

Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells

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Park, John M., Joseph G. Borer, Michael R. Freeman, and Craig A. Peters. Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C1247–C1254, 1998.—Cultured rat bladder smooth muscle cells (SMC) were grown on collagen-coated silicone membranes and subjected to continuous cycles of stretch-relaxation. Semiquantitative RT-PCR analysis revealed a time-dependent increase in heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) mRNA levels after stretch, with maximal levels appearing after 4 h. Immunostaining for proHB-EGF revealed higher levels of HB-EGF protein in the stretched than in the nonstretched SMC. The ANG II receptor type 1 antagonist losartan markedly suppressed stretch-activated HB-EGF expression. ANG II levels were 3.3-fold higher in the stretch- than in the non-stretch-conditioned media. Stretch stimulation of bladder SMC that had been transiently transfected with an HB-EGF promoter-luciferase expression construct resulted in an 11-fold increase in reporter activity. Mechanical stretch induced a 4.7-fold increase in tritiated thymidine incorporation rate, and this was reduced by 25% in the presence of losartan. We conclude that mechanical stretch activates HB-EGF gene expression in bladder SMC and that this is mediated in part by autocrine ANG II secretion.

cell growth; bladder outlet obstruction; cyclic strain; angiotensin II; epidermal growth factor

CELLS RESPOND TO MECHANICAL stimuli by increasing rates of gene expression, protein synthesis, and cell proliferation (27, 36). In other situations, cells may respond by differentiating (25), rearranging the cytoskeleton or focal contacts (10), or altering the composition of the extracellular matrix (38). Such may be the case for urinary bladder smooth muscle cells (SMC) (3). Repetitive physical deformation is a prominent feature of the bladder environment during filling and emptying of urine. Disease states of bladder outlet obstruction in which mechanical forces may change, such as benign prostatic hyperplasia and posterior urethral valves, produce a significant increase in local tissue mass, in part due to SMC proliferation (8, 22, 24, 31). Although several growth factors have been suggested to mediate bladder SMC growth, it is not certain which of these factors are physiologically important in the setting of increased mechanical stimulation, primarily because the critical link between mechanical forces and growth factor regulation has not been demonstrated.

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Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is an activating ligand for the EGF receptor, originally cloned and purified from macrophage-like U-937 cells (12). HB-EGF is a potent mitogen for fibroblasts, keratinocytes, and SMC (7, 12). In cultured SMC, HB-EGF gene expression can be rapidly induced by a variety of growth-promoting agents, including serum, phorbol esters, thrombin, tumor necrosis factor- α , interleukin-1 β , ANG II, platelet-derived growth factor, and basic fibroblast growth factor (bFGF) (7, 18, 19, 33). These findings support a physiological role for HB-EGF as an SMC mitogen. We recently reported that HB-EGF is synthesized in human bladder SMC *in vivo*, suggesting the possibility that it is a physiologically relevant SMC growth factor in the urinary tract (9).

We performed the present studies to test the hypothesis that HB-EGF expression might be regulated by mechanical forces in bladder SMC. This question was motivated by previous studies demonstrating that mechanical stretch can induce SMC proliferation (23, 30). We provide evidence that mechanical stretch is a potent stimulus for HB-EGF gene expression in cultured bladder SMC and that this mechanoinduction process involves autocrine signaling by ANG II.

MATERIALS AND METHODS

Bladder SMC cultures. Rat bladder SMC were isolated using an enzymatic dispersion method modified from that described by Gunther et al. (11). Bladders were harvested from 1-wk-old Lewis rats (Charles River Laboratories), and the muscle layers were cleared of epithelium and other extraneous tissues under a dissecting microscope. The tissues were cut into 1- to 3-mm pieces and incubated for 90 min at 37°C with 0.125 mg/ml elastase (type III, 90 U/mg), 1.0 mg/ml collagenase (type I, 150 U/mg), 0.250 mg/ml soybean trypsin inhibitor (type 1-S), and 2.0 mg/ml crystallized BSA (all purchased from Sigma Chemical). The resulting tissue suspension was triturated several times using serological pipettes, filtered through a 100- μ m cell strainer, and centrifuged. The cell pellet was resuspended in medium 199 (GIBCO) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Cells were maintained in a humidified 5% CO₂-95% air atmosphere at 37°C. All experiments were performed on cells between passages 3 and 5. Cells were characterized as SMC by morphological criteria (spindle shape, hill-and-valley pattern) and by their expression of smooth muscle α -actin and desmin (>95% of the cells stained positive for both markers).

Application of cyclical stretch-relaxation. Bladder SMC were grown to near confluence on six-well silicone elastomer-bottomed culture plates that had been coated with collagen type I (Bioflex, Flexcell, McKeesport, PA). Cells were rendered quiescent by incubation for 48 h in medium 199 supplemented with 0.5% FBS. They were then subjected to

continuous cycles of stretch-relaxation using a computer-driven, vacuum-operated stretch-inducing device (strain unit FX-3000, Flexcell). Each cycle consisted of 5 s of stretch and 5 s of relaxation (0.1 Hz). The vacuum induced an ~25% maximum radial stretch at the periphery of the membrane. In selected plates described below, 10 μM losartan (Merck), 10 μM PD-123319 (donated by Parke-Davis), and 30 μM PD-98059 (Biomol) were added to each well 30 min before the start of the stretch-relaxation process.

