DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells

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Using DNA microarray screening (GeneFilter 211, Research Genetics, Huntsville, AL) of mRNA from primary human umbilical vein endothelial cells (HUVEC), we identified 52 genes with significantly altered expression under shear stress [25 dynes/cm² for 6 or 24 h (1 dyne = 10 μN), compared with matched stationary controls]; including several genes not heretofore recognized to be shear stress responsive. We examined mRNA expression of nine genes by Northern blot analysis, which confirmed the results obtained on DNA microarrays. Thirty-two genes were up-regulated (by more than 2-fold), the most enhanced being cytochromes P450 1A1 and 1B1, zinc finger protein EZF/GKLF, glucocorticoid-induced leucine zipper protein, argininosuccinate synthase, and human prostaglandin transporter. Most dramatically decreased (by more than 2-fold) were connective tissue growth factor, endothelin-1, monocyte chemotactic protein-1, and spermidine/spermine N1-acetyltransferase. The changes observed suggest several potential mechanisms for increased NO production under shear stress in endothelial cells.

During the past 15 years, over 40 genes have been identified as being regulated by shear stress in endothelial cells (1–4). Shear stress responsive genes are involved in cell proliferation, differentiation, maintenance of vascular tone, thrombosis, cell–matrix and cell–cell adhesion, and modulation of the inflammatory/immune system. The identification of such genes is important not only for developing a fundamental understanding of how endothelial cells work, but also for understanding and treating pathological conditions that are influenced by shear stress, such as thrombosis, restenosis, and atherosclerosis (5, 6).

Most of the genes that have been shown to be regulated by shear stress were identified by using traditional techniques such as Northern blot analysis or reverse transcriptase PCR (7–9). The main limitation of these techniques is that only one gene or at best a handful of genes can be studied in one experiment. When multiple genes are studied by using traditional methods, the experiments usually require a reiteration of the detection procedure for each gene. Investigators must therefore be very selective in the genes they choose to study, necessitating *a priori* information linking the chosen genes to shear stress. Thus, these experiments generally tend to validate or disprove specific hypotheses and do not lead to the discovery of unexpected differentially expressed genes. However, DNA microarray technology allows researchers to study several thousands of genes at one time. In addition to identifying unexpected genes, this technology also has the power to lead to the development of new hypotheses concerning how cells respond to shear stress and identification of coregulated pathways responsive to the mechanical environment of the cell.

We used DNA GeneFilter GF211 from Research Genetics (Huntsville, AL; ref. 10), which contains over 4,000 named human genes, to identify genes altered by shear stress in primary human umbilical vein endothelial cells (HUVEC) exposed to 25 dyn/cm² (1 dyne = 10 μN) for 6 or 24 h. We observed significant increases in mRNA levels for 32 genes and significant decreases in expression for 20 genes. The microarray results were confirmed by Northern blot analysis for nine genes. In addition to detecting changes in the levels of several genes known to be affected by shear stress, we identified several previously unreported shear stress responsive genes. From this information we have developed several hypotheses on how shear stress regulates the synthesis of nitric oxide in endothelial cells.

Methods

Cell Culture. Human umbilical veins were flushed with PBS, then filled with PBS containing collagenase (200 mg/ml) and incubated for 30 min at room temperature (11). Then the veins were washed with PBS, and the collagenase solution and the wash were collected and pooled. Cells were pelletted (200 × *g* for 10 min) and resuspended in Medium 199 (Sigma) supplemented with 10% defined FBS (HyClone), penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium). The cells were seeded on glass slides (≈1.8 × 10^6 cells per slide) coated with glutaraldehyde cross-linked gelatin (12). The cells were washed daily with PBS, fed with complete medium, and incubated at 37°C in the presence of humidified 95% air and 5% CO_2. Cells were cultured for 3 days before exposure to shear stress. HUVEC were pooled from ≈30 umbilical veins for each harvest. This study is based on three harvests for each experiment.

Exposure of Cells to Shear Stress. HUVEC were shear stressed for 6 or 24 h, using a parallel plate flow chamber connected to a constant pressure drop flow loop (13). The flow loop apparatus was maintained at 37°C, and gassed continuously with a humidified mixture of 5% CO_2 in air. For each experiment three to four flow loops were run for each time point. Matched control cells were cultured under static conditions in parallel.

