oxide synthase (eNOS or NOS-3) and cyclooxygenase-2.^{11,12} Oscillatory and sinusoidal flows induce expression of eNOS to a lesser extent than uniform laminar flow,^{13,14} as well as production of hydrogen peroxide,¹⁵ which is shown to induce eNOS expression.¹⁶ *Ex vivo* modeling of oscillatory shear stress resulted in increased endothelial cell dysfunction and reduced eNOS expression compared with freshly harvested arteries or arteries that are subjected to uniform laminar shear stress.¹⁷ Disturbed hemodynamic forces may induce a unique pattern of EC gene expression that predisposes these arterial regions to atherosclerotic lesion formation if appropriate systemic risk factors are present.¹⁸ Possibilities include down-regulation of atheroprotective genes and induction of genes that modulate pro-inflammatory signal transduction or produce matrix components that enhance trapping of lipoproteins.

eNOS is the major enzyme responsible for nitric oxide (NO) production in vascular endothelium.^{19,20} eNOS activity is regulated through various posttranslational modifications, including phosphorylation, myristoylation, and palmitoylation. Phosphorylation of different residues can activate or deactivate eNOS. The best characterized residues are the activation site Ser¹¹⁷⁷ and inhibitory sites Ser¹¹⁶ and Thr⁴⁹⁵.²¹ Several kinases that phosphorylate Ser¹¹⁷⁷ have been identified, including Akt/phosphokinase B and phosphokinase A.^{22–25} eNOS participates in diverse vascular processes, including control of blood pressure,²⁶ local vascular tonus,^{27,28} and remodeling in response to altered shear stress.²⁹ eNOS and its product NO are highly relevant to atherogenesis, and are generally considered to be protective. Hypercholesterolemia decreases eNOS activity and expression, which may contribute to EC dysfunction.^{30,31} In hypercholesterolemic mice, administration of L-arginine and antioxidants induces increased expression of eNOS in atherosclerosis-prone and atherosclerosis-resistant regions of the vessel.³² Increased NO production by L-arginine supplementation during hypercholesterolemia can reduce the formation of atherosclerotic lesions.^{33,34} In murine studies, deficiency of eNOS in the ApoE-null background results in increased atherosclerotic lesion formation, which can only partially be attributed to hypertension.^{35–37} eNOS gene transfer studies in rats have shown that NO can also inhibit various atherosclerotic events, including platelet-derived growth factor-induced smooth muscle cell proliferation and migration^{38,39} and inhibition of vascular smooth muscle cell migration through inhibition of matrix metalloproteinase 2 and 9 activity.³⁹

NO may exert its atheroprotective effects through a variety of cellular pathways. It can reduce EC apoptosis and activation, the latter through inhibition of nuclear factor- κ B (NF- κ B) activity and inflammatory gene expression.^{40,41} A well-established anti-inflammatory property of NO is its ability to inhibit leukocyte-EC adhesive interactions, exocytosis of Weibel-Palade bodies, which contains von Willebrand's factor and P-selectin, and platelet aggregation.^{42–46} Although eNOS is generally accepted to be atheroprotective, murine studies using a high cholesterol-cholate-containing diet⁴⁷ or transgenic mice overexpressing eNOS⁴⁸ yielded conflicting results. This outcome may be due to "uncoupling" of eNOS from NO production as a consequence of substrate or cofactor deficiency, resulting in the generation of superoxide and/or oxidation of NO or cofactors by reactive oxygen species.^{48,49} Supplementation of mice with the eNOS cofactor tetrahydrobiopterin reduced atherosclerosis.⁴⁸

Previously, we mapped regions of the mouse ascending aorta and arch with high and low probability regions for developing atherosclerosis,⁵⁰ which are found in the lesser and greater curvature (LC and GC) regions of the ascending arch, respectively. We demonstrated that the expression levels of p65, a component of the NF- κ B signal transduction pathway, and its inhibitors I κ B α and I κ B β , were 5- to 18-fold higher in the high probability region of normal C57BL/6 mice.¹ Despite higher expression of critical components of the NF- κ B signaling pathway, NF- κ B nuclear signaling was activated in only a minority of ECs, as measured by nuclear translocation of p65. Importantly, activation of NF- κ B by systemic administration of lipopolysaccharide or feeding low-density lipoprotein receptor-deficient (*ldlr*^{-/-}) mice an atherogenic diet resulted in translocation of NF- κ B into the nucleus and up-regulation of NF- κ B-inducible genes preferentially in high probability region endothelium. These data suggested that high steady-state expression levels of NF- κ B/I κ B components promote regional activation of endothelial NF- κ B signaling in response to systemic stimuli and may contribute to the localization of atherosclerotic lesions.

In the current study, we evaluated the expression of eNOS and p65 in ECs located in atherosclerosis-prone and -protected regions of the normal mouse arteries. Expression of eNOS protein and mRNA was reduced in prone relative to protected regions, which was opposite to the topography of p65 expression. eNOS promoter- β -galactosidase reporter transgenic mice demonstrated that regulation of transcription contributed to differential regional expression of eNOS in arterial ECs.

