cDNA microarray analysis of endothelial cells subjected to cyclic mechanical strain: importance of motion control

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Physiol Genomics 21: 124–130, 2005. First published January 4, 2005; doi:10.1152/physiolgenomics.00029.2003. —Microarrays were utilized to determine gene expression of vascular endothelial cells (ECs) subjected to mechanical stretch for insight into the role of strain in vascular pathophysiology. Over 4,000 genes were screened for expression changes resulting from cyclic strain (10%, 1 Hz) of human umbilical vein ECs for 6 and 24 h. Comparison of t-statistics and adjusted P values identified genes having significantly different expression between strained and static cells but not between strained and motion control. Relative to static, 6 h of cyclic stretch upregulated two genes and downregulated two genes, whereas 24 h of cyclic stretch upregulated eight genes but downregulated no genes. However, incorporating the motion control revealed that fluid agitation over the cells, rather than strain, is the primary regulator of gene expression. Furthermore, no gene exceeded a threefold change when comparing cyclic strain to either static or motion control. Quantitative real-time polymerase chain reaction confirmed the dominance of fluid agitation in gene regulation with the exception of heat shock protein 10 at 24 h and plasminogen activator inhibitor 1 at 6 h. Taken together, the small number of differentially expressed genes and their low fold expression levels indicate that cyclic strain is a weak inducer of gene regulation in ECs. However, many of the differentially expressed genes possess antioxidant properties, suggesting that oxidative mechanisms direct EC adaptation to cyclic stretch.

AS THE INNERMOST LINING of the vascular system, endothelial cells are directly subjected to the hemodynamic forces resulting from blood flow. Cyclic circumferential strain, which ranges from 2 to 18% in vivo, is a result of arterial wall expansion and contraction in response to pulsatile pressure changes (13). Hypertension is a major risk factor for atherosclerosis, and large wall strains are often associated with sites of atherosclerotic plaque development in vivo (40). In vitro, cyclic strain of endothelial cells has been shown to increase oxidative stress (42), induce DNA synthesis and proliferation (38), modulate secretion of vasoactive materials (6), and alter cell morphology (23). To accomplish these changes, endothelial cells signal through the ERK, JNK, and p38 pathways to alter gene expression (1). Unlike traditional, low-throughput methods of gene expression detection, microarrays have made possible the expression analysis of thousands of genes simultaneously. By comparing the expression profiles of cell populations subjected to hemodynamic forces with cell populations held static, the microarrays are used to screen for genes that influence the cellular response to mechanical force. This laboratory previously used microarray technology to show that a flow-induced laminar shear stress of 25 dyn/cm² on vascular endothelial cells resulted in significant expression changes of many genes related to cell proliferation and differentiation (29). Microarrays have also been used to show large differences in gene expression profiles of endothelial cells exposed to steady vs. pulsatile, nonunidirectional shear forces (3), laminar shear and turbulent shear forces (19), and a steady, laminar shear stress of 12 dyn/cm² (9). Microarray analysis of vascular smooth muscle cells subjected to cyclic strain resulted in a very small number of differentially expressed genes, of which most had extracellular matrix or vasoregulatory functions (16).

This study focused on the gene expression profiles of vascular cells exposed to mechanical strain. We used microarray technology to determine the expression of over 4,000 genes after human umbilical vein endothelial cells (HUVECs) were cyclically stretched at 10% strain at 1 Hz compared with both static and motion controls. This resulted in significant expression changes in a small number of genes, most of which were not previously associated with cyclic strain. Most of these genes, however, appeared to be at least partially modulated by the fluid agitation inherent in the strain apparatus rather than by cyclic strain alone.

MATERIALS AND METHODS

All cell culture reagents were obtained from GIBCO, DNA microarrays and microarray reagents were from ResGen, and quantitative real-time polymerase chain reaction (qRT-PCR) primers were from Sigma-Genosys.

Cell culture. As previously described, endothelial cells were collagenase derived from the veins of human umbilical cords and seeded at confluence onto silicone membranes (5 × 10^3-in. thickness; Specialty Manufacturing) that were coated with 1% gelatin cross-linked with glutaraldehyde (37). Cells were seeded in Medium-199 containing 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 µg/ml streptomycin, and 300 µg/ml l-glutamine and maintained in 95% air-5% CO₂ at 37°C. Approximately 16 h after the seeding, the HUVECs were washed three times in phosphate-buffered saline (PBS) and given fresh medium and then washed and fed again after ~24 h. Immediately after the feeding on the third day, the cells were used for experimentation.

