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Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: Cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress

(atherosclerosis/vascular endothelium/hemodynamic forces/differential display)

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ABSTRACT **Early atherosclerotic lesions develop in a topographical pattern that strongly suggests involvement of hemodynamic forces in their pathogenesis. We hypothesized that certain endothelial genes, which exhibit differential responsiveness to distinct fluid mechanical stimuli, may participate in the atherogenic process by modulating, on a local level within the arterial wall, the effects of systemic risk factors. A differential display strategy using cultured human endothelial cells has identified two genes, manganese superoxide dismutase and cyclooxygenase-2, that exhibit selective and sustained up-regulation by steady laminar shear stress (LSS). Turbulent shear stress, a nonlaminar fluid mechanical stimulus, does not induce these genes. The endothelial form of nitric oxide synthase also demonstrates a similar LSS-selective pattern of induction. Thus, three genes with potential atheroprotective (antioxidant, antithrombotic, and antiadhesive) activities manifest a differential response to distinct fluid mechanical stimuli, providing a possible mechanistic link between endothelial gene expression and early events in atherogenesis. The activities of these and other LSS-responsive genes may have important implications for the pathogenesis and prevention of atherosclerosis.**

Vascular endothelium, the single-cell-thick lining of the cardiovascular system, forms a multifunctional, dynamically mutable interface, that is responsive to a variety of pathophysiologic stimuli. Dysfunction of endothelial cells (EC), induced by systemic biochemical risk factors (e.g., hypercholesterolemia, hyperhomocysteinemia, and diabetes), is thought to play a critical role in the development of atherosclerotic vascular disease and its clinical complications (1–3). The strikingly nonrandom distribution of the earliest lesions of atherosclerosis in both humans and experimental animals has suggested to many that hemodynamic forces might be acting as local "biomechanical risk factors"; however, the exact nature of the biomechanical stimuli involved and their influences on EC pathobiology remain ill-defined (4–6). Arterial bifurcations and curvatures, where disturbed flow patterns (flow separation, flow reversal, low amplitude, and fluctuating wall shear stresses) occur, typically are "lesion-prone areas," whereas geometries associated with uniform laminar flow (oscillatory without flow reversal) and relatively constant (time-averaged) wall shear stresses, such as the straight tubular portions of the aorta and its primary tributaries, tend to be "lesion-protected areas" (7–9). Interestingly, these patterns are retained even in genetically modified animals in which systemic risk factors, such as markedly elevated levels of atherogenic plasma lipoproteins, are present (10). These observations indicate that EC may respond differentially to their local fluid mechanical environment, and thus contribute to the characteristic pattern of atherosclerotic lesion development.

Although the molecular mechanisms responsible for atherosclerotic lesion initiation have yet to be defined, most hypotheses to date have focused on the up-regulation of putative pathogenic effector molecules at sites of lesion predilection. Given the ability of vascular EC to elaborate multiple "anti-atherogenic" effector molecules (1, 2), and the recent demonstration by our group and others (11, 12) that fluid mechanical forces can modulate EC gene expression, we have formulated an alternative hypothesis: The uniform laminar shear stresses (LSSs) characteristically associated with lesion-protected areas selectively induce the expression of one or more "atheroprotective genes" in EC, which then act locally to offset the effects of systemic atherogenic factors. To begin to identify these genes, we have applied a PCR-based differential display strategy to examine, in an unbiased fashion, the pattern of genes regulated in cultured EC by distinct fluid mechanical stimuli. Using this experimental approach, we have identified three endothelial genes, manganese superoxide dismutase (Mn SOD), cyclooxygenase (COX)-2 and the endothelial isoform of nitric oxide (NO) synthase (ecNOS, NOS-3), that demonstrate a differential response to laminar versus nonlaminar fluid mechanical stimuli, and whose known activities (antioxidant, antithrombotic, and antiadhesive) are consistent with a protective role in the atherosclerotic disease process. These, and other yet-to-be-characterized, EC genes that exhibit differential regulation by uniform LSS may provide new insights into the dysregulation of critical protective functions in the arterial wall during atherogenesis.

METHODS

EC Culture. Human umbilical vein ECs (HUVECs) were isolated from multiple segments of normal-term umbilical cords, pooled, and cultured in medium 199 supplemented with

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Abbreviations: EC, endothelial cell; HUVEC, human umbilical vein EC; LSS, laminar shear stress; TSS, turbulent shear stress; Mn SOD, manganese superoxide dismutase; COX, cyclooxygenase; ICAM-1, intercellular adhesion molecule-1; PDGF-B, the B chain of platelet-derived growth factor; ecNOS, the EC isoform of NO synthase (NOS-3); RT, reverse transcriptase; IL, interleukin; rhIL-1β, recombinant human IL-1β.

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Endothelial Cell Growth Supplement (50 μg/ml, Collaborative Research), heparin (50 μg/ml, Sigma), antibiotics, and 20% fetal bovine serum (Sigma). Cells at passage level 2 or 3 were replicate-plated on 0.1% gelatin-coated standard Petri dishes or specially designed plates fabricated from the same tissue culture plastic (Costar), and allowed to grow to confluent densities before experimental use.