Materials and Methods

Animals

C57BL/6 mice, 129 $\times$ 1/SvJ, 129, C3SW-H2b/SnJ congenic strain (C3H), B6.129P2-Nos3^{tm1Unc}/J (eNOS^{-/-}) strain (Jackson Laboratory, Bar Harbor, ME), and eNOS-promoter- β -galactosidase-reporter transgenic mice⁵¹ in a mixed C57BL/6-SJL strain were used at an age of 2 to

6 months. Mice were fed a standard laboratory chow diet, bred and housed at the University Health Network Animal Facility, and maintained in accordance with guidelines of the Canadian Council on Animal Care. *Ldlr*^{-/-} mice (C57BL/6 strain; Jackson Laboratory) were fed a cholesterol-enriched diet, and staining with oil red O was performed as described previously.⁵⁰

In Vitro Flow Chambers

Two different chamber designs were used for *in vitro* experiments: parallel plate/step and conventional parallel plate flow chambers. Porcine aortic ECs (passages 3 to 7) were used in parallel plate/step flow chambers experiments because they adhere very well to glass, and unlike human cells, they withstand exposure to disturbed flow for several days. Porcine ECs were cultured to confluence on glass coverslips (no. 1; 22 × 40 mm) in M199 medium supplemented with 10% fetal bovine serum and antibiotics as described previously.⁵² Coverslips were placed in modified Series 30 parallel plate flow chambers (Warner Instruments, Hamden, CT). To produce a step in the flow path, two 250- μ m-thick silicon gaskets with different flow channel lengths were stacked together so that the flow channel height before and after the step was 250 and 500 μ m, respectively (Figure 2A). The width of the flow path was 15 mm. A flow of 56 ml/min was maintained for 48 or 72 hours, resulting in a shear stress of 10 dynes/cm² in the region of uniform laminar flow downstream of the step. Human aortic endothelial cells (HAECs) (passages 4 to 7) (Clonetics, San Diego, CA) were used in conventional parallel plate flow chamber experiments, because the nucleotide sequence of human genes required for the design of PCR primers was readily available. Human ECs were cultured to confluence on sterile glass slides (Corning, Corning, NY) coated with human fibronectin (100 μ g/ml) (BD Biosciences, Mississauga, ON, Canada) in complete MCDB-131 medium (Vec Technologies, Rensselaer, NY). Slides were placed in parallel plate flow chambers, as previously described⁵³ and exposed to 10 dynes/cm² shear stress for 24 hours. In all experiments, each conventional or parallel plate/step flow chamber was incorporated into a closed-loop media circulation system consisting of a media reservoir chamber with humidified 5% CO₂, a peristaltic pump, and a flow dampener.

Immunofluorescence Staining

Immunofluorescence staining of mouse aortic and cultured porcine ECs was performed as described previously.^{1,50} Aortas of 8- to 10-week-old mice were perfused with PBS followed by 2% paraformaldehyde for 10 minutes, whereas EC monolayers cultured on glass coverslips were washed with PBS briefly and fixed with 2% paraformaldehyde for 15 minutes immediately after flow experiments. After fixation, specimens were incubated in 3% hydrogen peroxide/PBS to quench endogenous peroxidase and permeabilized with 0.2% Triton X-100. Primary antibody incubations were performed for 2 hours at room temperature or overnight at 4°C.

Antibodies included the following: rabbit anti-phospho-eNOS (phospho-Ser¹¹⁷⁷) (Cell Signaling Technology, Danvers, MA), rabbit anti-eNOS (Transduction Laboratories, Lexington, KY), rabbit anti- β -galactosidase (Cortex Biochem, San Leandro, CA), goat anti-p65, mouse anti- β -catenin, and goat anti- β -catenin (Santa Cruz Biotechnology, Santa Cruz, CA). A 30-minute incubation with a secondary antibody followed a brief wash. The following antibodies were used: for eNOS and p65, a biotin-conjugated donkey anti-rabbit or anti-goat IgG (Molecular Probes, Eugene, OR); for β -galactosidase, a biotin-conjugated donkey anti-rabbit IgG or Alexa Fluor 546-conjugated goat anti-rabbit; and for β -catenin, a Cy3-conjugated rabbit anti-mouse or donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Biotinylated secondary antibodies were visualized using streptavidin-conjugated horseradish peroxidase in conjunction with FITC-conjugated Tyramide Signal Amplification kit (PerkinElmer Life Sciences, Boston, MA). Nuclei were counterstained with propidium iodide or TOTO-3 (Molecular Probes). Negative controls included addition of blocking peptide to the primary antibody, and nonimmune goat or rabbit IgG. Aortic specimens were mounted for confocal microscopy (MRC-1024ES; Bio-Rad, Hercules, CA), and LC and GC regions were located using three anatomical landmarks.¹ After immunostaining, coverslips with cultured ECs were mounted on a glass slide using Vecta Shield (Vector Labs, Burlingame, CA) and examined with a confocal microscope or a conventional fluorescence microscope (Nikon Eclipse TE300). Digital images were acquired using Confocal Assistant (shareware) and analyzed using Adobe Photoshop (Adobe, San Jose, CA). Gene expression levels were quantified by calculating the percentage of pixels with fluorescence signals that were greater than the background and by assessing the intensity of the positive signals, as described previously.¹

Isolation of Endothelium from the LC and GC of the Mouse Ascending Aortic Arch and Preparation of cDNA

Ascending aortas were harvested from C57BL/6 mice and opened en face.⁵⁰ The endothelium was placed against a nitrocellulose-coated slide (Molecular Probes) for 5 minutes, and the media and adventitia were peeled away leaving the endothelial monolayer adherent to the nitrocellulose-coated slide. The monolayer was fixed immediately with 70% ethanol, and nuclei were stained with RNase-free HistoGene LCM staining solution (Arcturus Inc., Mountain View, CA). LC and GC regions were identified by their location and nuclear density. Cells were scraped directly into RNA extraction buffer, and mRNA was isolated using PicoPure RNA Isolation kit (Arcturus Inc.) and reverse-transcribed into cDNA using random decamers (Ambion, Austin, TX) and a Powerscript kit (Clontech, Palo Alto, CA). For each experiment, LC and GC regions from two to four mice were pooled.