Experimental procedure. HUVECs were subjected to 10% cyclic mechanical strain at a frequency of 1 Hz (60 cycles/min) for 6 or 24 h in a previously described strain unit (Fig. 1; Ref. 6). A silicone membrane was suspended slightly above and parallel to the bottom of a polycarbonate box between two sets of stainless steel plates. Each set consisted of two plates that clamped the ends of the membrane. For
the cyclic strain condition, one set of plates was fixed to the end of the box, while the second set was attached to a cam-driven movable piston at the opposite end of the box, allowing for cyclic unidirectional elongation of the membrane. For the motion control condition, one set of plates was fixed to the end of the box, while the other end was attached to plates fixed around a stainless steel plate that prevented the membrane from stretching. The same cam as that driving the strain condition drove a piston plate assembly at the template end, resulting in similar fluid motion without stretching the cells. For the static condition, the assembly was identical to the motion control and strain conditions, but the piston was not moved. For each condition, four membranes were used in each experiment. Each experiment consisted of all three conditions (strain, static, and motion control). Three experiments were performed for each time point. In the following sections, the term “experiment” will refer to data from a single pool of cells.

Immediately after the membranes were removed from the strain apparatus, the HUVECs were washed in ice-cold PBS. Total RNA was extracted using TRIzol, according to manufacturer’s specifications. The quantity and integrity of the RNA were verified using the spectrophotometer, accepting only RNA with a 260/280 ratio > 1.9, and gel electrophoresis. Acceptable RNA was aliquoted into single-use volumes and stored at -80°C.

cDNA microarray hybridization and imaging. Three micrograms of total RNA were reverse transcribed with AMV-reverse transcriptase and labeled using [α-33P]dCTP (NEN). The resulting cDNA was hybridized to the Human Named Genes GeneFilter 211 (ResGen GF211) nylon filter microarray for 16 h, as per manufacturer’s instructions. The microarray was then washed two times with 2× SSC-1% SDS at 50°C for 20 min each and one time with 0.5× SSC-1% SDS at 50°C for 15 min. Three filters were hybridized with aliquots of identical RNA for each condition; a total of nine microarrays were used for each experiment at each time point. Because these microarrays can be stripped and reused, the same filters were used for the same conditions in subsequent experiments.

After overnight exposure, the microarrays were imaged with a Storm phosphorimagery (Molecular Dynamics) at 50-μm resolution. The resulting gel files were imported into Pathways 4 (ResGen) software. Background, as calculated by the average intensity of a spotless strip in the middle of the filter, was subtracted from the intensity of each spot. This intensity was then divided by the average intensity over all spots (except the total genomic DNA control spots) to obtain the normalized intensity of the spot. Figure 2 represents spot intensities of four genes for static, strain, and motion control.

qRT-PCR. qRT-PCR was performed on seven genes to verify microarray results, using the following primers: heat shock 10-kDa protein-1 (HSP10; 5‘-CTCACCACCTTTCCTTTAGAACC-3‘ and 5‘-GAGTGCTGCTGAAACTGTAACC-3‘), heat shock 90-kDa protein-1β (HSP90; 5‘-CATCCAATCTCTTGTCAGAAG-3‘ and 5‘-CCCCTGCTGCTTCTAGTGT-3‘), plasminogen activator inhibitor-1 (PAI-1; 5‘-GAAAACCCCAAGAGGAAGG-3‘ and 5‘-ATGGGGAGAATTGCAATAGTCGAGG-3‘), endothelin-1 (ET-1; 5‘-TCTCTTCTCTCTCTCTCTCTCCT-CC-3‘ and 5‘-CTTCACTGGCTTCCCATCAG-3‘), catalase (CAT; 5‘-ATACACCCCCATTTTGTCTCAG-3‘ and 5‘-AGTCTCGCCGCACTCTCAAC-3‘), cyclooxygenase-2 (COX2; 5‘-AAACCAGGTGTATGATGATGATG-3‘ and 5‘-TTTAGCCATAGTCAGCATTG-3‘), and forhead box O1A (FOXO1A; 5‘-GAGTGCTGCTGAAACTGTAACC-3‘), heat shock 90-kDa protein-1 (HSP90), and plasminogen activator inhibitor-1 (PAI-1) or 24 h [endothelin-1 (ET-1)] show qualitative levels of expression. Each spot measured 600 μm in diameter, with centers spaced 750 μm apart. Per membrane, 16.67 μCi (3.67 × 104 dpm/membrane) of [α-33P]dCTP were loaded. MC, motion control.