Shear Stress Apparatus. Confluent monolayers grown on 17.8-cm diameter "maxiplates" ($\approx 10^7$ cells per plate) were introduced into a cone-plate flow cuvette (13, 14), consisting of a stainless steel cone rotating over a stationary baseplate. The culture medium present between the cone and the plate was gradually replenished during experiments, and the entire apparatus was maintained in a humidified 5% CO_2/95% air atmosphere. The equations and calculations for describing the shear stresses generated in this cone-plate apparatus have been reported in detail (13, 14). For LSS at 10 dyne (1 dyne = 10 μN)/cm^2, we used a 0.5° cone at a rotational velocity of 100 rpm. As described by Sdougos *et al.* (14), the parameter $\tilde{R}$ is a function of the local radius from the center of the cone, the angular velocity of the cone, the angle of the cone itself, and the fluid kinematic viscosity of the media, and at values <1, predicts uniform laminar flow conditions. Equivalent time-averaged shear stresses in turbulent flow can also be generated in this system by manipulating these variables to achieve $\tilde{R}$ values of >4. Using a 3.0° cone angle, a rotational velocity of 135 rpm, and radii of ≥3.5 cm, the $\tilde{R}$ values are >5, and turbulence is predicted as well as observed experimentally.

Differential Display and Quantitative Reverse Transcriptase (RT)–PCR. Cells were harvested from maxiplate or tissue culture dishes by washing with sterile PBS containing 1 mM EDTA and scraping with a rubber policeman. For the maxiplates [static, LSS, and turbulent shear stress (TSS)], cells within a 3.5-cm radius of the center of the plate were not harvested. Suspended cells were pelleted and lysed in 4 M guanidinium, 20 mM sodium acetate, 0.1 mM dithiothreitol, 0.5% sarkosyl, and RNA was pelleted through cesium chloride. For some of the experiments, RNA was isolated by guanidinium/phenol with identical results. Differential display analysis was performed essentially as described (15), using 12 poly(dT)-based 3′ primers and ≈40 randomly selected 5′ primers. Bands that appeared to be induced were excised and subcloned. These were then screened by Northern blotting, quantitative RT-PCR, and direct sequencing. For quantitative RT-PCR analysis, aliquots of equal amounts of RNA from the various conditions were annealed with random hexamer primers by heating to 95°C and slowly cooling to 37°C and then reverse transcribed in 50 mM Tris·HCl (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 0.02 mM DTT, and 200 μM of each dNTP for 30 min at 37°C. Controls omitting the reverse transcriptase were routinely performed. These samples were then extracted with phenol/chloroform and ethanol precipitated twice using ammonium acetate followed by sodium acetate. Equal amounts of the resulting cDNA (between 0.1–0.5 μg) were subjected to PCR with gene specific primers in a 50 μl volume containing 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 25 μM each primer, 200 μM dNTP, and 2.5 units *Taq* polymerase. A monoclonal antibody against the *Taq* polymerase (*Taq* Start, CLONTECH) that is irreversibly denatured by the first denaturing cycle of the PCR was used to "hot start" the reactions. Control reactions that varied the number of PCR cycles with a fixed amount of starting cDNA were carried out for every primer pair. We found that 25–30 cycles usually yielded a detectable amount of reaction product (see below) and avoided the nonlinear (plateau) phase of the reaction (typically observed at 35–40 cycles) (see Fig. 1*B*). All reaction products were analyzed by agarose gel analysis with 100-bp ladders as size standards and blotted to nylon membranes. These were then hybridized with gene specific probes and visualized by autoradiography. To confirm that this method yielded a quantitative reflection of the amount of starting RNA, aliquots of the cytokine and shear stressed RNA preps were combined, and reverse transcribed as above. Known, relative amounts (i.e., 1×, 5×, and 10×) of the reaction products were then subjected to PCR with all of the gene specific primers for 25–30 cycles (identical conditions as the test samples) and analyzed as above.

Probes and Primers. Primers for intercellular adhesion molecule (ICAM)-1 and glyceraldehyde-3-phosphate dehydrogenase were purchased from Stratagene. The remaining primers were as follows: platelet-derived growth factor (PDGF)-B, 5′-CTGTCCAGGTGAGAAAGATCGAGATT GTGCGG-3′ and 5′-GCCGTCTTGTCATGCGTGTGCTTG AATTTCCG-3′; the cellular form of transcription factor fos, 5′-AAGGAGAATCCGAAGGGAAAGGAATAAGATGG CT-3′ and 5′-AGACGAAGGAAGACGTGTAAGCAGTGC AGCT-3′; Mn SOD, 5′-GAGATGTTACAGCCCAGATAG C-3′ and 5′-AATCCCCAGCAGTGGAATAAGG-3′; COX-1, 5′-GTGCATCAACACAGGCGCCTCTTC-3′ and 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′; COX-2, 5′-TT CAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGAT CATCTCTGCCTGAGTATCTT-3′; ecNOS, 5′-ATGGGCA ACTTGAAGAGCGTGGCCC-3′ and 5′-GCCTGGCCCTT ACCTGTGTTCTGGCGCTGGTGGGAGTAG-3′. These primer pairs span introns present in the genomic sequences. Probes consisted of restriction fragments derived from cDNAs or generated by PCR and labeled with random hexamers, or oligonucleotides complementary to the species of interest.