Table 1. Real-Time PCR Primers for Quantification of mRNA

Gene	Sequence
mu eNOS F*	5'-TCCGGAAGCGTTTGATC-3'
mu eNOS R*	5'-GCCAAATGTGCTGGTCACC-3'
mu p65 F*	5'-GCTCAGCGGGCAGTATTCC-3'
mu p65 R*	5'-TGTTCCCTGGTCCCTGTGTAGCC-3'
mu ICAM-2	5'-ACCATTGAGTGCACGGTGTG-3'
mu ICAM-2	5'-GCTCCCCAAAGGTCTGATT-3'
mu CD31 F*	5'-TCAGAACCCTCAGGAGTGAATACG-3'
mu CD31 R*	5'-TGCTTGGAGGTGGCTACAATCG-3'
hu eNOS F*	5'-TGGACCTGGATACCCGGAC-3'
hu eNOS R*	5'-TGGTGACTTTGGCTAGCTGGT-3'
hu p65 F*	5'-CAACCCCTTCCAGTTCCTATAGA-3'
hu p65 R*	5'-CCTGCCTGATGGGTCCC-3'
hu $\text{I}\kappa\text{B}\alpha$ F*	5'-CCAACAGCCAGAAATTGCT-3'
hu $\text{I}\kappa\text{B}\alpha$ R*	5'-TGTTGGCTTGAAGAACAAAG-3'
hu/mu HPRT F*	5'-CAAGCTTGGCTGAAAGGA-3'
hu/mu HPRT R*	5'-TGAAGTACTCATTATAGTCAAGGCATATC-3'

F, forward; HPRT, hypoxanthine-guanine phosphoribosyltransferase; hu, human; ICAM, intercellular adhesion molecule; mu, murine; R, reverse.

*Indicates that the primer pair spans an intron.

Quantification of eNOS and p65 mRNA and Heterogeneous Nuclear RNA in Primary Cultured Endothelial Cells Exposed to Shear Stress in Vitro

The monolayer of HAECs on glass slides was removed from the parallel-plate flow chamber and briefly rinsed with cold PBS, and the cells were scrapped and pelleted by centrifugation. mRNA isolation was performed using either Tri Reagent (Invitrogen, Carlsbad, CA) or RNeasy kit (Qiagen, Valencia, CA), according to the protocols provided by the manufacturer. Subsequently, the isolated mRNA was reverse-transcribed into cDNA using random decamers (Ambion) and Powerscript kit (Clontech). Quantitative RT-PCR was performed as described below to measure relative abundance of eNOS and p65 mRNA and heterogeneous nuclear RNA (hnRNA).

Real-Time PCR

For all real-time PCR experiments, primers were designed using MacVector (Accelrys, San Diego, CA) and Primer Express (Applied Biosystems, Foster City, CA). For mRNA quantification, the primers spanned across an intron whenever possible, whereas for hnRNA quantification, primers were designed to target introns (see Figure 4A; Supplemental Figures 3A and 5A, see at <http://ajp.amjpathol.org>). Primer sequences are listed in Tables 1, 2, and 3. Real-time PCR reactions were performed using the default PCR cycle on a Prism 7900 HT Sequence Detection Systems (Applied Biosystems), and amplified DNA was detected by SYBR green incorporation, with the exception of 18S and eNOS proximal promoter, which were detected using TaqMan probes (Applied Biosystems). Dissociation curve analyses were performed to confirm specificity of the SYBR green signals in each experiment, and controls without reverse transcriptase were used when primers targeting hnRNA were used. Quantification was performed using the com-

Table 2. Real-Time PCR Primers for Quantification of Human hnRNA

Gene	Sequence
$\text{I}\kappa\text{B}\alpha$ F	5'-TCTGACCCTGGGACGTAGCT-3'
$\text{I}\kappa\text{B}\alpha$ R	5'-TAAAGACCTCACCAAATCAGTGGAA-3'
eNOS no. 1 F	5'-GGAGAGGACTGGGAAGAACC-3'
eNOS no. 1 R	5'-GAGAGTTTGAGCCAGGGTTG-3'
eNOS no. 2 F	5'-CCAAGGTGGAGATGAGGAA-3'
eNOS no. 2 R	5'-CCCTCCTTTGGTGTTTTCA-3'
eNOS no. 3 F*	5'-TTATTATTTTGGAGAACCTATTACGTTGC-3'
eNOS no. 3 R*	5'-TGAAGTTATTTAATCTATGTTGGCATCTAA-3'
p65 no. 1 F	5'-ATCTTTGACAATCGTGAGTAGCGAG-3'
p65 no. 1 R	5'-CAAATACGCAAGATGATTGAATGAA-3'
p65 no. 2 F	5'-AGGCACTTCACTGGATTACCTGAT-3'
p65 no. 2 R	5'-CTGCTACTTACTAGCCACGTGAACC-3'
p65 no. 3 F*	5'-GGTTACAGTGGCTAGTAAGTAGCAG-3'
p65 no. 3 R*	5'-GGGCTGTATGGTAGTGACAAGACA-3'
p65 no. 4 F	5'-CACCGTGGCACTTAATTTCTATATTG-3'
p65 no. 4 R	5'-AGGTGATTTTGTGTTCCCCAA-3'

F, forward; R, reverse.

*Relates to information found in Supplemental Figure 5A (available at <http://ajp.amjpathol.org>).

parative standard curve method (Sequence Detection Systems Software 2.0; Applied Biosystems).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using the Upstate ChIP Assay kit (Upstate Biotechnology, Charlottesville, VA), as was described previously.⁵⁴ HAECs grown on fibronectin-coated glass slides were exposed to 24 hours of uniform laminar flow (10 dynes/cm²) or cultured in the absence of flow. The glass slides were then placed in a 1% formaldehyde solution, and cells were fixed for 10 minutes at 37°C. After two washes with ice-cold PBS, cells were scraped, and the cell pellet was lysed in SDS lysis solution on ice for 10 minutes. Sonication was performed using five sonications (10 seconds at 30% of maximum power of a Sonics and Materials sonicator with a 3-mm tip). For each ChIP assay, chromatin was isolated from a single slide of cells cultured under static conditions. Chromatin was pooled from two slides exposed to uniform laminar flow, because the gasket of the flow chamber apparatus reduced the surface area of cultured cells by approximately 40%. Chromatin was also harvested for controls, in which the immunoprecipitating antibody was omitted (no antibody control). Each sample of chromatin was pre-cleared, and immunoprecipitation was performed overnight at 4°C with 2 μg of anti-RNA polymerase II antibody (N-20; Santa Cruz Biotechnology). A 1% input sample was removed before immunoprecipitation. After extensive washing, im-

