Mechanical stretch is a highly selective regulator of gene expression in human bladder smooth muscle cells

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Mechanical stretch is a highly selective regulator of gene expression in human bladder smooth muscle cells. Physiol Genomics 20: 36–44, 2004. First published October 5, 2004; doi:10.1152/physiolgenomics.00181.2004.—Application of mechanical stimuli has been shown to alter gene expression in bladder smooth muscle cells (SMC). To date, only a limited number of “stretch-responsive” genes in this cell type have been reported. We employed oligonucleotide arrays to identify stretch-sensitive genes in primary culture human bladder SMC subjected to repetitive mechanical stimulation for 4 h. Differential gene expression between stretched and nonstretched cells was assessed using Significance Analysis of Microarrays (SAM). Expression of 20 out of 11,731 expressed genes (~0.17%) was altered ~2-fold following stretch, with 19 genes induced and one gene (FGF-9) repressed. Using real-time RT-PCR, we tested independently the responsiveness of 15 genes to stretch and to platelet-derived growth factor-BB (PDGF-BB), another hypertrophic stimulus for bladder SMC. In response to both stimuli, expression of 13 genes increased, 1 gene (FGF-9) decreased, and 1 gene was unchanged. Six transcripts (HB-EGF, BMP-2, COX-2, LIF, PAR-2, and FGF-9) were evaluated using an ex vivo rat model of bladder distension. HB-EGF, BMP-2, COX-2, LIF, and PAR-2 increased with bladder stretch ex vivo, whereas FGF-9 decreased, consistent with expression changes observed in vitro. In silico analysis of microarray data using the FIRED algorithm identified c-jun, AP-1, ATF-2, and neurofibromin-1 (NF-1) as potential transcriptional mediators of stretch signals. Furthermore, the promoters of 9 of 13 stretch-responsive genes contained AP-1 binding sites. These observations identify stretch as a highly selective regulator of gene expression in bladder SMC. Moreover, they suggest that mechanical and growth factor signals converge on common transcriptional regulators that include members of the AP-1 family.

platelet-derived growth factor; smooth muscle; oligonucleotide array

CHRONIC OVERDISTENSION of the urinary bladder beyond its normal physiological limits is characterized by thickening of the bladder wall. This adaptive response, although initially protective, is associated with a progressive loss of muscle contractility and ultimately leads to decompensation and organ failure. Based on both clinical and experimental evidence, hypertrophic expansion of the bladder wall is believed to result from increases in cell number (hyperplasia) and cell size (hypertrophy) as well as alterations in extracellular matrix synthesis and deposition (reviewed in Ref. 46). However, the molecular events that underlie pathological tissue remodeling in the lower urinary tract remain poorly understood. By analogy with hypertrophic muscle growth in other hollow organs, such as the heart and the vasculature, excessive mechanical forces experienced by the hyperdistended bladder are thought to promote changes in gene expression that contribute to the pathological increase in tissue mass associated with bladder hypertrophy.

The process whereby mechanical forces impinging on the cell surface are converted to chemical information within the cell is termed mechanotransduction. Mechanical signals received by the cell in the form of distension or compression are transduced through transmembrane receptors such as integrins to intracellular signaling mechanisms. Downstream events include cAMP release, initiation of phosphorylation cascades and gene expression changes (reviewed in Ref. 17). To identify a mechanistic link between mechanical stimulation and growth of bladder muscle, several groups including our own have investigated the expression of known smooth muscle cell (SMC) mitogens and other growth regulatory molecules in bladder SMC following overdistension secondary to bladder outlet obstruction in vivo or in response to repetitive mechanical stimulation in vitro (5, 6, 8, 9, 30, 32, 38, 40).

From these studies, the gene encoding heparin-binding epidermal growth factor-like growth factor (HB-EGF) has emerged as a candidate mediator of SMC proliferation in the urinary tract (27, 30). HB-EGF, a member of the EGF-like growth factor family and an activating ligand for the epidermal growth factor receptor (EGFR)/ErbB1, is a potent mitogen for visceral SMC (35). Our laboratory demonstrated that exposure of rodent or human bladder SMC to defined cyclic stretch-relaxation in vitro induced rapid and transient expression of HB-EGF mRNA, in a pattern characteristic of immediate early genes (27, 30). Similarly, in a murine model of acute bladder outlet obstruction, HB-EGF mRNA was selectively increased in the SMC compartment of the bladder wall with minimal changes observed in the mucosa (3). In support of a role as a mediator of muscle cell hyperplasia, HB-EGF-induced proliferation of bladder SMC in culture. Moreover, log-phase growth of SMC was markedly inhibited in the presence of the nonotoxic diphtheria toxin analog, CRM197, a specific HB-EGF inhibitor (3, 18, 27). Collectively, these studies implicate stretch-induced expression of HB-EGF in the proliferative response of bladder muscle to prolonged mechanical stimulation.