Nuclear Runoff Analysis. Four maxiplates ($\approx 10^7$ cells per plate) were simultaneously prepared as above. One served as a control, one was treated with 10 units/ml of recombinant human interleukin 1β (rhIL-1β) for 3 hr, and the other two were subjected to 3 hr of LSS or TSS as above. Cells outside a 3.5-cm radius of the plate center were scrape harvested, washed with PBS, and lysed in 10 mM Tris (pH 8.4), 1.5 mM $MgCl_2$, and 140 mM NaCl, by slowly adding Nonidet P-40 to a final concentration of 1%. The nuclei were pelleted and resuspended in 24 mM Tris (pH 8.0), 24% glycerol, 166 mM KCl, 12 mM $MgCl_2$, 1.2 mM $MnCl_2$, 0.1% 2-mercaptoethanol, 1 mM each of GTP, ATP, and CTP, and 500 μCi (1 Ci = 37 GBq) of [α-^{32}P]UTP (3000 Ci/mmol), and incubated for 30 min at 30°C to label nascent RNA. RNA was isolated by guanidinium/phenol and precipitated with isopropanol. The labeled RNA was subjected to partial hydrolysis by incubating with 75 mM NaOH for 10 min on ice, and reprecipitated with ammonium acetate. Aliquots of each sample were counted, and equal numbers of counts were used as probes. Membranes carrying immobilized, denatured plasmids containing the cDNA of interest or a control plasmid (RSV-CAT) were prepared and probed with the labeled RNA. After 48 hr of hybridization at 65°C, the membranes were washed in 2× 0.18 M NaCl, 10 mM phosphate (pH 7.4), and 1 mM EDTA, followed by 0.2 × 0.18 M NaCl, 10 mM phosphate (pH 7.4) and 1 mM EDTA containing 10 μg/ml RNase A, and subjected to autoradiography.

Western Blot Analysis. Monolayers of HUVEC that had been subjected to shear stress or cytokine stimuli as described above were washed with sterile PBS, and scraped harvested. After pelleting at low speed, the cells were lysed in 150 mM NaCl, 10 mM Tris (pH 8.0), 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.2 unit/ml aprotinin. Insoluble material was pelleted and the lysate was frozen at −70°C. Proteins were separated on 10 or 12% denaturing acrylamide gels and transferred to nitrocellulose by electroblotting. A monoclonal antibody to COX-2 (Transduction Laboratories, Lexington, KY) was used at 0.1 μg/ml final concentration, and a polyclonal serum generated in sheep against human Mn SOD (Calbiochem) was used at 1/500 dilution. The blots were developed with the appropriate secondary antibody linked to

horseradish peroxidase and developed with a chemiluminescent reporter system (Enhanced Chemiluminescence kit, Amersham). As a control for protein loading, a monoclonal antibody raised in our laboratory termed E1/1 (16), now known to recognize endoglin, a cell surface type β transforming growth factor binding protein (17), was used. The level of this protein is not significantly altered by cytokine stimulation of HUVEC (16).

RESULTS AND DISCUSSION

To examine their differential responsiveness to defined fluid mechanical forces, monolayers of cultured HUVECs were subjected to a physiologic level (10 dyne/cm^2) of steady LSS, or a (nonlaminar) TSS of comparable time-averaged magnitude. Steady LSS was used as a simplified model of the nondisturbed flow (oscillatory LSS without flow reversal) that occurs in the unbranched arterial geometries that tend not to develop atherosclerotic lesions. Although true turbulence is limited in its occurrence within the vasculature (7–9), it does represent a distinct, nonlaminar fluid mechanical stimulus that can be achieved in a reproducible manner *in vitro*, and elicits distinct cell biological responses in cultured EC (18). In addition, turbulent flow contains many of the characteristics of the disturbed or separated laminar flows, including fluctuations in the frequencies, direction, and magnitude of the shear stress, that are typically encountered in lesion-prone arterial geometries *in vivo*. To produce these distinct flow patterns *in vitro*, we used a modified cone-plate viscometer that has been described previously (13, 14). By varying the cone angle and angular rotational velocity within this apparatus, various defined fluid mechanical conditions, ranging from steady laminar flow to complex secondary flows and turbulence, can be generated (13), while media composition and other culture conditions are kept constant. For comparison, a well-studied cytokine activation stimulus (rhIL-1β, 10 unit/ml) (19) was applied to static HUVEC cultures in parallel. RNA samples were isolated from HUVEC subjected to no flow (static conditions), 1 and 6 hr of laminar flow, turbulent flow or static cytokine treatment, and then displayed using multiple RT-PCR primer pairs. Partial cDNAs that appeared to be differentially regulated were isolated and characterized by sequencing and quantitative RT-PCR.