Table 3. Real-Time PCR Primers Used in ChIP Assays

ChIP assay primers	Sequence
eNOS proximal F	5'-GTGGAGCTGAGGCTTTAGAGC-3'
eNOS proximal R	5'-TTTCCTTAGGAAGAGGGAGGG-3'
eNOS proximal TaqMan	FAM-5'-CCAGCCGGGCTTGTTCCTGTCC-3'-BHQ
p65 exon 1 F	5'-CGGCCATGGACGGTGAGG-3'
p65 exon 1 R	5'-GCAGGAAGGGCGGGAAG-3'
CD31 F	5'-AGCCAGGCCCATTTGTTCC-3'
CD31 R	5'-GAAACCCGCCCTGTGAAAGC-3'

F, forward; R, reverse.

mune complexes were eluted, cross-links were reversed, and after phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in 30 μ l of water. Real-time PCR was performed in triplicate with 2 μ l of bound chromatin, 2 μ l of no antibody control, and 2 μ l of a diluted input sample. The number of copies of the genes of interest was determined by serial dilutions of human genomic DNA (where 1 ng of genomic DNA equals approximately 300 copies of a single-copy gene). Immuno-precipitated DNA was calculated by subtracting the number of copies in the no antibody control from the number of copies in the bound chromatin and dividing by the number of copies in the diluted input sample. Real-time PCR primers were located in the eNOS proximal promoter, p65 exon 1, and CD31 promoter regions (Table 3). Values obtained for the eNOS and p65 were normalized to CD31.

β -Galactosidase Staining

Aortas harvested from transgenic mice were rinsed in PBS; fixed for 4 to 6 hours in 0.2% glutaraldehyde, 2% formaldehyde, 5 mmol/L EGTA, 5 mmol/L $MgCl_2$, and 100 mmol/L sodium phosphate, pH 7.3; and stained in aqueous X-Gal solution (Boehringer Mannheim, Mannheim, Germany) as previously described.⁵¹

Statistical Analyses

Statistical differences between two groups were determined using a paired Student's *t*-test. In experiments consisting of multiple groups, analysis of variance and Fisher's protected least significant difference were used. All values are expressed as mean $\pm$ SEM.

Results

Expression of eNOS Protein and mRNA Is Reduced in the Lesser Curvature of the Ascending Aortic Arch

En face immunoconfocal microscopy was used to compare EC expression of eNOS in different regions of wild-type C57BL/6 mouse arteries. The atherosclerosis-prone region that was studied is located in the LC region of the aortic arch, whereas atherosclerosis-resistant regions are found in the GC region and in straight segments of the descending thoracic aorta (DTA) (Supplemental Figure 1, see <http://ajp.amjpathol.org>). Immunoreactive eNOS expression and phosphorylation of eNOS on Ser¹¹⁷⁷ (phospho-eNOS) were readily detected in the GC where staining of cell membranes and the perinuclear Golgi apparatus was prominent, consistent with the well-characterized pattern of *in vitro* expression (Figure 1A). Similar images were obtained in DTA (data not shown). In contrast, eNOS and phospho-eNOS staining was much less intense in the LC region of the arch. Morphometric analysis of images (percentage of pixels positive for eNOS and phospho-eNOS staining) revealed a significant difference between the LC and GC regions of ascending arch (Figure 1B). Similar eNOS expression pattern was

seen in atherosclerosis-predisposed regions of the brachiocephalic trunk (not shown). This asymmetric circumferential distribution of eNOS in the *in vivo* setting has not previously been noted.

Relative levels of eNOS and p65 mRNA were assessed in the C57BL/6 aorta using real-time PCR. Significantly lower levels were found in the LC region relative to the GC region (Figure 1C). The expression of eNOS mRNA in LC and GC regions was also determined in strains of mice with different susceptibilities for developing atherosclerosis,^{55,56} and comparable eNOS expression patterns were observed in the C57BL/6, 129, and C3H strains (Figure 1D). Expression levels of p65 mRNA, the main subunit of NF- κ B complex, were also assessed in the LC and GC regions. In contrast to eNOS, levels of p65 mRNA were 2.4- to 2.6-fold higher in the LC region (Figure 1C).

Exposure of Cultured Endothelium to Disturbed and Uniform Laminar Flow Induces eNOS and p65 Expression Patterns That Mimic Those Found in the LC and GC Regions in Vivo

Atherosclerotic lesions form at arterial branch points and curvatures, a feature suggesting that complex hemodynamics in these regions may promote atherosclerosis. In contrast, the laminar flow evident in straight segments may protect the artery wall. To determine whether disturbed and uniform laminar flows can differentially modulate EC expression of eNOS and p65, we used an established parallel plate/step model (Figure 2A).^{57,58} A two-dimensional computer simulation was performed to characterize the flow dynamics of the "stepped" flow path (Supplemental Figure 2, see <http://ajp.amjpathol.org>).^{59–62} Flow recirculation in the disturbed laminar flow (DLF) region could be visualized by phase contrast microscopy when particles were introduced into the flow system (data not shown). Porcine aortic ECs were cultured on the floor of the flow chamber and exposed to flow for 72 hours to allow cells to acclimatize to their hemodynamic environment and to minimize the effects of signaling in response to the initiation of flow. In the region of DLF (approximately 0 to 400 μ m downstream of the step), ECs maintained a polygonal morphology (Figure 2B). Further downstream, the cells were elongated in the direction of flow (Figure 2B), which is typical of endothelium exposed to uniform laminar flow (ULF)⁶³ and consistent with the *in vivo* morphology of ECs in the GC or the DTA region of the arterial tree.