RT-PCR. Semiquantitative RT-PCR assays were performed to assess relative mRNA levels. RNA extraction was performed using Tri-Reagent (Molecular Research) according to the manufacturer's instructions. RT was performed using Maloney's murine leukemia virus RT (Ambion) with oligo(dT) (GIBCO) as the first-strand primer. cDNA was precipitated with linear acrylamide, ammonium acetate, and ethanol and was redissolved in Tris-EDTA buffer. Primers were selected from the previously published rat HB-EGF (1) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (34) sequences, both found through GeneBank database search. An ~413-bp HB-EGF product was amplified using the following primer pair: sense 5'-TCC CAC TGG AAC CAC AAA CCA G-3' (nt 157-178) and antisense 5'-CCC ACG ATG ACA AGA AGA CAG AC-3' (nt 570-548). An ~571-bp GAPDH product was amplified using the following primer pair: sense 5'-TCA CCA TCT TCC AGG AGC G-3' (nt 245-263) and antisense 5'-CTG CTT CAC CAC CTT CTT GA-3' (nt 816-797). PCR reactions were performed in a total volume of 25 μl containing 22 μl of PCR SuperMix (GIBCO), 0.5 μl each of sense and antisense primer (20 pmol/ μl), 0.1 μl of [^{32}P]dCTP (3,000 Ci/mmol, Amersham), and 2 μl of cDNA. PCR amplification was performed for 28-30 cycles at 94°C (denature), 58-60°C (anneal), and 72°C (extend) for 40 s each. PCR products were subjected to size separation by PAGE. Additional confirmation of PCR products was made by sequence-specific restriction enzyme digestion. All samples were normalized to GAPDH expression, and a limiting dilution method was used to make semiquantitative comparisons. Band intensity was determined with ImageQuant Software on a PhosphorImager System (Molecular Dynamics).

Immunohistochemistry. Immunostaining for proHB-EGF expression was performed using the avidin-biotin detection system, as previously described (9). A polyclonal antibody raised in chicken directed against the cytoplasmic domain of the human HB-EGF precursor was used. After a brief wash with PBS, 24-h stretched and nonstretched (control) bladder SMC were fixed with 3.7% formaldehyde in PBS and ice-cold 100% methanol for 5 min each. Cells were incubated with the primary antibody at 1:100 dilution for 1 h at room temperature. Immunostaining without the primary antibody was used as a negative methodological control. Nuclear counterstains were performed with hematoxylin.

Effects of exogenous ANG II, serum, bFGF, and stretch-conditioned media on HB-EGF expression. Quiescent bladder SMC grown on Bioflex plates were incubated with 1 μM human ANG II (Peninsula Laboratory) for 0, 1, and 2 h without stretch. Selected cells were incubated with ANG II in the presence of losartan (10 μM) and PD-123319 (10 μM). Similarly, quiescent bladder SMC were incubated with 10 and 0.1 nM ANG II, FBS (20% vol/vol), and bFGF (25 ng/ml). To test the effect of stretch-conditioned media on HB-EGF expression, quiescent bladder SMC were subjected to stretch-relaxation for 1 h, and the culture media were collected. Non-stretch-conditioned media were prepared as controls. Quiescent bladder SMC were incubated with stretch- and non-stretch-conditioned media for 2 h. In selected samples, cells were incubated with stretch-conditioned media in the

presence of losartan (10 μM) and PD-123319 (10 μM). Cells were analyzed for HB-EGF expression by semiquantitative RT-PCR.

ANG II enzymatic immunoassay. ANG II enzymatic immunoassay (EIA; Peninsula Laboratories) was performed according to the manufacturer's instructions. Bladder SMC were grown to near confluence on Bioflex plates and rendered quiescent. Culture medium (100 μl , from the total volume of 6 ml/well) was collected from each well. One group of cells was subjected to stretch stimulation, while the other group was incubated alongside the first one but without stretch. After 1 h, 100 μl of culture medium were again collected from each well. According to the manufacturer's specifications, the minimum detection limit of this ANG II EIA assay was 1-2 pg/well. The intra- and interassay variations were <5% and 14%, respectively.