In a similar study, Chaqour and colleagues (5) demonstrated increased expression of the gene encoding insulin-like growth factor-I (IGF-I) following cyclic strain of fetal bovine bladder
SMC. Consistent with the in vitro observations, increased expression of IGF-I has also been identified in hypertrophic bladder muscle in vivo following urethral ligation (8). Stretch-stimulated induction of HB-EGF and IGF-I was found to be partially dependent on the angiotensin II-mediated activation of the angiotensin II type I (AT₁) receptor (5, 30), since expression of both genes was attenuated in the presence of the AT₁-specific blocker, losartan. This observation highlights the cooperativity that exists between mechanical and biochemical (i.e., peptide growth factor) signals in regulating growth responses of muscle cells to hypertrophic stimuli. In support of this concept, platelet-derived growth factor-BB (PDGF-BB) was shown recently to be a potent inducer of DNA synthesis in bladder and ureteral SMC isolated from rat and human (1, 39). Interestingly, PDGF-BB was considerably more potent than either HB-EGF or fibroblast growth factor-2 (FGF-2) in these studies (1, 39). In a comparative analysis, cyclic stretch and PDGF-BB both activated the phosphoinositide-3-kinase (PI3K)/Akt and p38 mitogen-activated protein kinase pathways with similar kinetics in rodent and human bladder SMC. Furthermore, stretch- or PDGF-BB-induced DNA synthesis was significantly inhibited with pharmacological antagonists of PI3K and p38 (1). Together these studies provide evidence that mechanical and growth factor signals converge on similar downstream targets and suggest that both PDGF-BB and stretch are physiologically relevant regulators of SMC hyperplasia in the lower urinary tract.

Although bladder distension and mechanical stretch of bladder SMC in vitro has been shown to alter expression of discrete sets of genes (5, 6, 8, 9, 30, 32, 38, 40), it remains to be determined whether stretch induces extensive or limited changes in gene expression in bladder SMC. To address this question, we have undertaken a large-scale DNA array analysis of primary culture human bladder SMC subjected to cyclic stretch-relaxation.

MATERIALS AND METHODS

Cell Culture

Primary culture human bladder SMC were isolated and propagated as described previously (28). Cells were maintained in Dulbecco’s modification of Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco/Life Technologies, Gaithersburg, MD), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air-5% CO₂. All experiments were performed on cells at passages 3 and 4.

Mechanical and Growth Factor Stimulation of Bladder SMC

Cyclic stretch-relaxation. SMC were seeded at 8 × 10^4 cells/well in six-well silicone elastomer-bottomed culture plates coated with type I collagen (Bioflex; Flexcell, Hillsborough, NC) and grown to ~80% confluence in DMEM/10% FBS. Cells were subjected to serum depletion (DMEM/1% FBS) for 48 h, and switched to fresh serum-free DMEM immediately prior to treatment. Cells were subjected to continuous cycles of stretch-relaxation, using a computer-driven, vacuum-operated stretch-inducing device (Strain Unit FX-3000, Flexcell) as described previously (30). Each cycle consisted of 5 s of stretch and 5 s of relaxation (0.1 Hz) under conditions of 20% maximum radial stretch at the periphery of the membrane. Cells incubated in parallel under identical conditions but without exposure to stretch served as control.

PDGF-BB treatment. SMC were grown to ~80% confluence in six-well plastic dishes and rendered quiescent as above. Cells were treated with 0.25 nM PDGF-BB in serum-free DMEM; cells receiving serum-free DMEM alone served as controls.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from control or treated SMC using TRIzol reagent according to the manufacturer’s instructions. Then, 1.5 μg of total RNA were reverse-transcribed using SuperScript II reverse transcriptase and oligo d(T)_{12–18} as first-strand primer. cDNAs were amplified using primers specific to human HB-EGF and GAPDH, as described previously (30). PCR products were resolved by acrylamide gel electrophoresis, and signals were visualized following exposure of dried gels to X-ray film. Normalization to GAPDH expression and limiting dilution of cDNA enabled semiquantitative comparisons to be made between samples following densitometric measurement of band intensities.