In this model, we examined expression levels of eNOS and p65 protein by immunostaining ECs and comparing with cells cultured under static conditions for the same time period. Expression patterns of eNOS and p65 mirrored those observed in LC and GC of the arch *in vivo* (Figure 3A). Protein expression levels of eNOS in cells exposed to a uniform laminar flow of 10 dynes/cm² were elevated approximately 2.5-fold ($P < 0.005$) relative to cells cultured under static conditions (Figure 3B). In contrast, eNOS expression in the DLF region in the parallel plate/step model was significantly lower compared with the uniform laminar flow region and was comparable with that in cells cultured under static conditions. Expression patterns

Figure 2. EC shape changes in response to disturbed laminar flow (DLF) and uniform laminar flow (ULF) in a parallel plate/step flow chamber. **A:** A schematic diagram of the chamber indicating regions of DLF and ULF. **B:** The shape of ECs along the flow path downstream of the step was quantified by assessing the elongation of nuclei. The index value is the mean ratio of nuclear dimensions parallel versus perpendicular to the direction of flow. A value of greater than 1 represents elongation in the direction of the flow. Representative confocal images of the DLF (**left**) and ULF (**right**) regions showing ECs immunostained with β -catenin, a protein localized at cell junctions. Nuclei were counterstained with propidium iodide.

of p65 were opposite to eNOS. Compared with static cells, p65 protein levels were reduced by flow but to a lesser extent in the DLF region relative to ULF region.

Shear Stress-Induced Modulation of eNOS Expression Involves Transcriptional Regulation

Because eNOS expression can be regulated by transcriptional activity, mRNA stability, and posttranslational modifications, we investigated whether changes in the rate of transcription contribute to increased ex-

Figure 1. EC expression of eNOS protein and mRNA is reduced in an atherosclerosis-prone region of the normal mouse aorta. **A:** Representative immunofluorescence images of eNOS and phospho-eNOS (Ser¹¹⁷) staining in the LC and GC of the ascending aorta from C57BL/6 mice fed standard laboratory chow. ECs in the LC display lower steady-state expression levels of eNOS and phospho-eNOS (green), compared with the GC. Nuclei were counterstained with propidium iodide (red). **B:** The extent of eNOS and phospho-eNOS staining in immunofluorescence images was quantified by assessing the percentage of positive pixels and comparing the LC with GC. These analyses confirm lower levels of eNOS and phospho-eNOS in the LC (* $P < 0.05$; ** $P < 0.01$; $n = 4$). **C:** Real-time PCR quantification of eNOS and p65 mRNA levels in the LC intima relative to GC. Data were normalized to intercellular adhesion molecule-2 or CD31. The mRNA levels of eNOS were significantly lower in the LC region relative to GC (dashed line), whereas p65 mRNA levels were significantly higher (* $P < 0.05$; $n = 7$). **D:** Real-time PCR quantification of eNOS mRNA levels in LC and GC regions of three strains of mice: C57BL/6, 129, and C3H ($n = 4$).

Figure 3. Expression of eNOS and p65 by porcine aortic ECs cultured in the DLF and ULF regions of the parallel-plate/step flow chamber, as well as under static conditions. **A:** Representative immunofluorescence images showing eNOS or p65 staining (green). Cell junctions were visualized by staining for β -catenin (red), and nuclei were counterstained either with propidium iodide (red) or TOTO-3 (blue). **B:** Quantification of eNOS and p65 expression (mean fluorescence intensities) in regions of DLF and ULF revealed opposing expression patterns. Data were standardized to expression levels in cells cultured under static conditions, which were assigned a value of 1. Boxed numbers above the bars represent the ratios of DLF to ULF. Significant differences are indicated (* $P < 0.05$; ** $P < 0.005$; $n = 4$).

pression of eNOS in response to chronic shear stress. To assess the endogenous transcription rate of eNOS and p65, we measured expression levels of hnRNA in cells exposed to shear stress and compared them with static controls. hnRNA consists of primary RNA polymerase II (Pol II) transcripts that have not yet undergone splicing into mRNA and is increasingly recognized as a surrogate measure of gene transcription activity. Typically hnRNAs have a short half-life, and their relative abundance has been correlated to the rate of transcription measured by nuclear run-off.⁶⁴ To establish a correlation between hnRNA expression and the rate of transcription in cultured endothelial cells, we followed $\text{I}\kappa\text{B}\alpha$ transcription in response to tumor necrosis factor α ($\text{TNF}\alpha$) stimulation. $\text{I}\kappa\text{B}\alpha$ is a well-established target gene of $\text{NF-}\kappa\text{B}$ that is induced by $\text{TNF}\alpha$ stimulation. Confluent HAEC monolayers grown on fibronectin-coated glass slides were stimulated with $\text{TNF}\alpha$ (100 ng/ml) for varying periods of time before isolation of RNA, reverse transcription, and real-time PCR. Primers for hnRNA amplified intronic regions, whereas mRNA primer sets were corresponded to exonic regions separated by a large intron (Table 1; Supplemental Figure 3A). A control for hnRNA amplification included absence of reverse transcription to ensure that genomic DNA contaminants in the isolated RNA did not contribute to PCR signals using hnRNA primers. $\text{I}\kappa\text{B}\alpha$ hnRNA expression peaked within 15 to 30 minutes after $\text{TNF}\alpha$ stimulation (seven- to eightfold increase over basal expression, $P < 0.05$; $n = 5$), whereas the induction of $\text{I}\kappa\text{B}\alpha$ mRNA was relatively delayed, with a peak at 1 hour (fivefold induction, $P < 0.05$; $n = 5$) (Supplemental Figure 3B). To ascertain further whether hnRNA expression reflects the rate of transcription, HAECs were pretreated with α -amanitin, a RNA Pol II inhibitor, before $\text{TNF}\alpha$ stimulation. This pretreatment resulted in abrogation of $\text{TNF}\alpha$ -mediated induction of both $\text{I}\kappa\text{B}\alpha$ hnRNA and mRNA in the expected time frame when compared with those found in cells that were stimulated with $\text{TNF}\alpha$ without α -amanitin pretreatment (Supplemental Figure 3C). Data were normalized to the 18S ribosomal RNA, transcription of which is not dependent on RNA Pol II and therefore is not inhibited by α -amanitin. Next, we performed a time-course experiment in which HAECs were exposed to varying duration of uniform laminar flow (Supplemental Figure 4, see <http://ajp.amjpathol.org>). These experiments show a gradual time-dependent induction of eNOS hnRNA and mRNA expression, consistent with previous studies.^{14,65,66} Because the rate of transcription may be dependent on transcription elongation,⁶⁷ we also tested numerous primers complementary to introns distributed across the p65 and eNOS genes (Table 1; Supplemental Figure 5A) and found that the results were comparable regardless of the primer locations (Supplemental Figure 5, B and C).