Fig. 1. Strain apparatus consists of 2 adjacent chambers. A: relaxed position. B: extended position. Top chamber in both A and B contains the stretched membrane, whereas bottom chamber in both A and B contains the motion control membrane. Because the membrane to be stretched is held by a fixed set of plates and by another set of plates connected to the piston, the cells attached to the membrane are stretched. For the motion control condition, the membrane remains unstretched, since it is fixed around a metal template. Cycling between the relaxed and stretched positions agitates the fluid.

Fig. 2. Representative microarray phosphorimages for each of 4 genes at either 6 h (heat shock 10-kDa protein-1 (HSP10), heat shock 90-kDa protein-1β (HSP90), and plasminogen activator inhibitor-1 (PAI-1)) or 24 h [endothelin-1 (ET-1)] show qualitative levels of expression. Each spot measured 600 μm in diameter, with centers spaced 750 μm apart. Per membrane, 16.67 μCi (3.67 × 104 dpm/membrane) of [α-33P]dCTP were loaded. MC, motion control.
For HSP10, HSP90, PAI-1, and ET-1 primer sets, standard curves were generated to check for linearity, and efficiencies from serial dilutions of RNA showed a linear profile within the dilution range (Fig. 3). Two micrograms of total RNA were reverse transcribed with MMLV-reverse transcriptase (Promega) in the presence of MMLV reaction buffer, RNase inhibitor, dNTP mix, and random primers, according to the manufacturer’s instruction. For each PCR, 1.0 μl of the resulting cDNA mixture were added to a mixture of PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂ (Ambion), 0.2 mM each dNTP, a 1:10,000 dilution of SYBR Green I (Molecular Probes), 100 nM PCR primers (300 nM of PAI-1), and 1.5 μl of Taq polymerase. The reactions were performed in a Rotor-Gene 3000 Multiplexing System (Corbett Research) with a 10-min initial denaturation at 95°C, 40 cycles of 95°C for 20 s, 60°C for 30 s, 72°C for 60 s, and a final anneal at 72°C for 10 min. The samples were subjected to a melt ramp from 70 to 99°C at a rate of 1°C/5 s. Fluorescence was excited at 470 nm and detected at 510 nm and at 585 nm with high-pass filters. Cycle threshold (Cₚ) values were determined with the comparative quantification feature (2nd-derivative method). Fold expressions relative to static were calculated using the ΔΔCₚ method compared with 18s ribosomal RNA (5'–GCAAT-TATTCCCCATGAACG–3' and 5'–GGCCCTACTAACCATC-CAA–3'). Comparison of the efficiencies of each primer set to that of 18s verifies the feasibility of using the ΔΔCₚ method for relative quantification. No differences in Cₚ values for 18s were found among stretch, static, and motion control samples.

For CAT, COX2, and FOXO1A, 1 μg of total RNA was reverse transcribed into cDNA with SuperScript II (Invitrogen), according the manufacturer’s instruction. The resulting cDNA was purified through Micro Bio-Spin P-30 Chromatography Columns (BioRad) to reduce manufacturer’s instruction. The resulting cDNA was purified through Micro Bio-Spin P-30 Chromatography Columns (BioRad) to reduce background fluorescence and diluted 1:100. For each reaction, 1 μl was added to a mixture of PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.25 mg/ml BSA (New England Biolabs), 4.0 mM MgCl₂, 0.2 mM dNTP, a 1:84,000 dilution of SYBR Green I, 100 nM PCR primers (50 nM of 18s; QuantumRNA Classical II, Ambion), and 0.10 U/μl Platinum Taq polymerase (Invitrogen). The qRT-PCR reactions and analyses were performed on a LightCycler 2.0 (Roche Applied Science), with a 1-min initial denaturation step at 94°C; 50 cycles (30 cycles for 18s) of 94°C for 5 s, 65°C for 10 s, and 72°C for 1 s per 25-bp product; and a ramped melting cycle. Absolute concentrations were quantified from Cₚ values with a linear standard curve.