Transfection and luciferase assay. The HB-EGF promoter-luciferase construct (kindly donated by Dr. Michael Klagsbrun) was made by ligating the 1.7-kb *Mbo* I-*Not* I fragment derived from the 5'-untranslated region of the murine HB-EGF genomic clone (corresponding to nt sequence -1837 to -155) into the 5'-end of the luciferase gene vector pGL2-basic (4). Cells ($\sim 2 \times 10^6$) were trypsinized and suspended in 20 ml of the standard culture medium (medium 199 with 20% FBS), and 400 μl of DNA-Superfect (Qiagen) mixture (total plasmid 2.5 μg) were slowly added to the cell suspension. Out of this cell suspension-DNA mixture, an equal number of cells ($\sim 1 \times 10^5$) was seeded onto each well of Bioflex culture plates. After 24 h of incubation, cells were rendered quiescent and subjected to stretch stimulation for 12 h. Cell lysates were harvested, and luciferase activity was measured using an LKB Rackbeta liquid scintillation counter immediately after the addition of luciferase substrate. A sample of cell suspension was similarly transfected with the promoterless pGL2-basic and served as control.

Tritiated thymidine incorporation assay. Bladder SMC were subjected to cyclical stretch-relaxation for 48 h, as described above. The control cells were incubated in parallel but without exposure to stretch stimulation; another group of cells was subjected to stretch in the presence of 10 μM losartan. During the last 8 h, 1 $\mu\text{Ci/ml}$ of [^3H]thymidine (5 Ci/mmol, Amersham) was added to the culture medium in each well. Cells were washed three times with PBS and treated with ice-cold 15% TCA for 30 min. After two additional washes with 15% TCA for 5 min each, cells were rinsed with methanol and air dried. To solubilize the TCA-precipitable materials, 1 ml of 1% SDS in 0.3 N NaOH was added to each well and incubated for 20 min. These were transferred to scintillation vials and mixed with 10 ml of scintillant. Counting was performed with an LKB Rackbeta liquid scintillation counter.

Statistical analysis. Values are means \pm SE. Comparisons between two means were made using nonparametric unpaired *t*-test.

RESULTS

Mechanical stretch induces HB-EGF mRNA and protein expression in bladder SMC. Quiescent bladder SMC were exposed to continuous cycles of stretch-relaxation for 0, 1, 2, 4, and 8 h ($n = 3$ for each time point), and HB-EGF mRNA levels were assessed using semiquantitative RT-PCR with normalization to GAPDH mRNA levels (Fig. 1). Compared with non-stretch-conditioned controls, bladder SMC that had been exposed to stretch stimulation demonstrated increased levels of HB-EGF mRNA in a time-dependent

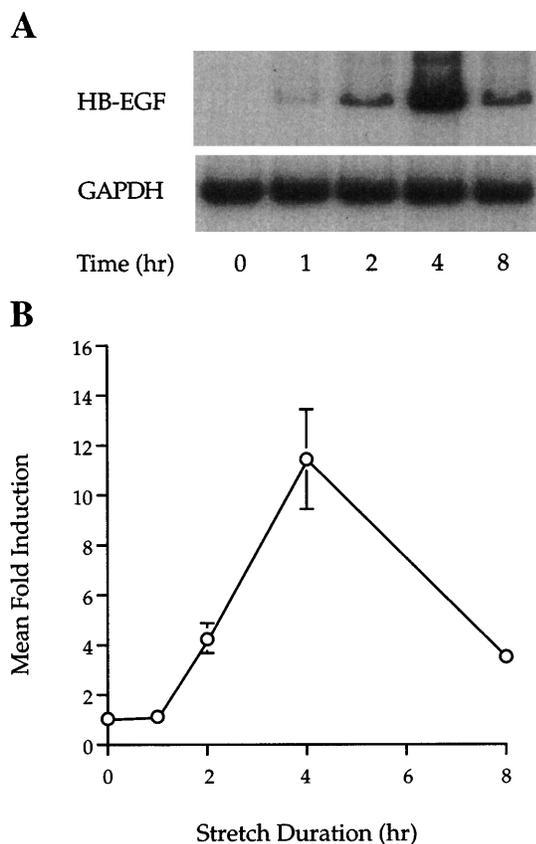


Fig. 1. Semiquantitative RT-PCR analysis of heparin-binding epidermal growth factor (HB-EGF) mRNA levels in cultured rat bladder smooth muscle cells (SMC) subjected to continuous cycles of stretch-relaxation for various durations. All samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. *A*: representative gel from 3 independently performed experiments. *B*: densitometric analysis ($n = 3$ for each time point).

manner. HB-EGF mRNA levels were low in all samples before stretch, increased significantly after 2 h, reached maximal levels after 4 h (10.2 ± 1.8 -fold increase compared with non-stretch-conditioned controls), and decreased toward the baseline after 8 h. Immunostaining for cytoplasmic membrane-anchored proHB-EGF revealed strong cytosolic stains in cells that had been exposed to 24 h of stretch stimulation. In contrast, proHB-EGF stains were weak in non-stretch-conditioned control cells (Fig. 2).