To determine the effect of shear stress on transcription of eNOS and p65, we assessed hnRNA expression in HAECs exposed to 10 dynes/cm² of uniform laminar flow for 24 hours compared with static controls. These experiments showed a 2.16-fold increase in eNOS mRNA ex-

Figure 4. Effects of uniform laminar shear stress on the rates of eNOS and p65 transcription. **A:** Diagrams of the eNOS and p65 genes indicating the location of real-time PCR primers for hnRNA and mRNA. Black boxes represent exons and lines represent introns (FP, forward primer; RP, reverse primer). **B:** Alterations of eNOS and p65 hnRNA and mRNA expression levels in HAECs exposed to uniform laminar flow for 24 hours. Real-time PCR data were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and compared with those in cells cultured under static conditions, which were assigned a value of 1. Significant differences are indicated (* $P < 0.05$; ** $P < 0.005$; $n = 4$). **C:** RNA Pol II ChIP assays showing rates of eNOS and p65 transcription in HAECs exposed for 24 hours to uniform laminar flow (ULF) relative to cells cultured under static conditions. eNOS proximal promoter, p65 exon 1, and CD31 promoter DNA bound to RNA Pol II was immunoprecipitated and quantified by real-time PCR. Data for eNOS and p65 were normalized to CD31. Statistically significant differences between ULF and control values are indicated (* $P < 0.05$; $n = 3$).

pression relative to static controls ($P < 0.005$; $n = 4$) and 1.5-fold increase in eNOS hnRNA expression ($P < 0.05$; $n = 4$) (Figure 4B). In contrast, both p65 mRNA and hnRNA expression were decreased by approximately one-third ($P < 0.05$; $n = 4$). These changes were comparable with the changes in protein expressions of eNOS and p65 observed in the DLF and ULF regions of the parallel plate/step flow chamber. Lastly, ChIP assays were performed using an antibody against RNA Pol II on HAECs exposed to 24 hours of shear stress and compared with static control cells to assess Pol II binding to eNOS and p65 genes. The ChIP assays showed an increased Pol II binding to the eNOS promoter region in HAECs exposed to shear stress. Pol II binding to the p65 gene was not significantly altered in response to shear stress (Figure 4C), which is not consistent with our hnRNA data and may reflect differences in the sensitivities of these assays, technical considerations, or mechanisms other than regulation of transcription controlling p65 expression.

Transcription of eNOS Is Decreased in Arterial Regions Predisposed to Atherosclerosis and Correlates Inversely with p65 Expression

To determine whether reduced transcription of eNOS contributes to relatively decreased levels of eNOS mRNA and protein in atherosclerosis-prone regions, we evaluated the topographic expression patterns of a 5.2-kb murine eNOS promoter-reporter construct (Supplemental Figure 6, see <http://ajp.amjpathol.org>).⁵¹ Expression of the nuclear-localized β -galactosidase reporter in these

transgenic mice reflects eNOS promoter activity because previous studies demonstrated that expression of the reporter was highly comparable with endogenous eNOS,⁵¹ which suggests that the transgenic promoter faithfully reflects transcriptional activity of endogenous eNOS. Histochemical staining revealed that nuclear localized expression of β -galactosidase was markedly diminished in the LC (Figure 5, A and B). We quantified the extent of nuclear β -galactosidase staining using high-resolution confocal microscopy and counterstained nuclear DNA (Figure 5C). A significant difference was found in the percentage of β -galactosidase-positive nuclei in the LC and GC (Figure 5D). Expression patterns were comparable in lines derived from two independent insertional promoter/reporter founders, and data were pooled.

To corroborate further the relationship between eNOS transcription and regional susceptibility to atherosclerosis, we assessed β -galactosidase expression in atherosclerosis-prone and -resistant regions located in the brachiocephalic trunk at the origin of the right brachial artery (Supplemental Figure 1C). We identified and mapped these regions by staining excised arteries of *ldlr*^{-/-} mice fed a cholesterol-enriched diet with oil red O (Figure 6, A and B). Immunofluorescence microscopy of eNOS promoter-reporter mice demonstrated a significant difference in the expression of nuclear-targeted β -galactosidase (Figure 6, C and D). The morphology of nuclear expression in each region was similar to that in the aortic arch LC and GC regions.

Previously, we found that expression of NF- κ B components was markedly elevated in high probability relative to low probability regions,¹ consistent with our quantification of mRNA levels using real-time PCR (Figure 1B). The precise spatial relationship between p65 and eNOS expression was evaluated by confocal microscopy. These experiments revealed an inverse relationship between endogenous p65 expression and staining for nuclear β -galactosidase in eNOS promoter transgenic mice (Figure 5E).

p65 Expression Patterns in Aortic ECs Are Not Altered in eNOS-Deficient Mice

Given the opposing effects of flow on eNOS and p65 expression and given that NO can exert anti-inflammatory effects through inhibiting NF- κ B binding to its target genes, such as vascular cell adhesion molecule-1 in ECs,⁶⁸ we investigated whether eNOS influences the topographic expression of p65. p65 immunostaining was evaluated in the GC and LC of aortas harvested from eNOS-deficient (eNOS^{-/-}) and age-matched wild-type mice. This experiment revealed comparable topography of p65 expression in eNOS^{-/-} and wild-type controls (Figure 7, B and C). The absence of eNOS expression in the eNOS^{-/-} mice was confirmed by immunostaining of the DTA (Figure 7A).