Statistical analysis. Each experiment yielded three strain arrays, three static arrays, and three motion control arrays. Let \( f = 1, 2, 3 \) denote experiment and \( j = 1, 2, 3 \) denote replicate; \( S_{ijk} \) is spot \( k \) in replicate \( j \) of strain experiment \( i \), where \( k = 1, \ldots, 4,324 \); \( C_{ijk} \) is spot \( k \) in replicate \( j \) of static control experiment \( i \); and \( M_{ijk} \) is spot \( k \) in replicate \( j \) of motion control experiment \( i \). To control for experiment-to-experiment variation, intensity ratios were calculated within each experiment. We used the log ratio of treatment to control expression values as the scale for sample comparison in these studies (4, 14). To have a common reference sample across all arrays, for each experiment \( i \) we defined a common reference \( R_{ik} \) comprised of all three static control arrays; for spot \( k \):

\[
R_{ik} = \frac{C_{ik1} + C_{ik2} + C_{ik3}}{3}
\]

This reference was used to calculate a log ratio for each spot \( (k) \) of strain replicate \( j \) in experiment \( i \): \( \log(S_{ijk}/R_{ik}) \). Similarly, a log ratio was calculated for the static replicates, \( \log(C_{ijk}/R_{ik}) \), and for the motion control replicates, \( \log(M_{ijk}/R_{ik}) \). Across the three experiments, we had nine strain log ratios, nine static log ratios, and nine motion control log ratios at each time point.

To evaluate differential expression between the strain sample and the static sample, we calculated a two-sample t-statistic for each array spot \( k \):

\[
t_i = \frac{\bar{S}_i - C_k}{SE(S_i - C_k)} = \frac{\bar{S}_i - C_k}{\sqrt{Var(S_i) + Var(C_k)}}
\]

where \( S \) is the average log ratio of the nine strain replicates, \( \log(S_{ijk}/R_{ik}) \); and \( C_k \) is the average of the nine static log ratios, \( \log(C_{ijk}/R_{ik}) \); \( SE \) is standard error. The variance of \( \bar{S}_i \) \( [Var(S_i)] \) appearing in the denominator of the t-statistic was estimated as the sample variance of the nine replicates of \( \log(S_{ijk}/R_{ik}) \) divided by the sample size \( (n = 9) \); the variance of \( C_k \) \( [Var(C_k)] \) was estimated similarly. The sign and magnitude of the t-statistic gave an indication of the strength of overexpression or underexpression for the given spot. Under the null hypothesis of equal expression at spot \( k \), the difference, \( S_i - C_k \), was expected to be zero. The t-statistics for motion control were calculated the same way.

After t-statistics (strain vs. static) of each gene were calculated, quantile-quantile plots compared the observed t-statistic quantiles with the standard normal quantiles (Fig. 4). As evidenced in these plots, the t-statistics were centered about zero, indicating that the expression levels of most genes were not significantly changed as expected in microarray experiments. The clear separation of genes in the upper and lower tails indicated genes that showed significantly different expression (plus signs).

Under standard assumptions of stochastic independence and normality of the underlying 18 log ratio values, the t-statistic would follow a t-distribution with 16 degrees of freedom (6). By use of a nominal significance level of 0.05 with a conventional t-table, our microarrays of over 4,000 genes could be expected to yield over 200 false-positive results even if all genes were equally expressed (5, 14). To account for this, we calculated adjusted P values for the two-sample problem, as discussed by Westfall and Young (43) and applied in the microarray context by Dudoit and colleagues (14, 15). The adjusted P values were calculated via a permutation distribution algorithm implemented in an R package (22), Statistics for Microarray Analysis (SMA), which may be downloaded from http://www.R-project.org. The significant genes indicated in Fig. 4 had adjusted P values of \( < 0.15 \) at both time points.

Significance for qRT-PCR data was determined using factorial ANOVA, followed by Fishers protected least significant difference test. Results are means ± SE. Values of \( P < 0.05 \) were considered significant.