In a large series of experiments, certain reproducible patterns of response were observed. A novel pattern of endothelial gene regulation, which we termed "LSS-selective up-regulation" (up-regulation in response to steady laminar but not TSS) was identified, and molecular species exhibiting this pattern of response were selected for further study. Two of these LSS-selective species were identified by sequence analysis as Mn SOD and COX-2, prostaglandin endoperoxide H synthase-2. These genes encode inducible isoforms of enzymes involved in intracellular antioxidant activity and the regulation of prostanoid synthesis, respectively. Both have been demonstrated to be up-regulated by inflammatory cytokines in a variety of tissues and cell types (20–23). However, neither gene has been reported to be differentially responsive to biomechanical stimuli in vascular endothelial cells.

As demonstrated in Fig. 1, the levels of both Mn SOD and COX-2 mRNAs were significantly increased after exposure to 1 and 6 hr of LSS. The levels of COX-1, the constitutive isoform of cyclooxygenase, and glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in glucose metabolism, showed negligible change. As controls for the RT-PCR method, certain genes previously demonstrated to be LSS-inducible in EC also were examined. Both ICAM-1 and the B chain of PDGF were observed to be induced by 1 and 6 hr of LSS, while the RNA level of the cellular form of transcription factor fos demonstrated an acute increase after 1 hr of LSS, but returned to near baseline levels by 6 hr, consistent with

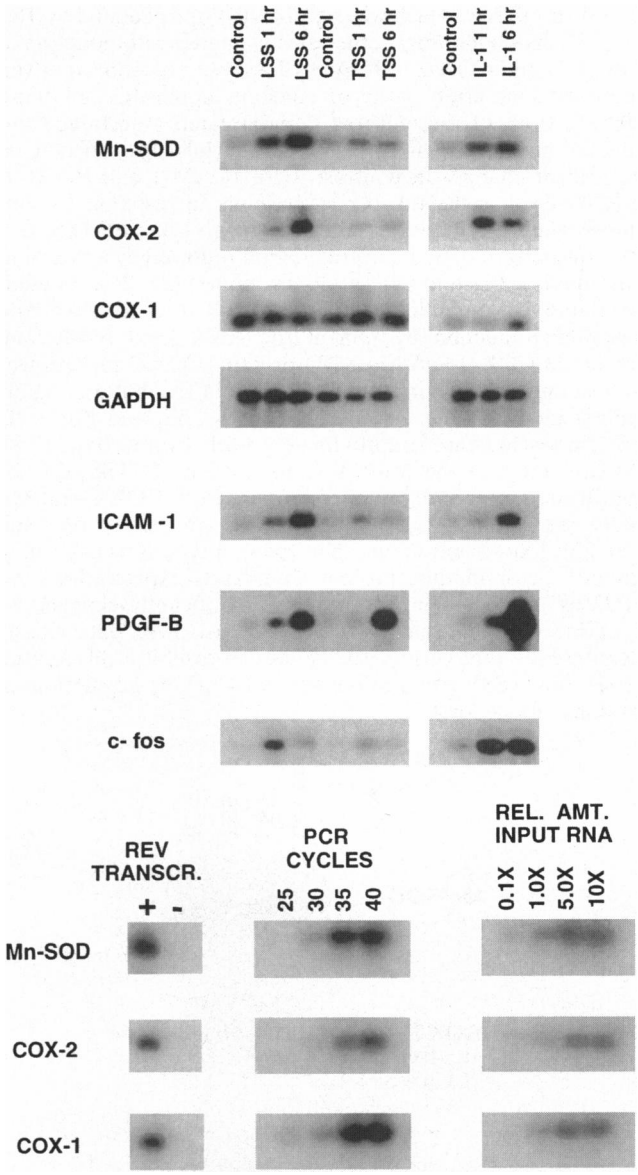

FIG. 1. (*A*) RT-PCR analysis of genes differentially regulated by shear stresses and cytokine stimulation in EC. Cultured HUVEC were subjected to no flow (control), LSS and TSS (10 dyne/cm^2), or 10 unit/ml of rhIL-1β, for 1 and 6 hr, and levels of specific mRNAs were determined by quantitative RT-PCR. (Note: the control sample in each set of stimuli is located to the left.) The products of the PCR reaction were separated by agarose gel electrophoresis and blotted to nylon membranes, with size standards. These were hybridized with gene-specific probes and visualized by autoradiography. (*B*) Examples of methodologic controls carried out for all primer pairs used. These consisted of omission of the reverse transcriptase enzyme ($\pm$RT) as well as variation of the PCR cycle number and relative amount of input RNA, thus insuring that the non-linear portion of the PCR cycle is avoided and that the resulting amount of product quantitatively reflects the amount of starting material (input RNA). Results shown are representative of at least three separate experiments.

previous reports (24–26). In contrast to the marked LSS-induced up-regulation of both Mn SOD and COX-2, exposure to a comparable time-averaged level of TSS did not elicit any significant change in either of their RNAs (Fig. 1). Although a small amount of induction (<2-fold) was typically seen after 1 hr of TSS, this elevation was not sustained at later time points, perhaps reflecting the acute transition from static (no-flow) culture conditions. In these short-term experiments, ICAM-1 behaved similarly to Mn SOD and COX-2, displaying little if any induction in response to TSS, in contrast to the