Affymetrix GeneChip Analysis

Total RNA was extracted from control SMC or SMC exposed to stretch-relaxation for 4 h using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). RNA concentration and purity were determined from OD_{260}/OD_{280} and the extent of degradation was evaluated by analysis of 18S and 28S ribosomal RNAs in formaldehyde cross-linked agarose gels. Five micrograms of total RNA was used for synthesis of labeled target, which was carried out in the Gene Expression and Bioinformatics Core Facility, Dept. of Genetics, Children’s Hospital Boston. Briefly, RNA was reverse-transcribed into cDNA using Superscript II (Invitrogen) with a T7-(dT)_{24} oligomer to prime first-strand synthesis, and DNA polymerase and DNA ligase were included in synthesis of the second strand. Following cleanup, cDNA was used as template for synthesis of biotin-labeled cRNA using the BioArray High Yield RNA Transcription Labeling kit (Affymetrix, Santa Clara, CA), followed by fragmentation of labeled cRNA prior to hybridization. Prior to GeneChip analysis, the quality of the labeled target was evaluated using the Test3 array, which determines representation of 5’, 3’, and intervening sequences within the target. Biotinylated cRNA (target) prepared from nonstretched and stretched bladder SMC was then hybridized to the human HG-U133A Affymetrix microarray, which represents ~14,500 human genes. GeneChips were processed using the Affymetrix GeneChip Fluidics Workstation 400, bound to streptavidin-phycoerythrin, and scanned with a GeneArray scanner (Hewlett-Packard, Palo Alto, CA). The cyclic stretch-relaxation experiment was repeated 4 times, yielding a total of 8 array data sets (nonstretched, n = 4; stretched, n = 4).

Analysis of Microarray Data

Prior to analysis, data from the eight hybridizations (4 nonstretch, 4 stretch) was processed using Microarray Suite software, v. 5.0 (MAS 5.0) to yield average difference values corresponding to signal intensity for each probe set. These values were calculated from the difference in signal intensity following hybridization with “perfect match” (PM) and “mismatch” (MM) oligonucleotides in each probe pair. Hybridization data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo) with GEO accession number GSE1595 (GSM27367–GSM27374). To allow for comparison of data on separate microarrays, the intensity values were first normalized according to the median signal intensity for a given array. The array with the highest correlation to all other arrays was selected as the reference against which all other arrays were normalized. Use of the median, in contrast to the mean or slope, minimizes skewing of the data by outliers. In addition, probe sets that gave an absent (A) call across all eight arrays were eliminated from subsequent analysis, leaving 11,731 of the initial 22,283 probe sets. To identify SMC genes that were differentially expressed in response to cyclic stretch-relax-
ation, we employed the Significance Analysis of Microarrays (SAM) statistical software package, developed at Stanford University (42). Data were permuted using the SAM software with the following parameters: two-class, unpaired, unlogged data, 1,000 permutations. In SAM analysis, each gene is assigned a numerical score that is derived from the change in gene expression relative to the variation (standard deviation) of repeated measurements across control and experimental data sets. The larger the score, the more likely that gene is to be differentially expressed between experimental groups.

Validation of Gene Expression Changes by Real-Time PCR

To confirm stretch-induced changes in gene expression, total RNA extracted from stretched and control SMC was reverse-transcribed using Omniscript reagent and random primers, (Qiagen, Chatsworth, CA) and subjected to semiquantitative real-time PCR. Primers were designed using Primer Express software (Applied Biosystems) to have annealing temperatures of 60°C and to generate products of 70 bp. Primers were confirmed to yield single products using melting curve analysis prior to analysis of SMC cDNA. Semiquantitative PCR was performed by real-time measurement of the changes in fluorescence of SYBR green dye, as described previously (36). The relative abundance of a given gene in control and treated SMC was estimated by comparing the cycle threshold (Ct) values for each sample following normalization to the housekeeping gene, GAPDH.