Stretch-activated HB-EGF expression is mediated by autocrine release of ANG II. Because previous studies have suggested that ANG II may be an important mediator of gene expression after mechanical stretch (28, 29) and that ANG II stimulates HB-EGF gene expression in vascular SMC (33), we examined the role of ANG II in stretch-activated HB-EGF gene expression in bladder SMC. Quiescent cells were subjected to stretch stimulation for 4 h in the presence of the ANG II receptor type 1 (AT_1) antagonist losartan ($10 \mu\text{M}$) and the ANG II receptor type 2 (AT_2) antagonist PD-123319 ($10 \mu\text{M}$) (13). By semiquantitative RT-PCR, HB-EGF mRNA levels were significantly suppressed by losartan but not by PD-123319 (Fig. 3). Previous studies also demonstrated that mechanical stretch, in part by auto-

crine secretion of ANG II, activates the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) signaling pathway (16, 26). We therefore performed experiments to see whether stretch-induced HB-EGF gene expression in bladder SMC was mediated by the ERK-MAPK pathway. Quiescent cells were subjected to stretch for 4 h in the presence of a specific ERK-MAPK kinase (MEK) inhibitor PD-98059 ($30 \mu\text{M}$) (2). This agent did not appreciably affect stretch-induced increase in HB-EGF mRNA levels (Fig. 3).

To confirm that AT_1 activation occurred via autocrine secretion of ANG II, we performed the following experiments. First, we demonstrated that bladder SMC were able to respond to exogenously added ANG II by increasing HB-EGF mRNA levels. Quiescent cells were exposed to ANG II ($1 \mu\text{M}$) in serum-free media for 0, 1, and 2 h. HB-EGF mRNA levels increased in a time-dependent manner in response to ANG II, reaching maximal levels after 2 h (10.6 ± 2.1 -fold increase compared with 0-h controls, $n = 3$ for each group). HB-EGF gene expression induced by ANG II was completely suppressed by losartan ($10 \mu\text{M}$) and unaffected by PD-123319 ($10 \mu\text{M}$), suggesting that the ANG

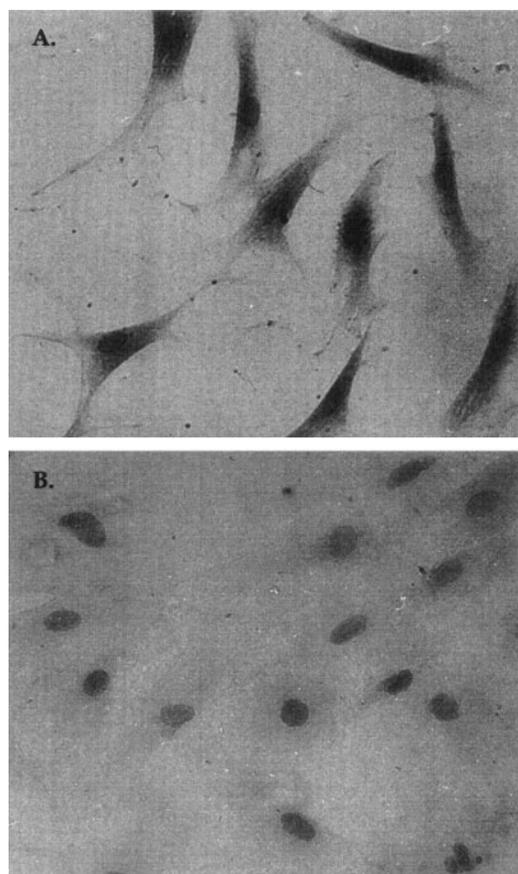


Fig. 2. Immunostaining for proHB-EGF in bladder SMC after exposure to mechanical stretch. A polyclonal antibody used in this experiment recognizes the carboxy terminus of membrane-bound proHB-EGF. Nuclear counterstains were performed with hematoxylin. Bladder SMC after 24 h of stretch stimulation demonstrate strong cytosolic stains (*A*) compared with non-stretch-conditioned control cells, which demonstrate weak stains (*B*).

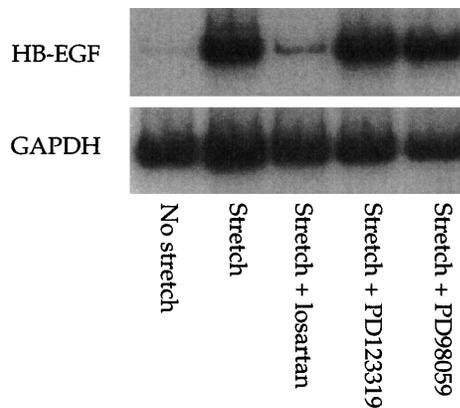


Fig. 3. Semiquantitative RT-PCR assay demonstrating effects of ANG II receptor antagonists and extracellular signal-regulated kinase (ERK) pathway inhibitor. First 2 lanes demonstrate HB-EGF mRNA levels in nonstretched and 4-h-stretched bladder SMC; last 3 lanes show HB-EGF mRNA levels in bladder SMC stretched for 4 h in presence of losartan (10 μ M), PD-123319 (10 μ M), and PD-98059 (30 μ M). Results are representative of 3 independently performed experiments.