RNA Isolation. After shear stress exposure, the cells were rinsed quickly in ice-cold PBS and RNA was isolated by using TRIzol Reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. RNA quality was insured by gel visualization and spectrophotometric analysis ($OD_{260/280}$). For each time point in each experiment, RNA from three to four slides were pooled. This provided sufficient RNA for both the DNA microarray and Northern analysis on the same sample. The RNA was quantitated by spectrophotometric analysis at 260 nm.

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Abbreviations: EC, endothelial cell(s); HUVEC, human umbilical vein EC; eNOS, endothelial NO synthase; PAI-1, plasminogen activator inhibitor-1; CTGF, connective tissue growth factor; ET-1, endothelin-1.

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Probe Preparation and cDNA Microarray Hybridization. Three micrograms of total RNA were reverse-transcribed in the presence of 300 units of SuperScript II RNase H^- Reverse Transcriptase (Life Technologies), 100 μCi (1 Ci = 37 GBq) of [α-^{33}P]dCTP (10 mCi/ml, 3,000 Ci/mmol; ICN) and 2 μg of oligo(dT) (10–20-mer; Research Genetics). Reactions were each carried out at 37°C for 90 min in 30 μl of buffer consisting of 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 3.3 mM DTT, and 1 mM each of dATP, dGTP, and dTTP. The resulting ^{33}P-cDNA probes were purified with Bio-Spin 6 Chromatography Columns (Bio-Rad) following the manufacturer's instructions. Human Named Genes GeneFilters (GF211 from Research Genetics) were used for differential expression screening. The membranes were first pretreated with boiled 0.5% SDS for 10 min. Prehybridizations were performed for 6 h at 42°C in MicroHyb hybridization solution (Research Genetics) with poly(dA) and denatured Cot-1 as blocking reagents. The column-purified and denatured probes were then added and hybridized at 42°C for 16 h. After hybridization, the membranes were washed twice in a solution containing 2× standard saline citrate (SSC) and 1% SDS for 20 min at 50°C followed by five additional washes performed at the same temperature in a washing solution consisting of 0.5× SSC and 1% SDS for 15 min each. The membranes were then exposed to phosphor image screens for 16 h. Images were acquired by using a Cyclone Phosphor System (Packard), and analyzed by using the PATHWAYS 2.1 (Research Genetics) and EXCEL (Microsoft). GeneFilters from the same manufacturing lot were used in all three separate experiments. The hybridization for each sample at each time point was done in triplicate using three separate GeneFilters. Normalized intensities were calculated from each GeneFilter by first subtracting a constant background value as reported by PATHWAYS and dividing each point by the average intensity for that GeneFilter and multiplying the result by 2,000. Gene expression ratios from each experiment were calculated by using the average normalized intensities from each of the GeneFilters for a specific RNA sample (shear stressed to control values). The expression ratios reported are the average and standard deviations from the three separate experiments. Only genes with average normalized intensities of 100 or above were studied.

Northern Analysis. Five micrograms of total RNA (from the same RNA isolates that were analyzed with DNA microarrays) were electrophoresed in a 1% formaldehyde agarose gel and vacuum transferred onto a nylon membrane. The RNA was immobilized on the membrane by UV-crosslinking. The blot was prehybridized at 60°C in QuickHyb hybridization solution (Stratagene) for 20 min and then hybridized for 1 h with 12.5×10^6 counts per minute of cDNA probe labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by using a random primer labeling kit (Stratagene). These probes were prepared from cDNAs with the same sequence as those bound to the GF211 DNA microarrays. The blot was washed as recommended by Stratagene. A phosphor imaging plate was then exposed to the blot for 10 min to 36 h. The plate was scanned and the fold changes in RNA levels were quantitated by normalizing relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Results

Genes with a ratio of 2.0 or above were considered positively regulated by shear stress, whereas those that had a ratio of 0.5 or below were considered negatively regulated (Tables 1 and 2). These tables also include genes that satisfied the threshold values in two of three experiments, but in which the average ratio was slightly above 0.5 or below 2.0. Using these criteria, we identified 52 genes that responded to shear stress. The expression of 32 genes increased, whereas the expression of 20 genes was decreased by shear stress.