Ex Vivo Model of Bladder Distension

The ex vivo model of bladder distension has been described previously (4). Briefly, 30- to 45-day-old (~300 g) female Wistar rats were subjected to general inhalational anesthesia with isoflurane. Following transurethral catheterization, bladders were distended with culture medium (M199/20% FBS) to 40 cmH2O using water manometry to monitor the degree of distension. Following distension and with the catheter in place, laparotomy was performed. The ureters and bladder neck were ligated with suture in situ, and the catheter was removed. The whole bladder was excised and incubated in culture medium (M199/20% FBS) in the distended state in a humified tissue culture incubator at 5% CO2, 37°C for the indicated times. Nondistended bladders incubated in parallel for equivalent times served as controls. At each time point, total RNA was extracted from bladders and processed for real-time RT-PCR essentially as described above, except that expression data were normalized to 18S rRNA. All procedures were performed in accordance with Animal Care and Use Committee Guidelines, Children’s Hospital Boston.

Identification of Putative Transcriptional Regulators in Bladder SMC

To determine which transcriptional regulators could explain the observed gene expression pattern, we reanalyzed the array data using the FIRED (“frequency-based identification of regulatory elements in differential expression”) algorithm developed by one of us (A. Nima gonkar). The expression data were normalized for overall intensity using linear regression (44), and each array was normalized to the reference array, i.e., the array with the highest correlation coefficient to all other arrays, using the central 95% of expression values. Only genes showing at least two “present” calls in either the control (nondistended) or treated (distended) groups were selected for further analysis. From this subset, 578 were differentially expressed by greater than 1.5-fold. Based on the presence of known transcription factor (TF) binding sites in the regulatory regions of genes that were differentially expressed, every TF was assigned a score, calculated by counting the number of differentially expressed genes that possess binding sites for that factor. TF binding sites were identified using the TRANSFAC database (http://transfac.gbf.de); for these studies, only experimentally validated binding sites were considered. To assess the significance of a given TF score from the list of differentially expressed genes, we performed randomization analysis (significance testing). To do this, 10,000 random gene sets of a given size were drawn from all the genes represented on the microarray. For each gene set, scores for all the TFs from the TRANSFAC database were computed and used to construct probability distributions for each TF. From these distributions, the probability of obtaining a particular score for a TF could be computed and compared with the observed score.

Statistical Analysis

Student’s t-test was used to compare Ct values obtained from real-time RT-PCR of cDNAs from bladder SMC subjected to cyclic-stretch relaxation or PDGF treatment in vitro. For analysis of real-time RT-PCR data of cDNAs from whole bladder distension ex vivo, the Wilcoxon signed rank test was used. In each case, P < 0.05 was considered significant.

RESULTS

Cyclic Stretch is a Selective Inducer of Gene Expression in Bladder SMCs

To evaluate genome-wide mRNA expression in bladder muscle, we employed oligonucleotide arrays to perform a large-scale analysis of gene expression changes occurring in human bladder SMC exposed to mechanical stimulation in vitro. SMC were subjected to cyclic stretch-relaxation for 4 h as described in the MATERIALS AND METHODS. Cells seeded in stretch plates but not exposed to the stretch stimulus served as controls. Total RNA was extracted from both groups of cells and processed for hybridization to Affymetrix HG-U133A oligonucleotide arrays as described. To identify genes in which the expression differed significantly between control and stretch treatments, we employed the SAM approach using software developed at Stanford University by Tusher and colleagues (42). Using SAM analysis with a threshold value of 0.419, we identified 20 genes out of a total of 11,731 (~0.17%) to be differentially expressed by twofold or greater in SMC exposed to stretch stimulation (Table 1). The number of stretch-responsive genes increased to 203 out of 11,731 (~1.73%) when the fold-change cutoff was reduced to 1.5.

To confirm stretch-induced changes in gene expression, total RNA extracted from stretched and control SMC was reverse-transcribed using Omniscript reagent and random primers, (Qiagen, Chatsworth, CA) and subjected to semiquantitative real-time PCR. Primers were designed using Primer Express software (Applied Biosystems) to have annealing temperatures of 60°C and to generate products of 70 bp. Primers were confirmed to yield single products using melting curve analysis prior to analysis of SMC cDNA. Semiquantitative PCR was performed by real-time measurement of the changes in fluorescence of SYBR green dye, as described previously (36). The relative abundance of a given gene in control and treated SMC was estimated by comparing the cycle threshold (Ct) values for each sample following normalization to the housekeeping gene, GAPDH.