Discussion

ECs form the interface between blood components and the artery wall, and regional differences in their gene

expression may have profound effects on their ability to protect the artery from atherosclerosis. Our data provide evidence for distinct and highly reproducible EC gene expression patterns in a well-characterized region of the normal mouse aorta that is predisposed to atherosclerosis. We showed that eNOS mRNA and protein levels were reduced in the LC of the ascending aortic arch, relative to the protected GC. Furthermore, the extent of eNOS phosphorylation on Ser¹¹⁷⁷, which is associated with enhanced eNOS activity, was also relatively reduced in the LC, suggesting a direct correlation between eNOS expression and activity. This is intriguing because eNOS, an “atheroprotective” gene, exhibits an expression pattern that is in clear contrast to that of p65, a proinflammatory and potential proatherogenic gene. We observed more pronounced regional differences in eNOS protein expression levels compared with mRNA. This may be real or may reflect differences in methodologies used to assess these parameters. For example, our immunostaining used a tyramide enzymatic amplification step that may have accentuated differences in protein expression between regions. We observed that eNOS mRNA expression patterns were comparable in strains of mice that have relatively high (C57BL/6), intermediate (129), and low (C3H) susceptibility to atherosclerosis.^{55,56} The cardiovascular anatomy and physiology as well as hemodynamics of different mouse strains are probably not drastically different, which may be why regional eNOS expression patterns are similar. In contrast to eNOS expression, the abundance of intimal dendritic cells in the LC region correlates with strain susceptibility to atherosclerosis.⁶⁹ Thus, our data suggest that eNOS may contribute to regional but not strain differences in atherogenesis.

Previously, we observed that ECs in the GC of the ascending aortic arch were elongated parallel to the direction of blood flow, whereas in the LC they were more polygonal and randomly oriented.^{1,69} This suggested that local hemodynamic environments are different in these regions. ECs can sense differences in mechanical shear forces, which may modulate gene expression patterns.^{10,70} Thus, we investigated if the type of flow (disturbed versus uniform laminar) in the LC and GC is responsible for differences in endothelial expression of eNOS and p65. Modulation of arterial hemodynamics *in vivo* by surgical approaches induces arterial remodeling

Figure 5. Analysis of eNOS transcription and endogenous p65 protein expression in eNOS promoter- β -galactosidase reporter transgenic mice. Representative low (**A**) and high (**B**) magnification views of an ascending aorta histochemically stained for β -galactosidase, which is under the transcriptional control of the eNOS promoter. Diminished nuclear staining is seen in the LC. **C:** Representative confocal microscope images of the ascending aorta LC and GC regions immunostained for β -galactosidase (green). Nuclei were counterstained with propidium iodide (red). A markedly lower number of endothelial cell nuclei in the LC stain positively for β -galactosidase, reflecting a lower rate of eNOS transcription. **D:** Quantification of β -galactosidase expression in the GC and LC regions of the ascending aorta (* $P < 0.001$, $n = 10$). **E:** Immunofluorescence images showing inverse expression patterns of β -galactosidase (red) and endogenous p65 (green) in the LC and GC regions. The abundance of endogenous p65 protein is higher in the cytoplasm of LC endothelial cells, whereas the number of β -galactosidase-positive nuclei is lower. Nuclei were counterstained with TOTO-3 (blue). In **C** and **D**, the blood flow was from left to right.

Figure 6. Mapping of atherosclerosis-prone (AP) and atherosclerosis-resistant (AR) regions in the brachiocephalic trunk (BCT) and analysis of eNOS transcription in these regions. **A:** Oil red O staining demonstrates reproducible atherosclerotic lesion formation at the origin of the right brachial artery from the BCT (arrowheads, posterior view) of four representative *ldlr*^{-/-} mice fed a cholesterol-rich diet for 6 to 8 weeks. **B:** En face view of oil red O-stained BCT and proximal right brachial artery from two representative *ldlr*^{-/-} mice fed a cholesterol-rich diet. The location of lesions was reproducible in five mice that were evaluated. **C:** Representative immunoconfocal images showing β -galactosidase staining in the AP and AR regions of the BCT in normocholesterolemic eNOS promoter- β -galactosidase reporter transgenic mice. **D:** Quantification of nuclei positive for β -galactosidase expression revealed a significant difference (* P < 0.05, n = 3) between the AP and AR regions.

Figure 7. Expression of p65 by aortic endothelial cells in LC and GC regions of wild-type and eNOS^{-/-} mice. **A:** Representative immunoconfocal images of the DTA from C57BL/6 wild-type (eNOS^{+/+}) and eNOS^{-/-} mice stained for eNOS (green) confirming the absence of eNOS expression in eNOS^{-/-} mice. Nuclei were counterstained with propidium iodide (red). **B:** Representative immunoconfocal images of the LC and GC stained for p65 (green) showing similar topography of endogenous p65 protein expression in eNOS^{+/+} and eNOS^{-/-} mice. Nuclei were counterstained with propidium iodide (red). **C:** Quantitative analysis of p65 staining in immunoconfocal images. The percentage of pixels positive for p65 staining was determined, and values of the GC and LC were compared (GC values were designated as 1). This analysis confirmed that the expression of p65 was comparable in eNOS^{+/+} and eNOS^{-/-} mice. * P < 0.05.