II effect was mediated specifically by AT₁ activation (Fig. 4). ANG II was able to stimulate HB-EGF expression in bladder SMC at lower concentration ranges (10 and 0.1 nM), and these effects were comparable to stimulation by other factors known to induce HB-EGF expression such as serum and bFGF (Fig. 5). Second, we investigated whether ANG II is a secreted factor capable of mediating HB-EGF gene expression after stretch. Bladder SMC demonstrated greater levels of HB-EGF mRNA after 2 h of incubation with the stretch-conditioned media than with the non-stretch-conditioned media. This inductive effect of the stretch-conditioned media was suppressed by losartan (10 μ M) but not by PD-123319 (10 μ M), suggesting that ANG II is the secreted factor responsible for HB-EGF gene expression after stretch (Fig. 6). Finally, to confirm that stretch stimulation caused ANG II secretion by bladder

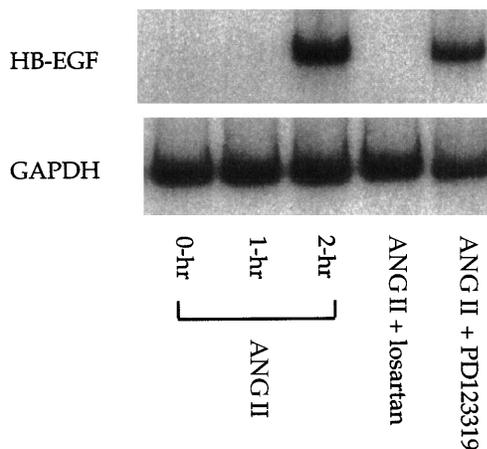


Fig. 4. Semiquantitative RT-PCR assay demonstrating effect of exogenous ANG II on HB-EGF gene expression in bladder SMC. Quiescent cells were incubated with 1 μ M ANG II for 0, 1, and 2 h in serum-free media. Last 2 lanes demonstrate HB-EGF mRNA levels in bladder SMC that were incubated with ANG II for 2 h in presence of losartan (10 μ M) and PD-123319 (10 μ M). Similar results were obtained in 3 independently performed experiments.

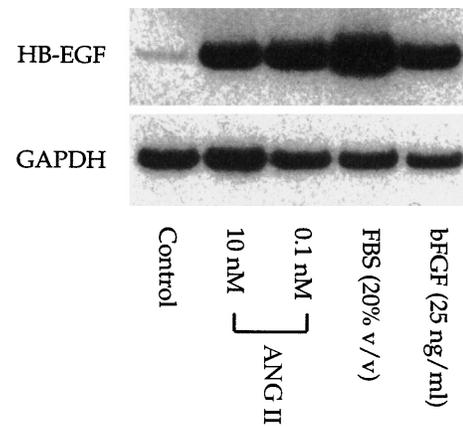


Fig. 5. Relative effects of ANG II, serum, and basic fibroblast growth factor (bFGF) on HB-EGF mRNA levels in bladder SMC. Quiescent bladder SMC were incubated for 3 h with ANG II, fetal bovine serum (FBS), and bFGF in absence of stretch. Relative HB-EGF mRNA levels were assessed by semiquantitative RT-PCR with normalization to GAPDH.

SMC, we measured ANG II levels in the stretch- and the non-stretch-conditioned media using a commercial high-sensitivity EIA kit. The media conditioned by nonstretched cells did not demonstrate any significant change in ANG II levels after 1 h (20.8 ± 3.9 vs. 19.8 ± 5.7 pg/ml, $n = 6$ for each group, $P = 0.9372$). In contrast, the media conditioned by stretch-stimulated cells demonstrated an ~ 3.3 -fold increase in ANG II levels after 1 h (24.5 ± 4.7 vs. 81.2 ± 1.7 pg/ml, $n = 6$ for each group, $P = 0.002$; Fig. 7).

Mechanical stretch stimulates HB-EGF promoter activity. To determine whether the mechanism of stretch-induced HB-EGF expression operates at the level of transcription, bladder SMC were transiently transfected with a plasmid DNA construct (pHB-EGF-Luc) containing 1.7 kb of the 5'-flanking region of the murine HB-EGF gene cloned into a luciferase reporter vector. This construct contains a functional HB-EGF promoter (4). Transfected cells were then subjected to continuous cycles of stretch-relaxation for 12 h. Blad-

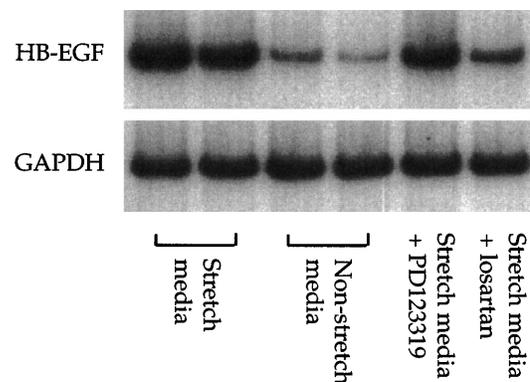


Fig. 6. Culture media conditioned by nonstretched and 1-h-stretched bladder SMC were applied to quiescent bladder SMC for 2 h, and HB-EGF mRNA levels were assessed by semiquantitative RT-PCR assay. Last 2 lanes demonstrate effect of stretch-conditioned media on HB-EGF gene expression in presence of losartan (10 μ M) and PD-123319 (10 μ M). Similar results were obtained in 3 independently performed experiments.