We examined the accuracy of the microarray analysis by choosing nine genes for Northern blot analysis that encompassed a wide range of expression ratios. Fig. 1 shows microarray ratios, Northern ratios, and representative blots on these genes. The data from both microarray and Northern analyses showed that the expression of the GAPDH gene is unresponsive to shear stress as previously shown (7); and expression ratios of Northern values on other genes were normalized to GAPDH.

The largest increases in gene expression were observed for the cytochrome P450 (CYP) 1A1 and 1B1 genes. The DNA microarray mRNA ratio for CYP1A1 at 6 h was 3.90 and at 24 h increased dramatically in expression, with a ratio of 11.15. These results agree with the Northern ratios of 3.47 at 6 h and 12.57 at 24 h. The expression level of CYP1B1 gene increased at both 6 and 24 h with microarray ratios of 7.55 and 9.70, respectively. The genes for connective tissue growth factor (CTGF) and endothelin-1 (ET-1) showed the largest decreases in mRNA levels due to shear stress. CTGF mRNA levels decreased drastically at 6 and 24 h with DNA microarray mRNA ratios of 0.13 and 0.10, respectively, and agree with the Northern blot ratios. ET-1 mRNA levels also dropped significantly with DNA microarray mRNA ratios of 0.24 and 0.20 at 6 and 24 h, respectively. Once again, these values agreed with those obtained from the Northern values of 0.22 and 0.17.

The genes for both NADH dehydrogenase and plasminogen activator inhibitor-1 (PAI-1) had microarray expression ratios that did not respond significantly to shear stress. NADH dehydrogenase had a ratio of $\approx$1 at both 6 and 24 h. Northern analysis confirmed that the NADH dehydrogenase gene did not respond to shear stress. The results were different for the PAI-1 gene, which had a microarray ratio that was slightly greater than 1 at 6 h, but was 0.65 at 24 h. PAI-1 has two transcripts, 2.3 and 3.2 kb in length, that derive from different polyadenylation sites (14). At 6 h, Northern analysis indicated that the 2.3-kb transcript increased with an mRNA ratio of 1.43, whereas the 3.2-kb transcript was not changed. The levels of both transcripts were significantly decreased at 24 h, with Northern ratios of 0.55 and 0.63; similar to the 0.65 ratio obtained by microarray analysis.

At 24 h, two genes, Prostaglandin H synthase 2 (PGHS-2, COX-2; ref. 4) and tissue plasminogen activator (tPA; ref. 7), that have been reported to respond to shear stress gave somewhat different results. PGHS-2 had a microarray mRNA ratio of 2.2 at 6 h, in agreement with the Northern mRNA ratio of 2.5. But at 24 h we found no significant differences in PGHS-2 mRNA levels between shear stressed and control cells, and the mRNA ratio obtained from Northern blotting (0.91; Fig. 1) confirmed the microarray result. For tPA, the microarray ratios were 1.03 and 1.28 at 6 and 24 h, respectively. However, the Northern ratio for tPA at 6 h was 2.23, twice that of the tPA Northern ratio at 24 h. This discrepancy in ratios for tPA, although not significant, may be due to the low expression level of this gene in HUVEC.

Discussion

The use of DNA microarray analysis has identified 32 genes that were up-regulated significantly by shear stress (Table 1) and 20 genes that were significantly down-regulated (Table 2). We confirmed the accuracy of the microarray results with Northern analysis of 9 genes (Fig. 1). Specifically, CYP 1A1 and CYP 1B1 were dramatically up-regulated, and ET-1 and CTGF were down-regulated in both analyses. The microarray results confirmed findings reached by other methods on ET-1 (15), MCP-1 (16), and PAI-1 (7). Shear stress did not change NADH dehydrogenase; although Ando *et al.* (17) found that a gene coding for a different subunit of this enzyme was down-regulated by shear stress. PGHS-2 mRNA was up-regulated at 6 h and returned to baseline by 24 h, which is consistent with our

Table 1. Genes that increase under shear stress detected by DNA microarray analysis