RESULTS

Cyclic strain evaluated with cDNA microarrays. Primary HUVECs were exposed to 10% cyclic strain or held static for

![Fig. 3. Standard curves generated from serial dilutions show a linear profile for all primer sets within the dilution range. Linear regression revealed efficiencies between 92 and 94% (with no significant difference compared with 18s) and correlation coefficients of at least 0.9941. Cₚ, cycle threshold.](image-url)
differentially expressed compared with static controls (Table 2), all of which were upregulated. Those most highly upregulated were H2A histone family member L and CAT, with strain-to-static ratios of $2.71 \pm 0.49$ and $2.66 \pm 0.49$, respectively.

**Motion control effects evaluated with cDNA microarray.** In addition to cyclic strain and static controls, the HUVECs were exposed to motion control to account for the fluid agitation that occurs during stretching of the cells. In the motion control environment, the medium is passed over the cells in an oscillatory manner while the cells are held static. Examination of motion control allowed for stretch responses to be separated from shear and mass transport responses. The ratio of cyclic strain to motion control was determined for each differentially expressed gene. As seen in Tables 1 and 2, many genes had a strain-to-motion control ratio near 1, indicating that fluid agitation contributed to the regulation of expression. For example, the strain-to-motion control ratio of HSP10 at 24 h was $1.26 \pm 0.17$, whereas the motion control-to-static ratio was $1.77 \pm 0.22$, indicating that the fluid agitation contributed to the strain-to-static ratio of 2.24 $\pm 0.29$ in HSP10 regulation. In contrast, CAT had a strain-to-motion control ratio of 2.49 $\pm 0.52$, very close to the strain-to-static ratio of 2.66 $\pm 0.49$. Therefore, fluid motion appeared to have had little effect on CAT gene expression.

**qRT-PCR verification of results.** On the basis of the microarray results, seven genes were further analyzed using qRT-PCR. We compared the microarray results with those of qRT-PCR for three genes (HSP10, FOXO1A, and CAT) that were differentially expressed, three genes (HSP90, PAI-1, and COX2) that were close to the significance threshold, and one gene (ET-1) that was not significant but hypothesized to change.

At 6 h, the strain-to-static fold expressions determined from qRT-PCR for HSP10 (2.42 $\pm 0.07$), HSP90 (1.95 $\pm 0.10$), PAI-1 (2.74 $\pm 0.10$), COX2 (2.30 $\pm 0.68$), and FOXO1A (0.51 $\pm 0.19$) agreed with those of the microarray (Fig. 5). Cyclic stretch for 24 h upregulated four genes compared with static: HSP10 (2.40 $\pm 0.22$), HSP90 (1.57 $\pm 0.14$), PAI-1 (3.99 $\pm 0.66$), and ET-1 (1.51 $\pm 0.21$). However, qRT-PCR analysis did not detect any significant changes in CAT expression between 24 h of cyclic stretch and static. As a positive control, hyperglycemic treatment (8) of HUVEC P1 seeded on gelatin-coated tissue culture dishes cross-linked with glutaraldehyde upregulated CAT expression (data not shown).