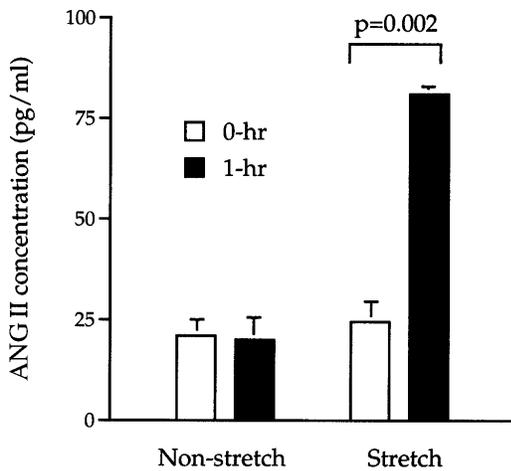


Fig. 7. Effect of mechanical stretch on ANG II levels measurable in culture media. Culture media were sampled at 0 and 1 h from nonstretched and stretched bladder SMC and analyzed for ANG II concentration ($n = 6$ for each group) using a high-sensitivity enzymatic immunoassay kit.

der SMC that had been transfected with a promoterless luciferase vector (pGL2-basic) demonstrated minimal luciferase activity after stretch stimulation. Bladder SMC transfected with pHB-EGF-Luc demonstrated a significant increase in luciferase activity after stretch compared with the nonstretch condition (10.7 ± 1.8 -fold increase, $n = 6$ for each group, $P = 0.002$). This increase was markedly suppressed when cells were subjected to stretch in the presence of $10 \mu\text{M}$ losartan (down to 3.6 ± 0.6 -fold increase, $n = 6$ for each group, $P = 0.002$; Fig. 8).

Stretch-induced increase in DNA synthesis rate is partially mediated by AT_1 activation. To determine whether mechanical stretch stimulates bladder SMC proliferation, we assessed DNA synthesis rate by performing a tritiated thymidine incorporation assay. Quiescent cells were subjected to continuous cycles of stretch-relaxation for 48 h. During the last 8 h, $1 \mu\text{Ci/ml}$ of [*methyl*- ^3H]thymidine was added to the culture media. Stretch-stimulated bladder SMC demonstrated an ~ 4.7 -fold increase in tritiated thymidine incorporation rate compared with non-stretch-conditioned controls. When stretch stimulation was performed in the presence of $10 \mu\text{M}$ losartan, bladder SMC demonstrated an $\sim 25\%$ attenuation of stretch-induced increase in tritiated thymidine incorporation rate (3.5- vs. 4.7-fold increase, $n = 6$ for each group, $P < 0.002$; Fig. 9).

DISCUSSION

In the current study we have demonstrated that mechanical stretch activates HB-EGF gene expression in cultured rat bladder SMC. In support of a physiological role for HB-EGF in SMC function, previous studies have documented that HB-EGF is synthesized in vascular SMC (7) and is a potent SMC mitogen (12). Our findings indicate that HB-EGF may be an autocrine mediator of SMC proliferation in response to mechanical stimuli.

Using early-passage bladder SMC derived from newborn Lewis rats, we have demonstrated that HB-EGF mRNA levels increased rapidly in a time-dependent manner in response to cyclical stretch stimulation. The temporal pattern of HB-EGF gene expression after mechanical stretch is consistent with that of the functional class of immediate early genes, as demonstrated in previous reports of HB-EGF gene expression (7, 33). We were also able to confirm that stretch-induced HB-EGF gene expression correlated with increases at the protein level. Immunohistochemistry using a polyclonal antibody directed against the carboxy terminus of the membrane-bound HB-EGF precursor proHB-EGF demonstrated strong cytoplasmic staining in bladder SMC that had been exposed to 24 h of stretch stimulation, in contrast to low levels of proHB-EGF staining in control cells that had not been exposed to stretch.