Group*	Gene	Ratio (SS/Cont)	
Antioxidants	Cytochrome P450 1B1	6 h	7.55 ± 1.28
	AA448157	24 h	9.70 ± 2.71
	Cytochrome P450 1A1	6 h	3.90 ± 0.48
	AA418907	24 h	11.15 ± 3.75
	Heme oxygenase-1	6 h	3.08 ± 0.84
	T71606	24 h	1.78 ± 0.18
	NAD(P)H:quinone oxidoreductase (NQO1)	6 h	1.28 ± 0.12
	AA458634	24 h	2.09 ± 0.32
Proliferation/differentiation	Zinc finger protein EZF/GKLF	6 h	4.56 ± 0.69
	H45668	24 h	2.78 ± 0.54
	Receptor tyrosine phosphatase	6 h	3.73 ± 0.77
	AA486403	24 h	1.82 ± 0.05
	TGF-β-stimulated clone 22 (TSC-22)	6 h	2.18 ± 0.23
	AA664389	24 h	2.18 ± 0.25
	Tyrosine kinase receptor precursor	6 h	2.31 ± 0.36
	TIE-2 H02848	24 h	1.80 ± 0.48
	Tyrosine kinase HTK	6 h	1.40 ± 0.05
	T51849	24 h	2.09 ± 0.79
	E2F transcription factor 5, p130 binding	6 h	2.05 ± 0.71
	AA455521	24 h	1.56 ± 0.64
	Human orphaned G protein coupled receptor	6 h	2.39 ± 1.17
	N53172	24 h	1.13 ± 0.31
	Glycyl tRNA synthetase	6 h	1.97 ± 0.56
	AA629909	24 h	1.08 ± 0.19
	Jagged 1 (Human HJ1)	6 h	2.22 ± 0.37
	R70685	24 h	1.82 ± 0.51
Vascular tone	Argininosuccinate synthetase	6 h	2.41 ± 0.21
	AA676466	24 h	3.04 ± 0.88
	α-Galactosidase A precursor	6 h	1.42 ± 0.02
	AA251784	24 h	2.56 ± 0.23
	Vasoactive intestinal polypeptide receptor	6 h	1.39 ± 0.20
	precursor (VIPR1) H73241	24 h	2.13 ± 0.28
	PGHS-2	6 h	2.20 ± 0.45
	AA644211	24 h	0.91 ± 0.16
ECM/cytoskeleton	Elastin	6 h	2.93 ± 0.42
	AA459308	24 h	2.26 ± 0.59
	Connexin 37	6 h	2.45 ± 0.88
	H44032	24 h	1.48 ± 0.38
	α-Spectrin	6 h	1.76 ± 0.17
	T60117	24 h	1.99 ± 0.51
	Galectin 3	6 h	1.22 ± 0.09
	AA630328	24 h	2.16 ± 0.56
Immune/inflammation	Podocalyxin-like protein	6 h	3.37 ± 0.55
	N64508	24 h	1.77 ± 0.21
	CD34	6 h	1.70 ± 0.20
	AA043438	24 h	2.41 ± 0.22
	IL-1 receptor, type 1 precursor	6 h	1.51 ± 0.38
	AA464526	24 h	2.22 ± 0.18
	Leukocyte elastase inhibitor	6 h	1.06 ± 0.11
	AA486275	24 h	2.11 ± 0.58
Transcription factor	Glucocorticoid-induced leucine zipper protein	6 h	3.35 ± 0.70
	AA775091	24 h	3.28 ± 0.79
Transport systems	Human prostaglandin transporter	6 h	3.33 ± 1.05
	AA037014	24 h	2.49 ± 0.19
	Heat shock protein 70	6 h	2.08 ± 0.31
	AA629567	24 h	0.76 ± 0.09
	Chromogranin A	6 h	1.05 ± 0.17
	R36264	24 h	2.07 ± 0.83
Protein modification	Paired basic amino acid cleaving system 4	6 h	1.74 ± 0.30
	AA251457	24 h	2.44 ± 0.21
RNA degradation	RNase A family, 1	6 h	0.91 ± 0.18
	AA485893	24 h	2.01 ± 0.06
Thrombosis	S100 calcium binding protein A10	6 h	1.27 ± 0.23
	AA444051	24 h	1.96 ± 0.32

*Genes were grouped based on function. Many genes can be assigned to more than one group (e.g., transcription factors).