PDGF-B mRNA, which was significantly up-regulated by TSS (Fig. 1). As seen in Fig. 2, the selective pattern of induction of Mn SOD and COX-2 mRNAs by LSS, but not TSS, was even more evident after 24 hr of constant application of these stimuli. Both of these RNAs demonstrated a sustained up-regulation after 24 hr of steady LSS, while TSS elicited no significant change. In contrast, both ICAM-1 and PDGF-B mRNA levels returned to near baseline in response to continued fluid mechanical stimulation (both LSS and TSS, Fig. 2). This pattern of acute up-regulation followed by a return to baseline is consistent with previous reports (24–26). As demonstrated by immunoblotting in Fig. 3, the selective and sustained induction by LSS at the RNA level resulted in increased COX-2 and Mn SOD protein. COX-2 protein was almost undetectable in unstimulated HUVEC, but was significantly induced after 24 hr exposure to LSS, and Mn SOD protein was increased approximately 5-fold over its basal level. As predicted by the mRNA results in Fig. 2, TSS did not significantly up-regulate either protein. Both COX-2 and Mn SOD showed cytokine inducibility as previously reported (20–23). Expression of endoglin (p96), a type β transforming growth factor binding protein abundantly expressed on the HUVEC surface (16, 17), was not significantly changed by cytokine or biomechanical stimuli. Thus, these data clearly demonstrate that certain EC genes can exhibit a differential (LSS, not TSS) and prolonged ($\geq$24 hr) up-regulation in response to steady LSS.

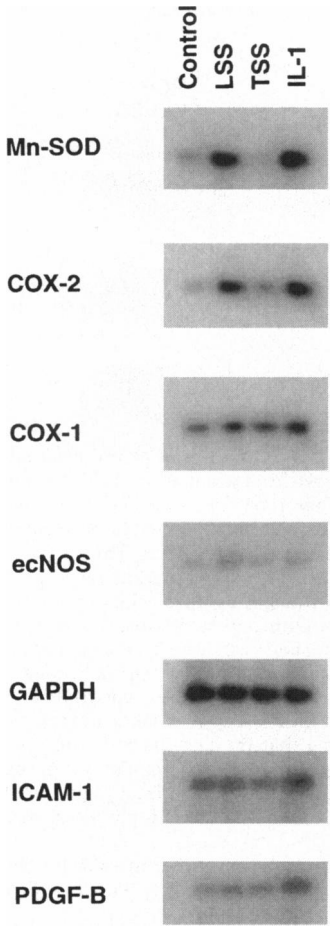

FIG. 2. Sustained up-regulation of Mn SOD, COX-2, and ecNOS by 24 hr of LSS. RNA was isolated from HUVEC monolayers continuously exposed to LSS or TSS (10 dyne/cm^2), or 10 units/ml of rhIL-1β for 24 hr. The relative levels of RNAs for Mn SOD, COX-2, COX-1, ecNOS (endothelial form of nitric oxide synthase), glyceraldehyde-3-phosphate dehydrogenase, ICAM-1, and PDGF-B were determined by quantitative RT-PCR, as in Fig. 1.

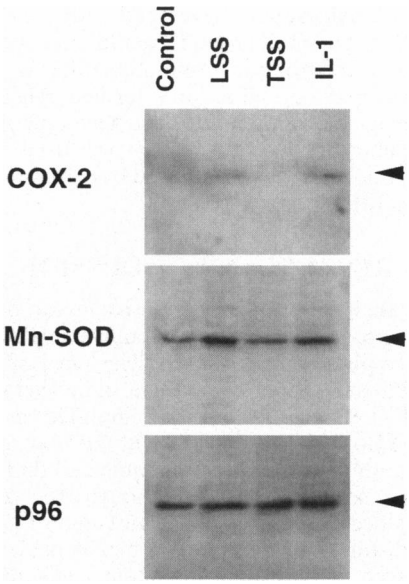

FIG. 3. LSS up-regulates COX-2 and Mn SOD protein in HUVEC. HUVEC monolayers were stimulated with LSS or TSS (10 dyne/cm^2), or 10 units/ml of rhIL-1β, for 24 hr. Whole cell lysates were prepared and equal amounts of protein from each condition were electrophoresed on 12% polyacrylamide denaturing gels and blotted to nitrocellulose membranes. A monoclonal antibody to COX-2 and a polyclonal antisera to human Mn SOD were used to probe the membranes. These Western blots were developed with the appropriate secondary antibody and a chemiluminescent reporter system. Quantitation was performed by densitometer. p96 is an endothelial cell surface protein identical to endoglin, a type β transforming growth factor binding protein (25).

Recent work in our laboratory has demonstrated that the LSS-induction of PDGF-B chain gene expression in cultured EC involves the interaction of a cis-acting promoter element, termed the "shear-stress-response element," with shear-inducible transcription factors (27, 28). Analogous shear-responsive transcriptional regulatory mechanisms have been described in other genes (29). To determine if transcriptional up-regulation was playing a role in the differential response of COX-2 and Mn SOD to steady laminar versus turbulent flows, nuclear runoff analysis was performed. As shown in Fig. 4, HUVEC stimulated by LSS for 3 hr demonstrated an increase in the transcriptional rate of the COX-2, Mn SOD, and ICAM-1 genes. A similar pattern was observed in response to IL-1β, whereas COX-1 and tubulin were not significantly altered by either LSS or cytokine. In contrast, exposure to TSS did not stimulate the transcriptional rate of any of these genes. Thus, the LSS-selective pattern of induction of COX-2 and Mn SOD genes in EC appears to be mediated, at least in part, at the transcriptional level.