Validation of Array Findings by Real-time RT-PCR

To verify the gene expression changes observed by array analysis, we employed real-time RT-PCR to examine expression of putative stretch-responsive genes in separate cultures of SMC exposed to stretch. Fifteen of the 20 genes shown to change by >2-fold on the array were tested in the validation experiments using real-time RT-PCR. As shown in Fig. 1A, stretch-induced changes in mRNA levels were validated for 14 of 15 genes tested; of these ELL-2 (elongation factor, RNA polymerase II, 2) did not show a statistically significant change in expression with stretch, and tropomyosin-4 (TPM4) was found to decrease with stretch, in contrast to increased expression by array analysis. The expression level of genes that increased ranged from 1.2- to 3.1-fold compared with nonstretched controls, which was comparable to the relative expression determined by array analysis. The range of expression for downregulated genes was 0.36- to 0.73-fold relative to control.
To test the hypothesis that stretch- and PDGF-induced signals converge at the level of transcription, we assessed whether stretch-responsive genes in bladder SMC were also regulated in response to PDGF treatment. As shown in Fig. 1B, PDGF-BB treatment for 4 h elicited gene expression changes in bladder SMC that were qualitatively similar to those observed in response to stretch. However, in all but four cases [soluble urokinase plasminogen activator receptor (SUPAR), ELL-2, calbindin-2, and Krüppel-like factor-6 (KLF-6)], PDGF was a more potent inducer of gene expression than stretch, with mRNA levels ranging from 1.0- to 6.4-fold over baseline at 4 h. Changes in gene expression following PDGF treatment were statistically significant, except for calbindin-2, KLF-6, tenascin-C (TNC), and TPM4. PDGF also suppressed gene expression, in the case of fibroblast growth factor-9 (FGF-9), to a greater extent than stretch. In addition, PDGF treatment elicited time-dependent changes in expression; for 9 of 15 genes tested, PDGF-stimulated changes in gene expression were greater at 8 h than at 4 h (Fig. 1B) and ranged from 2.4- to 23.9-fold over baseline. With the exception of TNC and TPM4, gene expression changes following PDGF treatment for 8 h were statistically significant.

We also compared the kinetics of induction of HB-EGF mRNA in cells exposed to stretch or PDGF treatment using a separate PCR-based method. As shown in Fig. 2, the two distinct stimuli elicited comparable patterns of HB-EGF expression in SMC with peak expression levels evident 4–8 h after initiation of treatment. Interestingly, a second peak of HB-EGF mRNA expression was evident 48 h following application of the stretch stimulus, but not in response to PDGF treatment. This suggests that sustained mechanical stimulation elicits signals that lead to delayed HB-EGF expression. Collectively, these observations are consistent with the conclusion that mechanical and growth factor stimuli converge on common signaling networks in bladder SMC.

### Table 1. Differential gene expression in bladder SMC exposed to cyclic stretch-relaxation

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<th>Probe Set ID</th>
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<th>Fold (r(i))</th>
<th>(Sd\ (i))</th>
<th>Mean NS</th>
<th>Mean S</th>
<th>(q) Value, %</th>
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Microarray data were analyzed using the Significance Analysis of Microarrays approach, as described. Genes are listed that were induced or repressed greater than twofold following cyclic stretch of human bladder smooth muscle cells (SMCs). Probe set ID numbers refer to HG-U133A GeneChips from Affymetrix. The \(q\) value for each gene is a measure of the probability of obtaining the corresponding score (\(d(i)\)) for that gene, assuming that the gene is not differentially expressed, and represents the false discovery rate. The score \(d(i)\) is the relative difference in expression for a given gene \(i\) between the nonstretched (NS) and stretched (S) conditions. Fold \(r(i)\) represents the arithmetic fold change in gene expression between the nonstretched and stretched conditions, and \(Sd\ (i)\), a measure of the gene-specific scatter, is the standard deviation of repeated measurements. Mean NS and Mean S refer to the average gene expression (intensity) values under nonstretched and stretch conditions, respectively.
HB-EGF, bone morphogenetic protein-2 (BMP-2), cyclooxygenase-2 (COX-2), leukemia inhibitory factor (LIF), protease-activated receptor-2 (PAR-2), and FGF-9] in bladders that were distended with culture medium and incubated for 30 min, 1 h, 4 h, 8 h, and 24 h. Nondistended bladders incubated for the same duration served as controls. As shown in Fig. 3, expression of the six genes varied in a time-dependent manner following bladder distension. HB-EGF showed the greatest increase in expression relative to control, with an 18.6-fold change in expression evident at 1 h of distension. Peak expression of COX-2 and LIF was delayed until 8 h with 10.5- and 4.3-fold changes, respectively. Expression of BMP-2 and PAR-2 increased modestly (2.5-3.5-fold) at 1 h and decreased thereafter. Distension-induced expression of the five upregulated genes returned to basal levels after 24 h. Consistent with our findings in vitro, FGF-9 expression decreased markedly at 1 h following distension and remained suppressed for the duration of the experiment. Taken together, these findings suggest that gene expression changes measured in bladder SMC exposed to mechanical stimulation in monolayer culture are pertinent to alterations in transcription induced by bladder overdistension in vivo.