**Table 1. Differentially expressed genes obtained with microarray analysis after 6 h of cyclic strain**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strain-to-Static Ratio</th>
<th>$P$ Value</th>
<th>Strain-to-Motion Control Ratio</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock 10-kDa protein-1 (AA448396)</td>
<td>$2.26 \pm 0.26$</td>
<td>0.005</td>
<td>$0.91 \pm 0.10$</td>
<td>1.000</td>
</tr>
<tr>
<td>Thrombomodulin (H59861)</td>
<td>$0.56 \pm 0.05$</td>
<td>0.008</td>
<td>$0.79 \pm 0.09$</td>
<td>0.999</td>
</tr>
<tr>
<td>Forkhead box O1A (AA448277)</td>
<td>$0.48 \pm 0.06$</td>
<td>0.029</td>
<td>$0.63 \pm 0.09$</td>
<td>0.872</td>
</tr>
<tr>
<td>Endothelial cell-specific molecule-1 (W 46577)</td>
<td>$2.17 \pm 0.29$</td>
<td>0.037</td>
<td>$1.41 \pm 0.20$</td>
<td>0.996</td>
</tr>
<tr>
<td>Collagen-binding protein-2 (R71093)</td>
<td>$1.71 \pm 0.19$</td>
<td>0.169</td>
<td>$0.95 \pm 0.10$</td>
<td>1.000</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1 (N54794)</td>
<td>$2.14 \pm 0.34$</td>
<td>0.201</td>
<td>$1.69 \pm 0.32$</td>
<td>0.980</td>
</tr>
<tr>
<td>Nidogen (R38383)</td>
<td>$0.86 \pm 0.03$</td>
<td>0.212</td>
<td>$0.92 \pm 0.04$</td>
<td>1.000</td>
</tr>
<tr>
<td>Cyclooxygenase-2 (AA644211)</td>
<td>$1.93 \pm 0.27$</td>
<td>0.217</td>
<td>$1.68 \pm 0.30$</td>
<td>0.965</td>
</tr>
<tr>
<td>Heat shock 90-kDa protein-1 beta (R44334)</td>
<td>$1.57 \pm 0.15$</td>
<td>0.217</td>
<td>$1.09 \pm 0.16$</td>
<td>1.000</td>
</tr>
<tr>
<td>Heat shock 70-kDa protein-10 (AA629567)</td>
<td>$1.60 \pm 0.16$</td>
<td>0.227</td>
<td>$1.15 \pm 0.13$</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Ratios are means $\pm$ SE. Gene accession nos. are in parentheses. Top: genes considered differentially expressed. Bottom: genes just below $P$ value threshold of significance.
Table 2. Differentially expressed genes obtained with microarray analysis after 24 h of cyclic strain

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strain-to-Static Ratio</th>
<th>P Value</th>
<th>Strain-to-Motion Control Ratio</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forkhead box F1 (AA112660)</td>
<td>1.75±0.13</td>
<td>0.007</td>
<td>1.20±0.12</td>
<td>1.000</td>
</tr>
<tr>
<td>Heat shock 10-kDa protein-1 (AA448396)</td>
<td>2.24±0.29</td>
<td>0.041</td>
<td>1.26±0.17</td>
<td>1.000</td>
</tr>
<tr>
<td>TGF-β-stimulated protein TSC-22 (AA664389)</td>
<td>2.10±0.26</td>
<td>0.047</td>
<td>1.29±0.17</td>
<td>1.000</td>
</tr>
<tr>
<td>Collagen-binding protein-2 (R71093)</td>
<td>1.81±0.18</td>
<td>0.060</td>
<td>1.09±0.10</td>
<td>1.000</td>
</tr>
<tr>
<td>Transmembrane 9, superfamily 2 (AA479100)</td>
<td>1.55±0.12</td>
<td>0.079</td>
<td>1.11±0.10</td>
<td>1.000</td>
</tr>
<tr>
<td>H2A histone family, member L (AA452933)</td>
<td>2.71±0.49</td>
<td>0.101</td>
<td>1.02±0.14</td>
<td>1.000</td>
</tr>
<tr>
<td>Catalase (H15685)</td>
<td>2.66±0.49</td>
<td>0.123</td>
<td>2.49±0.52</td>
<td>0.459</td>
</tr>
<tr>
<td>Clathrin assembly protein, 180 kDa (AA441930)</td>
<td>1.41±0.09</td>
<td>0.128</td>
<td>0.94±0.06</td>
<td>1.000</td>
</tr>
<tr>
<td>Hypothetical protein FLJ10986 (N95121)</td>
<td>0.55±0.07</td>
<td>0.245</td>
<td>0.84±0.13</td>
<td>1.000</td>
</tr>
<tr>
<td>Heat shock 70-kDa protein-10 (AA629567)</td>
<td>1.48±0.12</td>
<td>0.253</td>
<td>1.20±0.12</td>
<td>1.000</td>
</tr>
<tr>
<td>Nonmetastatic cells 1 (AA644092)</td>
<td>1.49±0.12</td>
<td>0.257</td>
<td>1.31±0.21</td>
<td>1.000</td>
</tr>
<tr>
<td>G protein, α-stimulating, peptide 1 (R43581)</td>
<td>1.57±0.15</td>
<td>0.262</td>
<td>1.01±0.10</td>
<td>1.000</td>
</tr>
<tr>
<td>Tax1 binding protein (AA598483)</td>
<td>1.52±0.13</td>
<td>0.294</td>
<td>1.27±0.17</td>
<td>1.000</td>
</tr>
<tr>
<td>Collagen, type III, alpha 1 (T98612)</td>
<td>1.59±0.16</td>
<td>0.300</td>
<td>0.94±0.07</td>
<td>1.000</td>
</tr>
<tr>
<td>Superoxide dismutase, soluble (AA599127)</td>
<td>1.68±0.18</td>
<td>0.304</td>
<td>1.18±0.12</td>
<td>1.000</td>
</tr>
<tr>
<td>Replication protein A2 (R39861)</td>
<td>2.57±0.52</td>
<td>0.326</td>
<td>1.51±0.23</td>
<td>0.995</td>
</tr>
<tr>
<td>TCF1 (AA459909)</td>
<td>1.66±0.18</td>
<td>0.334</td>
<td>1.16±0.15</td>
<td>1.000</td>
</tr>
<tr>
<td>Heat shock 90-kDa protein-1, beta (R44382)</td>
<td>1.76±0.21</td>
<td>0.335</td>
<td>1.07±0.13</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Ratios are means ± SE. Gene accession nos. are in parentheses. Top: genes considered differentially expressed. Bottom: genes just below P value threshold of significance.