Transient transfection experiments using an HB-EGF promoter-luciferase reporter construct provided additional evidence that HB-EGF gene expression was stimulated by mechanical stretch. Our results also suggest that the stretch-induced increase in HB-EGF mRNA levels occurs primarily as a result of mechanisms operating at the transcriptional level. A 1.7-kb

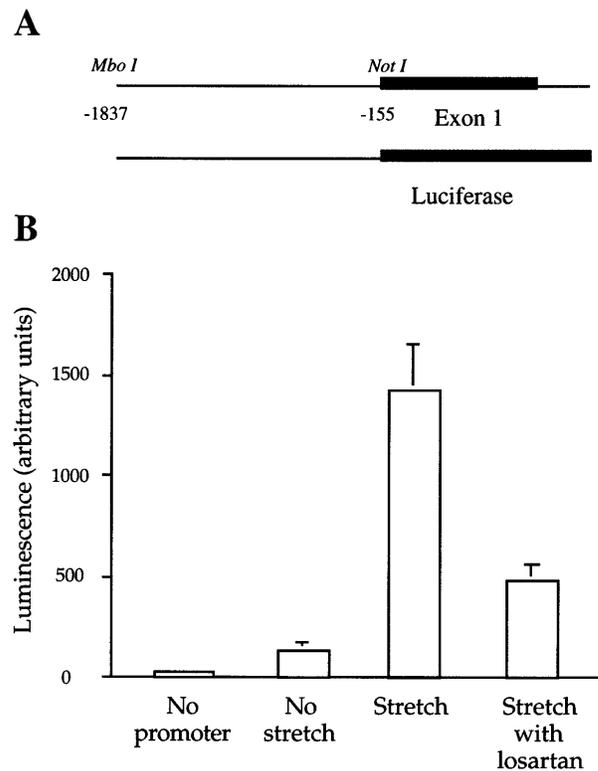


Fig. 8. Effect of mechanical stretch on bladder SMC after transient transfection with an HB-EGF promoter-luciferase reporter construct (pHB-EGF-Luc). *A*: schematic depiction of pHB-EGF-Luc. A 5'-flanking region between *Mbo*I restriction site at nt $-1,837$ and *Not*I site at nt -155 was linked upstream to a luciferase reporter gene vector. *B*: stimulation of HB-EGF promoter activity in bladder SMC after exposure to mechanical stretch. Transfected cells were subjected to no stretch, stretch, and stretch in presence of losartan ($10 \mu\text{M}$). After 12 h, cell lysates were assayed for luciferase activity. A promoterless luciferase construct was used as a negative control.

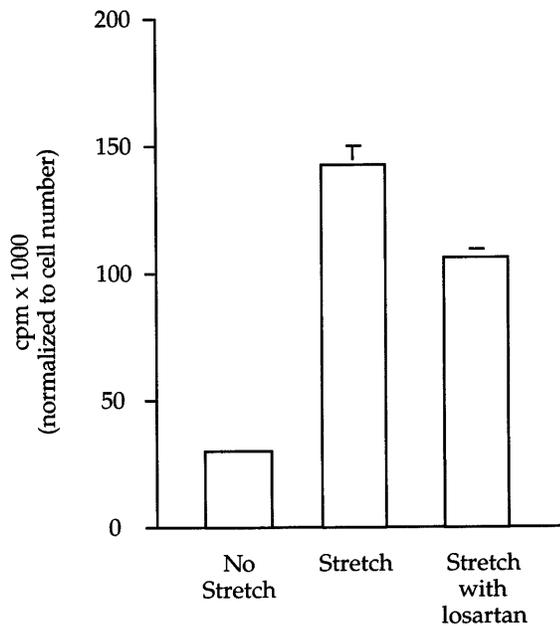


Fig. 9. Effect of mechanical stretch on bladder SMC proliferation. DNA synthesis rate was assessed by measuring [*methyl*-³H]thymidine incorporation rate in nonstretched and stretched bladder SMC. To study effect of AT₁-receptor inhibition, stretch stimulation was also performed in presence of 10 μ M losartan; *n* = 6 for each group.

DNA fragment corresponding to the 5'-flanking region of the murine HB-EGF gene responded to stretch stimulation with a degree of reporter gene activation similar to direct analysis of HB-EGF mRNA levels by semiquantitative RT-PCR. The mechanism of transcriptional activation by stretch is incompletely understood, but several candidate regulatory elements have been identified in previous studies. Mechanical stretch has been shown to activate several immediate early genes, including *c-fos* and *c-jun* in cultured rat cardiomyocytes (27). Corresponding increases in AP-1 nuclear binding activity were seen after cardiomyocyte stretch (27). By mutational analysis of the *c-fos* promoter, it was shown subsequently that the stretch response maps to a serum response element and a binding site for p62^{TCF} in the 5'-flanking region (26). In this study it was also suggested that an AP-1 binding site may be important in mediating stretch-induced gene expression. The 1.7-kb HB-EGF promoter fragment that was used in our study is known to contain several putative transcription factor binding sites, including two E-boxes, and nuclear factor- κ B, AP-1, SP-1, and Pit-1 sites (4).