Identification of Putative Transcriptional Regulators of Stretch-Responsive Gene Expression

To identify the TFs that may underlie the gene expression response observed in bladder SMC exposed to stretch, we employed an informatics approach using the FIRED algorithm (Nimgaonkar et al., unpublished observations), as described in the MATERIALS AND METHODS. As shown in Table 2, analysis of the array data using this algorithm identified 10 TFs with significance \( P < 0.05 \), that most strongly correlated with the stretch-induced gene expression profile. Of these, c-Jun, cAMP response element binding protein-1 (CRE-BP1, also known as ATF-2), activator protein-1 (AP-1), and neurofibromin-1 (NF-1) were the most significant.

DISCUSSION

The pathological tissue remodeling that occurs in hollow organs in response to mechanical or hemodynamic overload is associated with characteristic gene expression patterns. Although selected genes have been linked to compensatory thickening of the bladder wall (reviewed in Ref. 45), large-scale evaluation of the extent of “stretch-responsiveness” in the
expressed genome of bladder cells or tissue has not been reported. In this study, we employed oligonucleotide arrays to interrogate the expression of more than 20,000 transcripts simultaneously in primary culture human bladder SMC exposed to a defined mechanical stimulus. Using this approach, we found stretch to be a highly selective regulator of gene expression in bladder SMC, with <0.2% of expressed (11,731) genes induced or repressed greater than twofold. Selective induction of gene expression was observed previously in vascular SMC exposed to biaxial strain with only 3 transcripts out of 5,000 induced greater than 2.5-fold (13). Although different genes were induced in our study and the study on vascular SMC, altered expression of a small fraction of the genome is consistent with a highly specific cellular response to the stimulus. Despite the selectivity of the mechanical stimulus, our cohort of stretch-responsive genes included two transcripts, HB-EGF and COX-2, that have previously been identified as mechanically regulated in bladder SMC in vitro (27, 30) and in vivo (3, 32), thereby increasing our confidence in the information obtained from the array analysis.

In this study, we compared gene expression in bladder SMC exposed to two “hypertrophic” stimuli: cyclic strain and PDGF-BB. Previous studies from our group have demonstrated that both activators induce DNA synthesis in rodent and human bladder SMC through common signaling cascades, including the PI3K/Akt and p38 MAPK pathways (1, 26). All of the stretch-responsive genes validated by real-time PCR, except for calbindin-2 and KLF-6, were also PDGF responsive, consistent with our previous demonstration that mechanical and growth factor signals converge on common intracellular effectors (1). In an earlier study, Hu and colleagues (16) showed that PDGF receptor-α (PDGFRα)-mediated signaling could be activated in a ligand (PDGF)-independent manner in vascular SMC by exposing the cells to mechanical stress. In that study, the patterns of PDGF- and stretch-induced tyrosine phosphorylation of PDGFRs were essentially indistinguishable. Moreover, the rapid kinetics of receptor activation reported in that paper precluded a role for stretch-stimulated secretion of PDGF in mediating PDGFRα phosphorylation. Similar patterns of rapid activation of the vascular endothelial growth factor (VEGF) receptor, Flk-1, and the ANG II type I receptor were observed in endothelial cells (7) and cardiomyocytes (47), respectively, in response to mechanical stimulation. In both cases, receptor activation occurred independently of ligand. Consistent with these observations, recent findings from our laboratory suggest that stretch-induced Akt phosphorylation and HB-EGF gene expression in human bladder SMC can be attenuated in the presence of AG1296, a pharmacological inhibitor of the PDGF receptor tyrosine kinase (RTK) (R. M. Adam and S. H. Eaton, unpublished observations). This, as well as previously published data on other RTK inhibitors (26), indicate that mechanical signals in bladder SMC may be transduced through classic growth factor receptors. Collectively, these studies support the concept that transmission of mechanical information into the cell involves integration between force sensors such as integrins, chemical transducers, e.g., receptor and non-RTKs, and the cytoskeleton. They also suggest that mechanical and chemical signals converge at multiple nodes throughout the cell.