Fold changes of motion control relative to static determined from qRT-PCR agreed with those from microarrays for all genes at both time points (Fig. 5). Fluid agitation significantly changed the fold expression of HSP10 (2.27 ± 0.16 at 6 h, 1.70 ± 0.15 at 24 h) and HSP90 (1.83 ± 0.06 at 6 h, 1.34 ± 0.11 at 24 h). Additionally, 6 h of agitation significantly upregulated PAI-1 over static (1.99 ± 0.13) but significantly less than the strain condition. Similarly, HSP10 at 24 h resulted in significant differential expression of the motion control from both strain and static. With the exception of PAI-1 upregulation by strain at 24 h, and the lack of response of CAT to cyclic strain at 24 h, qRT-PCR and microarray analyses gave similar fold changes.

DISCUSSION

We subjected primary HUVECs to 10% cyclic strain at 1 Hz for 6 and 24 h and examined differential gene expression using DNA microarrays. We found 11 genes of 4,000 that were differentially expressed in response to cyclic strain using conservative criteria for significance. Our microarray results showed that cyclic strain, per se, may not be as potent as the fluid agitation that characterizes the motion control condition in mediating endothelial gene expression. Although 11 genes are differentially expressed when comparing cyclic strain with static conditions, a comparison of cyclic strain and motion control with microarrays revealed only CAT at 24 h to be regulated by cyclic strain. The differences in gene expression in motion control are probably due to the shear force exerted on the cells, even though the estimated shear stress in this system is very low, < 0.5 dyn/cm². Very low oscillatory shear stresses have been shown to have an influence on gene expression (3).

Examination of Tables 1 and 2 suggests that differentially expressed genes to either cyclic strain or motion control conditions may be a response to oxidative stress. Three heat shock proteins, HSP10, collagen-binding protein-2 (HSP47), and HSP90B, were upregulated. Heat shock proteins generally protect the cells from stresses by ensuring correct protein folding and preventing protein aggregation (25), while oxida-
tive stress results in increased misfolding and aggregation of proteins (17, 26). The response of HSP10 to oxidative stress has not been well studied in endothelial cells; however, the protein synthesis of HSP60, with which HSP10 is often coexpressed and complexed, is stimulated by oxidative stress (24, 30). HSP47, which is often found in fibrotic tissues including atherosclerotic lesions, showed upregulation of both mRNA and protein expression under oxidant stress (20, 41). HSP90 protein production was also enhanced in response to oxidative stress (18, 28). Therefore, heat shock proteins may be upregulated in endothelial cells as a protection against oxidative stress induced either by cyclic strain, fluid agitation, or both (21).