In cardiomyocytes and vascular SMC, ANG II has been implicated as an important mediator of cell responses to mechanical stimuli (28, 29). Mechanical stretch leads to phosphorylation of ERK in part by autocrine secretion of ANG II (37). ANG II potentiates stretch-induced proliferation of cultured vascular SMC by augmenting platelet-derived growth factor secretion (30). There is evidence that ANG II and mechanical stretch provide a synergistic stimulation for gene expression in vascular SMC (20, 21). It has also been shown that ANG II receptor activation participates in cell-extracellular matrix interactions via integrins. In aor-

tic SMC, ANG II promotes phosphorylation of paxillin and tyrosine kinase pp125^{Fak}, the proteins that colocalize with integrin molecules at the termini of actin stress fibers in the cell attachment structures known as the focal adhesion complex (35). Our findings indicate that this paradigm of autocrine ANG II as a mediator of stretch-induced cell response is not restricted to vascular cells but also applies to visceral cells such as bladder SMC. Recently, evidence has been presented indicating that ANG II is functionally important in the bladder. ANG II has been shown to mediate contractile responses in the rat bladder muscle strips by activating AT₁ receptors (32). ANG II has also been suggested to modulate bladder SMC proliferation in vivo and in vitro (5, 6). In our study, stretch-induced HB-EGF gene expression was significantly suppressed in the presence of losartan but not PD-123319, an AT₂ receptor inhibitor, indicating that specific activation of the AT₁ receptor was involved in this mechanoinduction process. In addition, we accumulated evidence that ANG II is a secreted factor that mediates HB-EGF gene expression in bladder SMC. First, bladder SMC demonstrated increased HB-EGF mRNA levels when subjected to exogenously added ANG II, and this was suppressed specifically by losartan, indicating the presence of an ANG II-responsive signaling pathway involving AT₁. Second, increased HB-EGF mRNA levels were seen after incubation with stretch-conditioned media, and this inductive effect was also suppressed by losartan. These findings suggest that stretch-induced HB-EGF expression is mediated by secreted autocrine factor(s) and that ANG II is one such factor. Third, pHB-EGF-Luc-transfected cells demonstrated a significant attenuation of stretch-induced promoter activity when exposed to stretch in the presence of losartan, indicating that ANG II-mediated HB-EGF gene expression is operative at the transcriptional level. Finally, measurement of ANG II levels revealed an approximately threefold increase in the stretch-conditioned media compared with the non-stretch-conditioned media, again consistent with autocrine secretion of ANG II after stretch.

ANG II may not be solely responsible for stretch-mediated cell growth and gene expression. We observed consistently that stretch-induced HB-EGF gene expression was significantly but partially suppressed by 10 μ M losartan, suggesting that mechanisms other than ANG II-mediated cell signaling may be involved. Inhibition of AT₁-receptor activation with losartan also resulted in a significant but partial attenuation of increase in tritiated thymidine incorporation rate after stretch. In contrast, ANG II-stimulated HB-EGF expression was completely suppressed by losartan. Similar findings have been reported in previous studies of other cell types. In vascular SMC, ANG II was partially responsible for activation of tyrosine kinase signaling pathways after stretch in cardiomyocytes (37). Mechanical stretch and ANG II have been shown to evoke differential patterns of gene expression and protein synthesis (14). Mechanical stretch activates the stress-activated protein kinase (SAPK)-c-Jun NH₂-terminal

kinase (JNK) signaling pathway in cardiomyocytes, and activation of this pathway does not involve ANG II (15). It has been suggested that ANG II by itself is a weak stimulator of cell growth and gene expression, but its effects become significantly more pronounced when combined with other factors, such as different extracellular matrices (30) and mechanical stimulation (20).

Mechanical stretch activates several signal transduction pathways associated with cell growth, including phospholipase C, protein kinase C, tyrosine kinases, ERK, and SAPK-JNK (17, 28). In view of the fact that HB-EGF has been postulated to play a role as an SMC mitogen, we were interested to see whether stretch-induced HB-EGF gene expression in bladder SMC involved signaling via the ERK-MAPK pathway. PD-98059, which selectively inhibits the ERK signaling pathway, did not affect stretch-induced HB-EGF gene expression, suggesting that alternate pathways are involved in this mechanoinduction process. One potential signaling cascade may be the SAPK-JNK pathway, which has been shown to regulate gene expression through activation of the transcription factor AP-1 (15).

We believe our study is the first demonstration of linkage between regulation of HB-EGF gene expression in SMC and mechanical stretch stimulation, and it supports a physiological role for HB-EGF as an autocrine mediator of bladder SMC proliferation. We recently reported that proHB-EGF is synthesized by bladder SMC in vivo (9). Collectively, our findings suggest that one potential mechanism by which bladder SMC proliferation occurs in urinary tract outlet obstruction may be by activation of an autocrine regulatory loop in which growth factor synthesis is stimulated. Bladder outlet obstruction leads to progressive wall distension due to incomplete emptying of urine, thereby resulting in increased local stretch of bladder SMC. This mechanical stimulation may cause bladder SMC to release ANG II, which then activates AT₁ receptors in an autocrine fashion. AT₁ receptor activation, along with other yet undefined factors, stimulates expression of SMC growth factors, such as HB-EGF, and may stimulate cell growth. We speculate that this paradigm of stretch-induced HB-EGF gene expression may also apply to other systems in which mechanical load is thought to play a critical role in pathophysiology, such as cardiac hypertrophy, vascular SMC proliferation, and renal mesangial cell proliferation.

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