A major effort in functional genomics, in addition to revealing genome-wide changes in gene expression under defined conditions, is the “reverse-engineering” of signaling networks within cells such that array data are used to infer potential upstream regulatory events. This concept is based on the premise that coexpressed genes are likely to share common transcriptional regulators (2). To address this question, we employed an informatics approach based on the FIRED algorithm, as described above. Of the TFs identified in this analysis, c-jun, CRE-BP1 (ATF-2), AP-1, and NF-1 were found to be the most significant (P < 0.05). The presence in this analysis of c-jun, c-fos, ATF-2, and AP-1, which comprises dimers of c-jun and c-fos family members, strongly supports published data from our group and others that members of the AP-1 family mediate transcriptional regulation of stretch-responsive genes in bladder SMC (29, 34). These data are also consistent with previous findings from our group that demonstrated stretch-stimulated phosphorylation of c-jun N-terminal kinase (JNK/SAPK), as well as stretch-mediated induction of c-jun protein levels in rodent bladder SMC (26). Upregulation
of JNK kinase activity in response to bladder SMC stretch was also shown by Kushida and colleagues (22). In that study, distension-induced JNK activation in bladder SMC was shown to be dependent on stretch-activated calcium channels. AP-1 and CRE-BP1 (ATF-2) have also been shown to lie downstream of JNK activation in response to altered hemodynamic load in a rodent model of pressure-overload cardiac hypertrophy (14).

Previous data from our group identified AP-1 and Ets as mediators of mechanically regulated gene expression in bladder muscle cells (26, 29). We found that stretch-induced expression of HB-EGF could be ablated by point mutations within a composite AP-1/Ets site in the HB-EGF promoter, implicating this site as a stretch-responsive element. In support of this hypothesis, in the study by Park et al. (29), we also found that matrix metalloproteinase-1 (MMP-1), which contains a consensus AP-1 binding site within its promoter, was robustly induced in bladder SMC by stretch, a response that was ablated in the presence of the AP-1 inhibitor curcumin. In contrast, the MMP-2 gene, which lacks AP-1 binding motifs within the 5′/flanking sequence, was not stretch responsive and was unaffected by the AP-1 inhibitor (29). To extend this hypothesis, in the current study we have analyzed published data on the promoter regions of genes that were shown by array analysis to be stretch-responsive in bladder SMC. This search revealed that, of 13 genes for which promoter sequence was available, 9 contained consensus TF binding sites for AP-1 and CRE-BP1 (ATF-2) have also been shown to lie downstream of JNK activation in response to altered hemodynamic load in a rodent model of pressure-overload cardiac hypertrophy (14).

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Table 2. Identification of putative transcriptional regulators

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Name</th>
<th>No. of Genes Regulated</th>
<th>Significance (P)</th>
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<tbody>
<tr>
<td>P05412</td>
<td>c-jun</td>
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<tr>
<td>P15336</td>
<td>CRE-BP1</td>
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<tr>
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<td>0.045</td>
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<tr>
<td>P21359</td>
<td>NF-1</td>
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</tr>
<tr>
<td>P01100</td>
<td>c-Fos</td>
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<td>0.075</td>
</tr>
<tr>
<td>P03372</td>
<td>ER-α</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
<td>P35869</td>
<td>AhR:Arnt</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
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<td>USF-1</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
<td>Q15853</td>
<td>USF-2</td>
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<td>0.04</td>
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<td>0.047</td>
</tr>
<tr>
<td>Q10586</td>
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<td>0.049</td>
</tr>
<tr>
<td>P10275</td>
<td>AR</td>
<td>1</td>
<td>0.053</td>
</tr>
</tbody>
</table>