In the developing atherosclerotic lesion, enhanced production of reactive oxygen species (e.g., peroxides, superoxides, and hydroxyl radicals), both in vascular endothelial and smooth muscle cells, as well as emigrated mononuclear leukocytes (foamy macrophages), results in a localized "oxidative stress" within the arterial wall (30, 31). Current models of atherogenesis envision products of the oxidative modification of low density lipoprotein (e.g., lysophosphatidylcholine) as well as intracellular redox state, *per se*, influencing key functions including gene expression in the dysfunctional EC (32–34). For example, intracellular redox-sensitive signals have been implicated in the up-regulation of vascular cell adhesion molecule-1, a mononuclear leukocyte-selective adhesion molecule induced on the surface of ECs during early atherosclerotic lesion development in certain animal models (35–38). Mn SOD is an intracellular antioxidant enzyme that might influ-

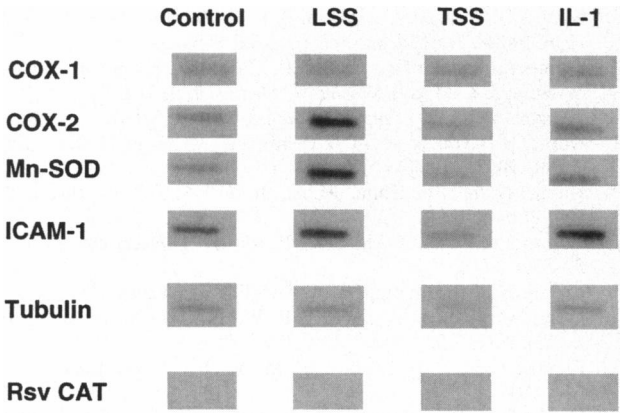

FIG. 4. Nuclear runoff analysis of HUVEC subjected to shear stress or cytokine stimulation. HUVEC monolayers were subjected to LSS or TSS (10 dyne/cm^2), or 10 units/ml of rhIL-1β, for 3 hr, nuclei isolated and nascent RNA labeled. The labeled RNA was isolated and used to probe membranes containing immobilized, denatured plasmids containing the indicated cDNAs. The intensity of specific bands is a direct reflection of the rate of transcription of that gene elicited by the test stimulus. RSV-CAT is a negative control plasmid, which contains no EC genes.

ence these processes. Although the role of Mn SOD in regulating cytosolic redox status under physiologic conditions is unknown, this enzyme resides within the mitochondrion, a site where excessive production of radicals can occur during oxidative stress. Its localization, as well as inducibility in response to injurious stimuli such as hyperoxia and phorbol esters, suggests that this molecule plays an important role in cellular defense. Indeed, overexpression of Mn SOD, both *in vitro* and *in vivo*, confers resistance to oxidative damage, or radiation and tumor necrosis factor-induced cytotoxicity, in a variety of cell types including endothelial cells (39, 40).

COXs (COX-1 and COX-2) are central enzymes regulating the synthesis of prostanoids from membrane-derived arachidonic acid. Although this pathway typically is proinflammatory in most cell types, in EC the major product is prostacyclin (PGI_2), a potent inhibitor of platelet aggregation, leukocyte activation and adhesion, vascular smooth muscle contraction, migration and growth, and cholesterol ester accumulation in vascular cells (41, 42). Decreased PGI_2 production has been reported in human atherosclerotic vessels, and this molecule has been used successfully to treat clinical complications of peripheral vascular disease (42). These observations suggest that PGI_2 may function as an endogeneous vasoprotective and anti-atherogenic molecule. COX-2, in contrast to COX-1, is an inducible enzyme, whose levels are quite low in most cell types including EC, but can be rapidly up-regulated by a variety of biochemical stimuli, such as lipopolysaccharide, cytokines, and growth factors (21, 22, 43, 44). Overexpression of COX-2 in cultured epithelial cells confers a resistance to chemically induced apoptosis (44), while genetically modified mice, lacking the gene for COX-2 exhibit significant renal pathology and are susceptible to peritonitis (46), suggesting that this enzyme may have a variety of pathophysiologic roles. Recently, lysophosphatidylcholine, a component of oxidized lipoproteins, was found to induce COX-2 in cultured EC, a finding interpreted as a potential vasoprotective response that could serve to limit the progression of atherosclerotic lesions *in vivo* (47).