Table 2. Genes that decrease under shear stress detected by DNA microarray analysis

Group	Gene	Ratio (SS/Cont)	
Vascular tone	Endothelin-1	6 h	0.21 ± 0.08
	H11003	24 h	0.20 ± 0.05
	Caveolin-1	6 h	0.49 ± 0.09
	AA055835	24 h	0.49 ± 0.13
Extracellular matrix	CTGF	6 h	0.13 ± 0.06
	AA598794	24 h	0.10 ± 0.02
	Cardiac gap junction (Connexin 43)	6 h	0.58 ± 0.24
	AA487623	24 h	0.39 ± 0.10
	Matrilin-2	6 h	0.81 ± 0.11
	AA071473	24 h	0.52 ± 0.08
Proliferation/differentiation	Spermidine/spermine N1-acetyltransferase	6 h	0.37 ± 0.11
	R58991	24 h	0.20 ± 0.07
	Cyr61	6 h	0.35 ± 0.15
	AA777187	24 h	0.41 ± 0.18
	S1-5 (fibrillin-like, FBNL)	6 h	0.65 ± 0.06
	AA875933	24 h	0.21 ± 0.05
	BMP-4	6 h	0.45 ± 0.03
	AA463225	24 h	0.58 ± 0.13
	Aldehyde dehydrogenase 1	6 h	0.50 ± 0.22
	AA664101	24 h	0.52 ± 0.18
	Adenylosuccinate synthetase	6 h	0.59 ± 0.29
	AA431414	24 h	0.74 ± 0.24
	Gene for H4 histone	6 h	0.80 ± 0.10
	AA868008	24 h	0.50 ± 0.05
Cytoskeleton	Nonmuscle myosin heavy chainB Smemb	6 h	0.48 ± 0.14
	AA490477	24 h	0.87 ± 0.20
	α-Tubulin	6 h	0.88 ± 0.14
	AA865469	24 h	0.50 ± 0.04
GTPases	Rho B	6 h	0.63 ± 0.10
	AA495790	24 h	0.27 ± 0.07
	Guanylate binding protein 1 (GBP1)	6 h	0.48 ± 0.11
	AA486850	24 h	0.96 ± 0.24
Transcription factor	SL3-3 enhancer factor 2 (SEF2-1A)	6 h	0.40 ± 0.10
	AA669136	24 h	0.52 ± 0.08
Protein modifier	Sialyltransferase 1	6 h	0.54 ± 0.13
	AA598652	24 h	0.46 ± 0.18
Chemotaxis	Monocyte chemotactic protein 1 (MCP-1)	6 h	0.35 ± 0.14
	AA425102	24 h	0.28 ± 0.07
Unknown	Myosin heavy chain homolog (Doc1)	6 h	0.39 ± 0.08
	W69790	24 h	0.58 ± 0.17

previous studies of PGHS-2 protein levels under similar shear conditions (18).

The most dramatically up-regulated gene expression was observed in cytochromes P450 (CYP) 1A1 and 1B1 (Fig. 1). Classically, the CYP gene families have been associated with cellular detoxification mechanisms. Of the 19 CYP genes present on the GF211, only CYP 1A1 and 1B1 were affected by shear stress in HUVEC. CYP 1A1 activity can be induced in human endothelial cells (EC) by toxic aromatic hydrocarbons, but not CYP 1B1 (19–21). CYP 1A1 has been postulated to participate in endogenous signaling of oxidative processes (22). Furthermore, there is evidence that the production of an endothelial-derived hyperpolarizing factor from arachidonic acid in endothelial cells is catalyzed by CYP 1A1; and, loss of this gene in cultured EC correlates with dedifferentiation (23). Expression of CYP 1B1 in EC is much less studied than CYP 1A1. A comparison of senescent fibroblast, epithelial, and HUVEC cell lines revealed CYP 1B1 to be up-regulated only in senescent HUVEC (24). The strong induction of these CYP genes by shear stress is consistent with the suggestion that physiological levels of shear stress are protective for endothelium.

CTGF, which was initially purified and identified from HUVEC-conditioned medium, is elevated in fibrotic lesions, and may play a role in the development of fibrotic diseases (25). It has also been shown to be highly expressed in vascular cells in atherosclerotic lesions, but not in normal arteries (26). These lesions are usually found in regions of low wall shear stress, often with complex, recirculating blood flow patterns (1). The striking down-regulation of CTGF expression that we observe in response to normal arterial levels of shear stress is consistent with these findings, and with the hypothesis that physiological arterial shear stress protects against fibrotic and atherosclerotic disease processes.