The frequency of transcription factor binding sites in the promoters of genes identified as differentially expressed by microarray analysis, was computed using the FIRED algorithm as described. Transcription factors listed are those that achieved statistical significance. AP-1, activator protein-1; CRE-BP1, cAMP response element binding protein-1; NF-1, neurofibromin-1; AhR:Arnt, dimer of aryl hydrocarbon receptor precursor and AhR nuclear translocator; ER-α, estrogen receptor-α; USF, upstream stimulatory factor; ATBF1-A, AT-binding transcription factor 1, isoform A; DBP, D-site binding protein; AR, androgen receptor.
sponsiveness to mechanical stimulation and the presence of AP-1 sites in the promoter. Moreover, they are also consistent with the informatic analysis using FIRED.

To assess the relevance of our in vitro findings to distension of the intact bladder, we employed an ex vivo model of stretch-induced bladder injury (4). Distension of the rat bladder ex vivo markedly upregulated expression of HB-EGF and COX-2 mRNAs, consistent with previous findings in models of acute bladder outlet obstruction in mice (3, 31, 32). We also observed time-dependent changes in expression of four additional transcripts in the ex vivo model: BMP-2, LIF, PAR-2, and FGF-9. BMP-2, a member of the TGF-β superfamily, has not been detected previously in bladder muscle. However, the BMP-2 gene is induced in response to activation of the PI3K pathway in cardiomyocyte precursor cells (15). Notably, using in vitro stretch conditions identical to those employed here, we demonstrated recently that mechanical stimulation of bladder SMC activated PI3K signaling (1), suggesting a potential mechanistic link between stretch and BMP-2 expression in bladder muscle. LIF, also not described previously in bladder muscle, is rapidly (i.e., within 60 min) upregulated in response to hemodynamic overload in the heart (43), a pathophysiological situation that is phenotypically analogous to bladder distension secondary to urinary tract obstruction. In that study, overload-induced expression of LIF was found to mediate both cytoprotective and hypertrophic effects in cardiac myocytes (43), suggesting it may fulfill similar functions in the bladder wall. PAR-2, a member of the protease-activated receptor family is expressed in bladder muscle (12). PAR-2 is activated following trypsin-mediated cleavage of its NH2-terminal domain, and consequently has been linked to conditions such as inflammation where trypsin-like levels increase dramatically (12). In addition, PAR-2, along with other members of the PAR family, has been implicated in regulation of smooth muscle tone (24). FGF-9, a member of the FGF family (25), was the only gene that was significantly downregulated in our analyses. Genetic studies have identified roles for FGF-9 in testicular embryogenesis, as well as lung branching morphogenesis; mice lacking FGF-9 expression display male-to-female sex reversal (10) and also die at birth from lung hypoplasia (11). From these studies, FGF-9 has been implicated as a modulator of mesenchymal proliferation (10, 11). Although FGF-9 has not been described previously in the bladder, our findings suggest that alterations in FGF-9 expression may also be relevant to growth regulation within the lower urinary tract. Collectively, the findings from the ex vivo and in vitro models are in close agreement and are also consistent with the known functions of these gene products in other organs.

In summary, we have employed high-density oligonucleotide arrays to perform genome-wide expression analysis in primary culture SMC exposed to a defined mechanical stimulus. These studies demonstrate that 1) mechanical stimulation is a highly selective regulator of gene expression in primary culture bladder SMC; 2) distinct inducers of bladder SMC hypertrophy/hyperplasia, namely stretch and PDGF-BB, elicit overlapping gene expression changes in bladder SMC; 3) mechanical stimulation of bladder SMC in vitro and whole bladder distension ex vivo result in qualitatively similar patterns of gene expression; and 4) in silico analyses implicate members of the AP-1 family as transcriptional regulators of stretch-induced gene expression. Collectively, these observations suggest that mechanical and biochemical (growth factor) signals converge on common transcriptional regulators to elicit gene expression changes that characterize the “hypertrophic response” of bladder muscle cells to overload.

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REFERENCES

STRETCH INDUCES GENE EXPRESSION IN BLADDER MUSCLE