Thus, both COX-2 and Mn SOD, based on their known activities, appear to represent "atheroprotective genes," whose selective up-regulation in EC by steady LSS may represent an important local vasoprotective mechanism. To further investigate the hypothesis that steady LSS may differentially regulate potentially atheroprotective genes, we examined the behavior of the endothelial isoform of NOS (NOS-3, ecNOS), whose product NO is thought to play a central role in vascular homeostasis (for reviews, see refs. 48 and 49). Specifically in the context of atherogenesis, NO has been demonstrated to inhibit thrombosis, cytokine-induced vascular cell adhesion molecule-1 expression, leukocyte adhesion to endothelium, and smooth muscle proliferation and migration (48–50). Together these observations suggest that NO is an important atheroprotective molecule. As demonstrated in Fig. 2, the mRNA for ecNOS was up-regulated approximately 3-fold over baseline by 24 hr of steady LSS, whereas turbulent shear and cytokine treatment had no effect. These results confirm and extend a previous report demonstrating LSS up-regulation of ecNOS mRNA in cultured bovine aortic endothelial cells (51), and indicate that ecNOS may constitute an additional atheroprotective gene differentially regulated by these fluid mechanical stimuli. Together with the observation that LSS acts to down-regulate the expression of vascular cell endothelial adhesion molecule-1, a potential pro-atherogenic leukocyte adhesion molecule (52), our data indicate that steady LSSs may exert a coordinated influence on EC phenotype, the net effect of which is anti-atherogenic.

Our differential display analysis also identified a number of other endothelial genes that exhibited various patterns of response to cytokine and fluid mechanical stimuli. Interestingly, however, none of these, which include macrophage chemotactic protein-1 (MCP-1), the endothelial-leukocyte adhesion molecules E-selectin, vascular cell adhesion molecule-1, and ICAM-1, as well as the platelet adhesion molecule von Willebrand factor, exhibit the sustained LSS-selective up-regulation described above (data not shown; refs. 24 and 29). These observations suggest that this differential pattern of response to fluid mechanical stimuli is limited to a subset of endothelial genes. Current efforts are directed to functionally characterizing certain novel EC genes that also demonstrated a LSS-selective pattern of response in our differential display analysis (unpublished observations).

In summary, we have found that a physiological level of steady LSS, an *in vitro* fluid mechanical stimulus that mimics the average wall shear typically encountered in lesion-protected areas of the arterial vasculature, can induce the selective and sustained up-regulation of three pathophysiologically relevant endothelial genes, COX-2, Mn SOD, and ecNOS. Based on their known activities, the products of each of these genes would be predicted to have "anti-atherogenic" or "atheroprotective" effects *in vivo*. Their coordinated and selective induction by steady LSS provides a possible mechanistic link between the local hemodynamic milieu, endothelial gene expression, and early events in atherogenesis, and thus may be one of the mechanisms contributing to the nonrandom localization of early atherosclerotic lesions. Further experimental validation of this hypothesis will involve the elucidation of the regional patterns of expression of these molecules in the arterial endothelial lining *in vivo*, and, ultimately, their targeted overexpression in appropriate animal models, as an attempt to modify the topographic distribution of atherosclerotic lesions.

Note Added in Proof. Since submission of this manuscript, Inoue *et al.* (53) have reported that laminar shear stress also stimulates the expression of the cytosolic copper/zinc-containing superoxide dismutase (Cu/Zn SOD), the major form of SOD outside of the mitochondria, in cultured human aortic endothelial cells.