Many of the other genes in Tables 1 and 2, whose biological functions are known, are associated with vascular biological pathways that are regulated at least in part by shear stress. By identifying additional genes in these systems it will be possible to further define the complex mechanisms by which shear stress controls them. For example, shear stress is considered by many to be the most important stimulus for NO production in EC (27). Several genes identified as shear stress responsive from our DNA microarray analysis may play a role in the regulation of NO production by shear stress, and suggest the following pathways for shear stress regulation of NO production in endothelial cells. Generating hypotheses in the absence of further supporting data may be considered excessive, but we believe they provide stimuli for further work.

	DNA Microarray	Northern
CYP1A1		
6 hr	3.90 ± 0.48	3.47 ± 0.47
24 hr	11.15 ± 3.75	12.57 ± 6.70
CYP1B1		
6 hr	7.55 ± 1.28	10.40 ± 4.16
24 hr	9.70 ± 2.71	15.33 ± 10.4
CTGF		
6 hr	0.13 ± 0.06	0.23 ± 0.05
24 hr	0.10 ± 0.03	0.14 ± 0.06
ET-1		
6 hr	0.24 ± 0.10	0.22 ± 0.06
24 hr	0.20 ± 0.05	0.17 ± 0.05
PGHS-2		
6 hr	2.20 ± 0.45	2.53 ± 0.29
24 hr	0.91 ± 0.16	0.70 ± 0.17
NADH Dehydrogenase		
6 hr	0.91 ± 0.22	0.83 ± 0.07
24 hr	1.29 ± 0.08	1.00 ± 0.09
PAI-1		
6 hr	1.32 ± 0.41	1.43 ± 0.15*
		1.26 ± 0.24†
24 hr	0.65 ± 0.06	0.55 ± 0.04*
		0.63 ± 0.04†
tPA		
6 hr	1.03 ± 0.21	2.23 ± 1.05
24 hr	1.28 ± 0.23	1.23 ± 0.23
GAPDH		
6 hr	0.98 ± 0.22	0.95 ± 0.08
24 hr	1.07 ± 0.15	0.91 ± 0.01

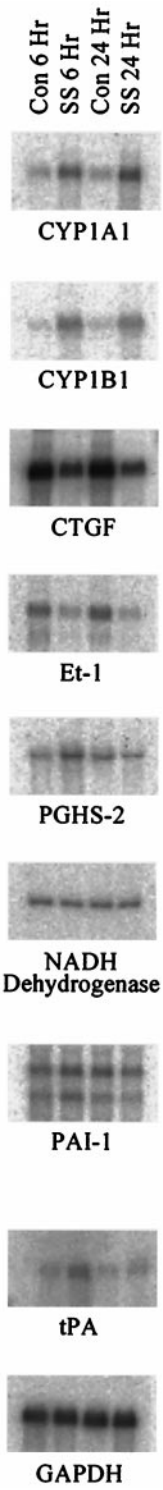

Fig. 1. Comparison of DNA Microarray intensity ratios and Northern blot densitometry ratios (based on GAPDH) for nine genes. Values are mean ± SD. Blots show representative results on each gene for control (Con) and shear stress (SS) conditions at 6 h and 24 h. *, 2.3-kb PAI-1 transcript; †, 3.2-kb PAI-1 transcript.

In EC, argininosuccinate synthetase catalyzes the rate-limiting step in the synthesis of L-arginine from L-citrulline, then endothelial NO synthase (eNOS) in turn recycles arginine back to citrulline, producing NO. Thus, available arginine is a prerequisite for NO production. Although the intracellular concentration of arginine is two to three orders of magnitude higher than both the K_m for NOS and the circulating arginine concentration, intracellular arginine can be rate limiting because of organellar sequestration or the inhibiting effects of other pathways that metabolize arginine (e.g., arginase; ref. 28). Both an increase in extracellular L-arginine levels and an increase in the synthesis of arginine from citrulline will increase NO production by endothelial cells, in the *absence* of synthesis of additional eNOS. It may be that the shear stress-induced increase in NO synthesis may also depend on an increase in L-arginine synthesis from L-citrulline, via increased argininosuccinate synthetase levels (Table 1).