with associated inflammation and EC gene expression. To assess directly the effects of diverse hemodynamics on EC gene expression, we used an established *in vitro* parallel plate/step model that creates spatial shear gradients, flow separation, and flow recirculation. These hemodynamic features are found in atherosclerosis-prone regions. Locations of the DLF and ULF regions were determined by endothelial cell morphology, two-dimensional computer simulation, and direct visualization of small particles introduced into the chamber. A pulse-dampener was incorporated to eliminate pulsations generated by the peristaltic pump. These experiments were maintained for at least 48 hours to allow cells to acclimatize to the local hemodynamic environment and to minimize the effects of acute signaling in response to the initiation of flow.⁸ In this model, ECs located in the region of DLF exhibited polygonal morphology, and further downstream in the region of ULF, they were elongated. Consistent with previous reports,^{11,71,72} eNOS expression was significantly increased in response to ULF compared with static conditions. In the DLF region, eNOS expression was also increased relative to static controls, but the increase was small relative to the ULF region and was not statistically significant. In contrast to eNOS, expression of p65 was reduced slightly in the DLF region and significantly in the ULF region compared with static control. These *in vitro* data recapitulated the morphology of EC *in vivo* as well as eNOS and p65 expression patterns and suggested that gene expression patterns in cells cultured under static conditions can be similar to those found in regions of disturbed flow.

The expression of eNOS is largely restricted to vascular endothelium of medium- and large-sized arteries.^{51,73,74} Constitutive expression is modulated by many factors, including laminar shear stress, cytokines, oxidized LDL, hypoxia, estrogen, and cell proliferation; and expression levels are regulated by transcription, mRNA stability, and epigenetics.^{75–78} To determine whether shear stress-induced modulation of eNOS expression involves regulation of transcription, HAECs were exposed to uniform laminar shear stress, and the rate of transcription was determined by measuring hnRNA levels. After 24 hours, transcription was induced by approximately 50% for eNOS relative to static controls and was reduced by 30% for p65. These changes were comparable with the changes in mRNA levels. Similar findings were obtained for eNOS with RNA Pol II ChIP assays. *In vivo* experiments using eNOS promoter- β -galactosidase reporter transgenic mice demonstrated localized down-regulation of eNOS transcription in regions predisposed to atherogenesis that correlated topographically with lower mRNA and protein levels. Our findings provide new mechanistic insights by demonstrating that hemodynamic forces regulate eNOS transcription both *in vivo* and *in vitro* and are in agreement with recent studies where hemodynamic perturbations were induced in the mouse carotid artery by placing a tapered cylinder around the vessel.⁷⁹

Characterization of the human eNOS promoter has revealed numerous *cis*-regulatory sequences, including six shear stress response elements; putative Sp1/Sp3 and GATA motifs; Ets family; MAZ, YY-1, and NF-1 ele-

ments; a cAMP-responsive element; and AP-1- and -2-binding sites.^{80,81} A large number of these elements are conserved in the mouse.⁸² Recent studies have shown that shear stress-responsive transcription factor Kruppel-like factor 2 (KLF2) can serve as a “molecular switch” to up-regulate eNOS expression and negatively regulate interleukin-1 β , vascular cell adhesion molecule-1, and E-selectin expression, possibly through its interactions with cofactors CBP/p300.^{83,84} In the human aorta, KLF2 expression is reduced in bifurcations and branch points that are susceptible to neointimal formation.⁸⁵ KLF2 also has “anti-inflammatory” properties in monocytes, where it suppresses NF- κ B activity and lipopolysaccharide-induced expression of inflammatory genes.⁸⁶ Consistent with the above findings, we have also observed increased KLF2 mRNA expression in cultured HAECs exposed to shear stress (data not shown). The up-regulation of KLF2 expression in endothelium by uniform laminar shear stress provides a potential mechanism for an atheroprotective milieu in specific regions of the artery wall. In addition to KLF2, it is likely that other mechanisms contribute to regional differences in EC gene expression that we and others⁸⁷ have observed. For example, it is possible that relatively higher expression of NF- κ B components and priming of NF- κ B signaling contributes to the higher susceptibility to atherosclerosis in regions of curvature.¹

Recently, Harrison and colleagues^{66,88} implicated NF- κ B as a potential positive regulator of eNOS transcription in the *in vitro* setting in response to shear stress. In studies using eNOS promoter-CAT reporter constructs, they demonstrated shear stress-induced NF- κ B binding to GAGACC sequence in the eNOS promoter. In contrast to these data, we showed that eNOS and p65 expression is modulated in an opposing manner by shear stress. In the LC of the ascending arch, where eNOS expression is low, we observed increased p65 expression and a low-level of NF- κ B activation (nuclear translocation in <10% of cells).¹ We observed even less activation of NF- κ B in the GC,¹ a region with relatively higher expression of endogenous eNOS mRNA and protein and a reporter gene under the transcriptional regulation of the eNOS promoter. Acute exposure of endothelium to flow induces activation of NF- κ B^{89,90} and many other signaling pathways, which subsequently become down-regulated as cells acclimatize to their new hemodynamic environment. In our *in vitro* experiments, exposure of endothelium to uniform laminar shear stress for several days resulted in a sustained increase in eNOS expression, yet p65 expression was decreased and confined to the cytoplasm at these time points, suggesting that NF- κ B signaling was quiescent. Collectively, our data are not consistent with NF- κ B being a positive regulator of eNOS transcription. Perhaps the contradictory observations on NF- κ B and eNOS expression can be reconciled by the fact that Harrison's group studied expression of an episomal promoter-reporter construct and a relatively short interval after exposure of endothelium to shear stress,⁶⁶ whereas we assessed the expression of a chromosomally integrated transgene *in vivo* and the endogenous eNOS gene in cultured cells 24 to 72 hours after introduction of shear

stress. Further experimentation will be required to attain a more complete understanding of how hemodynamic forces and various signaling pathways modulate eNOS transcription.

In summary, we demonstrated that eNOS transcription and levels of steady-state mRNA and protein are regulated in regions of mouse arteries predisposed to atherosclerosis, and similar patterns of eNOS mRNA expression are found in strains of mice with differing susceptibility to atherosclerosis. We showed that distinct hemodynamic conditions modulate eNOS and p65 expression differently and that eNOS deficiency does not influence the regional aortic expression pattern of p65 or its cellular localization in ECs.

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