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1. Ross, R. (1993) *Nature (London)* **362,** 801–809.
2. Gimbrone, M. A., Jr. (1995) in *Molecular Cardiovascular Medicine*, ed. Haber, E. (Sci. Am. Med., New York), pp. 49–61.
3. Gimbrone, M. A., Jr., Cybulsky, M. I., Kume, N., Collins, T. & Resnick, N. (1995) *Ann. N.Y. Acad. Sci.* **748,** 122–132.
4. Fry, D. L. (1973) *CIBA Found. Symp.* **12,** 96–118.
5. Caro, C. G., Fitzgerald, J. M. & Schroter, R. C. (1969) *Nature (London)* **223,** 1159–1161.
6. Cornhill, J. F. & Roach, M. R. (1976) *Atherosclerosis* **23,** 489–499.
7. Karino, Y. & Motomiya, M. (1983) *Biorheology* **20,** 119–127.
8. Zarins, C. K., Giddens, D. P., Bharadvaj, B. K., Sottiurai, V. S., Mabon, R. F. & Glagov, S. (1983) *Circ. Res.* **53,** 502–514.
9. Ku, D. N., Giddens, D. P., Zarins, C. K. & Glagov, S. (1985) *Arteriosclerosis* **5,** 293–301.
10. Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. & Ross, R. (1994) *Arterioscler. Thromb.* **14,** 133–140.
11. Davies, P. F. (1995) *Physiol. Rev.* **75,** 519–560.
12. Resnick, N. & Gimbrone, M. A., Jr. (1995) *FASEB J.* **9,** 874–882.
13. Bussolari, S. R., Dewey, C. F., Jr., & Gimbrone, M. A., Jr. (1982) *Rev. Sci. Instrum.* **53,** 1851–1854.
14. Sdougos, H. P., Bussolari, S. R. & Dewey, C. F. (1984) *J. Fluid Mech.* **138,** 379–404.
15. Liang, P. & Pardee, A. B. (1992) *Science* **257,** 967–971.
16. Bevilacqua, M., Stengelin, S., Gimbrone, M. A., Jr., & Seed, B. (1989) *Science* **243,** 1160–1165.
17. Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeus, C., Massague, J. & Letarte, M. (1992) *J. Biol. Chem.* **267,** 19027–19030.
18. Davies, P. F., Remuzzi, A., Gordon, E. J., Dewey, C. F., Jr., & Gimbrone, M. A., Jr. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2114–2117.
19. Pober, J. S. & Cotran, R. S. (1990) *Physiol. Rev.* **70,** 427–451.
20. Pritchard, K. A., O'Bannion, M. K., Miano, J. M., Vlasic N., Bhatia, U. G., Young, D. A. & Stemerman, M. B. (1994) *J. Biol. Chem.* **269,** 8504–8509.
21. Jones, D. A., Carlton, D. P., McIntyre, T. M., Simmerman, G. A. & Prescott, S. M. (1993) *J. Biol. Chem.* **266,** 9049–9054.
22. Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T. & Hla, T. (1994) *J. Biol. Chem.* **269,** 11769–11775.
23. Suzuki, K., Tatsumi, H., Satoh, S., Senda, T., Nakata, T., Fujii, J. & Taniguchi, N. (1993) *Am. J. Physiol* **265,** H1173–H1178.
24. Nagel, T., Resnick, N., Atkinson, W. J., Dewey, C. F. Jr., & Gimbrone, M. A., Jr. (1994) *J. Clin. Invest.* **94,** 885–891.
25. Hsieh, H. J., Li, N. Q. & Frangos, J. (1991) *Am. J. Physiol.* **260,** H642–H646.
26. Hsieh, H., Li, N. & Frangos, J. A. (1992) *J. Cell. Physiol.* **150,** 552–558.
27. Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F., Jr., & Gimbrone, M. A., Jr., (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4591–4595.
28. Khachigian, L. M., Resnick, N., Gimbrone, M. A., Jr., & Collins, T. (1995) *J. Clin. Invest.* **96,** 1169–1175.
29. Shyy, J. Y., Lin, M. C., Han, J., Lu, Y., Petrime, M. & Chien, S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8069–8073.
30. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witzum, J. L. (1989) *N. Engl. J. Med.* **320,** 915–924.
31. Alexander, R. W. (1995) *Hypertension* **25,** 155–161.
32. Kume, N., Cybulsky, M. I. & Gimbrone, M. A., Jr. (1992) *J. Clin. Invest.* **90,** 1138–1144.
33. Kume, N. & Gimbrone, M. A., Jr. (1994) *J. Clin. Invest.* **93,** 907–911.
34. Offermann, M. K. & Medford, R. M. (1994) *Heart Dis. Stroke* **3,** 52–57.
35. Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W. & Medford, R. M. (1993) *J. Clin. Invest.* **92,** 1866–1874.
36. Cybulsky, M. I. & Gimbrone, M. A., Jr. (1991) *Science* **251,** 788–791.
37. Li, H., Cybulsky, M. I., Gimbrone, M. A., Jr., & Libby, P. (1993) *Arterioscler. Thromb.* **13,** 197–204.
38. Collins, T. (1993) *Lab Invest.* **68,** 499–508.
39. Wispe, J. R., Warner, B. B., Clark, J. C., Dey, C. R., Nwuman, J., Glasser, S. W., Crapo, J. D., Chang, L.-Y. & Whitsett, J. A. (1992) *J. Biol. Chem.* **267,** 23937–23941.
40. Lindau-Shepard, B., Shaffer, J. B. & Vecchio, P. J. D. (1994) *J. Cell. Physiol.* **161,** 237–242.
41. Pomerantz, K. B. & Hajar, D. P. (1989) *Arteriosclerosis* **9,** 413–429.
42. Vane, J. R. & Botting, R. M. (1995) *Am. J. Cardiol.* **75,** 3A–10A.
43. Hla, T. & Neilson, K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7384–7388.
44. Vane, J. R., Mitchell, J. A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J. & Willoughby, D. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2046–2050.
45. Tsujii, M. & Dubois, R. N. (1995) *Cell* **83,** 493–501.
46. Morham, S. G., Langenbach, J. R., Loftin, C. D., Tiano, H. F., Vouloumaos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A. & Smithies, O. (1995) *Cell* **83,** 473–482.
47. Zembowicz, A., Jones, S. L. & Wu, K. K. (1995) *J. Clin. Invest.* **96,** 1688–1692.
48. Dusting, G. (1995) *J. Vasc. Res.* **32,** 143–161.
49. Wennmalm, A. (1994) *J. Intern. Med.* **235,** 317–327.
50. DeCaterina, R., Libby, P., Thannickal, V. J., Peng, H.-B., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S. & Liao, J. K. (1995) *J. Clin. Invest.* **96,** 60–68.
51. Nishida, K., Harrison, D. G., Nawas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Nerem, R. M., Alexander, R. W. & Murphy, T. J. (1992) *J. Clin. Invest.* **90,** 2092–2096.
52. Ando, J., Tsuboi, H., Korenaga, R., Takada, Y., Toyama-Sorimachi, N., Miyasaka, M. & Kamiya, A. (1994) *Am. J. Physiol.* **267,** C679–C687.
53. Inoe, N., Ramasamy, S., Fukai, T., Nerem, R. M. & Harrison, D. G. (1996) *Circ. Res.* **79,** 32–37.