Vasoactive intestinal polypeptide (VIP) has been reported to increase NO production (29). Our microarray data indicate that shear stress increased expression of the VIP receptor precursor gene. Thus, shear stress, through up-regulating the VIP receptor, may increase the availability of receptors for VIP, leading to increased binding of the ligand to EC and increasing the production of NO. Another potential autocrine/paracrine system is the binding of elastin peptide to its receptor, elastin-laminin receptor. *In vivo*, elastin peptides circulate because of the breakdown of the extracellular matrix by elastase. These peptides, via binding to elastin-laminin receptors on EC, increase intracellular calcium and lead to NO release (30). Shear stress, by increasing elastin gene expression, may increase the binding of elastin peptides to the elastin-laminin receptor, leading to an increase in intracellular Ca^{2+} concentration, and an increase in the activation of eNOS.

An additional pathway through which shear stress may regulate the production of NO is by controlling the inactivation and sequestering of eNOS by caveolin-1 (31). With the onset of shear stress, eNOS dissociates from caveolin-1, binds to calmodulin, and is activated, increasing NO production. Inhibition of caveolin-1 has been shown to decrease shear stress signaling, which activates ERK (32). Shear stress may further regulate this pathway by decreasing the expression of the caveolin-1 gene (Table 2), thereby decreasing the amount of caveolin-1 present in the cells, and thus decreasing the amount of eNOS that could be sequestered in an inactive state. The amount of active eNOS in the cell would then remain elevated, which may in part be responsible for the long-term increase in NO production by EC in response to shear stress.

Still another possible pathway for shear stress to affect the NO output by EC is in the synthesis of ceramide from sphingolipids (which are enriched in the caveolae; ref. 33). IL-1β selectively increases the metabolism of ceramide from sphingomyelin in the glycosphingolipid-rich caveolae of cells. Alpha-galactosidase A catalyzes the breakdown of glycosphingolipids to ceramide. Ceramide is able to activate eNOS in a Ca^{2+}-independent manner. Shear stress-induced sustained NO production by endothelial cells appears to be independent of intracellular calcium (34). Perhaps shear stress may increase the catabolism of glycosphingolipids to ceramide in caveolae by increasing α-galactosidase A gene expression. Then ceramide, in a calcium-independent manner, could activate eNOS in the caveolae that is not bound to caveolin-1, and sustain production of NO by shear stress.

There is clear evidence that NO production (27) is elevated under shear stress in HUVEC. However, the evidence that eNOS mRNA expression is up-regulated is less convincing in HUVEC (4, 35), although eNOS mRNA expression is clearly up-regulated by shear stress in bovine aortic EC (35–37). Although under the hybridization conditions reported herein we saw no increase in eNOS expression under shear stress, we do observe increases in eNOS expression when using higher stringency washes (unpublished results).

Other posttranscriptional changes might impact NO production, including phosphorylation of eNOS or an associated reg-

ulatory protein (38) and decreased mRNA stability (28). Still another possible influence may be the matrix on which the cells are seeded. Gloe *et al.* (39) demonstrated that shear stress increases eNOS mRNA expression, protein, and activity in porcine aortic EC cultured on laminin I, but not on fibronectin, collagen I, or uncoated glass (39). Explaining the regulation of eNOS expression and NO production by shear stress requires further investigation.

The expression of zinc finger protein EZF/GKLF, glucocorticoid-induced leucine zipper protein, and human prostaglandin transporter genes were strongly up-regulated by shear stress; whereas expression of CTGF, ET-1, MCP-1, and spermidine/spermine N1-acetyltransferase were strongly down-regulated. Understanding the importance of these results for vascular biology is clearer in some cases (e.g., ET-1 and MCP-1), but for other genes requires further study.

The use of DNA microarray technology has provided results that allow us to generate hypotheses, and to elucidate how groups of genes, which we know from other studies are regulated by shear stress, may be related. In addition, we have discovered changes in the expression levels of genes (e.g., CYP 1A1, CYP 1B1, CTGF) that would not normally have been selected for study. The powerful technique of DNA microarray analysis will accelerate our progress in understanding the complex mechanotransduction pathways in vascular cells.

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