When an oscillation of ±0.1 dyne/cm² is added to a steady but low shear stress of 0.3 dyne/cm², endothelial cells maintain superoxide production at 24 h, resulting in a sustained antioxidative defense (36). Removal of this oscillation eliminates the superoxide production and presumably the antioxidative defense, supporting our hypothesis that the shear stress effect of fluid agitation predominates in regulating antioxidative mechanism genes in endothelial cells subjected to cyclic stretch.

qRT-PCR confirmed the microarray findings, except for PAI-1 and CAT at 24 h (Fig. 5). Despite the conservative $P$ value criteria, microarray analysis did not reveal any differential regulation by cyclic strain when fluid agitation was taken into account. The lack of differential expression in CAT using qRT-PCR suggests that its contribution to an antioxidative defense is regulated at the level of either translation or activity (27). However, the shear stress across these cells may not be the only factor in the regulation of gene expression in the motion control condition; local mass transport alterations due to fluid agitation could also result in a differential response.

PAI-1 and COX2 showed changes in gene expression consistent with their corresponding protein level or activity. PAI-1 secretion from HUVECs increased in response to 12% cyclic strain (10). Prostacyclin, a product of arachidonic acid metabolism by COX2, levels increased in response to 24 h of 10% cyclic strain at 1 Hz (6).

In vascular smooth muscle cells, cyclic strain downregulates thrombomodulin and upregulates PAI-1 mRNA and protein expressions (16). Oxidative stress is hypothesized to direct either cyclic strain- or fluid-agitation-induced procoagulant genes. In support of this idea, PAI-1 secretion from endothelial cells subjected to cyclic strain was modulated by reactive oxygen species (10). Moreover, protease-activated receptor-1 (not included on the GF211 microarrays we used) upregulation in vascular smooth muscle cells subjected to cyclic strain was a response to oxidative stress (32).

The inclusion of a motion control condition in this study showed the importance of accounting for fluid agitation in the regulation of gene expression. With any in vitro cyclic strain model, cells are concomitantly exposed to low fluid motion-induced shear stress and convective transport due to fluid agitation. Previous studies from our laboratory (6) have shown that fluid motion from the motion control condition increased production of ET-1 and prostacyclin, albeit not as much as in strained cells. In more recent work (32), the motion controls gave results similar to the static controls in studies of protease-activated receptor-1 (PAR-1) gene expression. The devices used in many strain studies do not allow for a motion control; therefore, many of the reported cellular responses to cyclic strain have not been confirmed as actual stretch responses.

HUVECs subjected to a defined shear stress showed a larger number of genes with higher magnitudes of expression change compared with cyclic strain when studied with the use of identical microarray technology (29). In response to shear stress we found 52 differentially expressed genes, whereas under cyclic strain we found 11, only one of which (CAT) was independent of the motion control. The largest expression change with cyclic strain was 3-fold over static cells, but in response to shear stress larger changes were seen, with the highest being >10-fold. Even when a twofold threshold was applied to the cyclic strain data, the number of differentially expressed genes totaled 30 and all ratios were less than fourfold (data not shown). The chosen level of stretch (10%) may have been a factor in the smaller changes seen with cyclic strain; however, preliminary microarray data at 20% strain also resulted in a small number of changed genes with small fold differences. Consistent with our results, microarray studies of vascular smooth muscle cells and monocytic THP-1 cells subjected to cyclic mechanical strain showed a low number of differentially expressed genes with relatively low fold ratios (16, 33). Additionally, Azuma et al. (1) found that shear stress, compared with cyclic strain, more quickly and strongly activated the ERK and p38 pathways.

We had hypothesized that these two mechanical forces, shear stress and cyclic strain, would give opposite gene expression results, based, for example, on our earlier studies of PAR-1 in vascular smooth muscle cells (32, 34). Additionally, shear stress and cyclic strain showed differing effects on the expression of tissue plasminogen activator (12, 39), ET-1 (11, 31), and monocyte chemotactic protein-1 (35, 44) in endothelial cells.

Because arterial levels of fluid-induced shear stress are believed to be protective and elevated levels of cyclic strain are believed to be pathogenic, the molecular differences in response to these forces may be important for the understanding of disease progression in the cardiovascular system. Although the effects of shear stress on endothelial cells have previously been evaluated with cDNA microarrays, we are among the first (2), to our knowledge, to use this powerful technique to examine cyclic strain modulation of endothelial cell gene expression.

GRANTS

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REFERENCES


