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Mechanical Regulation of IGF-I and IGF-Binding Protein Gene Transcription in Bladder Smooth Muscle Cells

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Abstract Mechanical forces are well known to modulate smooth muscle cell growth and synthetic phenotype. The signals controlling this process are complex and potentially involve changes in the expression of peptide growth factor genes such as those of the insulin-like growth factor (IGF) system. This study was designed to investigate the mechanical regulation of IGF-I and the binding proteins for IGF (IGFBPs) in smooth muscle cells cultured on a deformable surface and subjected to cyclic stretch. Using the RNase protection assay, we found that the application of a cyclic biaxial strain to cells induced a 2.5- to 4-fold increase in IGF-I mRNA levels after 8 h and an even greater increase after 16–24 h of stretch. This change was not affected by variations in the magnitude of the applied strain but was attenuated (~40%) when cells were treated with antagonists for angiotensin II receptors. Furthermore, the transcript levels of the three major IGF binding proteins produced in smooth muscle cells, e.g., IGFBP-2, IGFBP-4, and IGFBP-5, varied between stretched and control cells. Both IGFBP-2 and IGFBP-4 mRNA levels were consistently reduced in stretched cells but remained comparable to those of the control cells when the angiotensin II transducing pathway was blocked by inhibitors prior to the application of mechanical strain. Conversely, the gene expression of IGFBP-5 was upregulated in stretched cells, and neutralizing antibodies to IGF-I blocked this activation. Similarly, pharmacologic inhibition of the phosphatidylinositol 3-kinase, an important component of the IGF receptor transduction pathway, inhibited IGFBP-5 gene expression in stretched cells. These results suggest that the downstream effects of mechanical strain on IGF-I and IGFBP transcript levels are mediated, to greater or lesser extent, either through an angiotensin II tranducing pathway or via a feedback loop involving the autocrine secretion of IGF-I itself. J. Cell. Biochem. 84: 264–277, 2002.

Key words: mechanical stretch; growth factors; binding proteins; gene expression; mechano-transduction

Smooth muscle cells play crucial roles in the control of blood pressure, enteric peristalsis, and bronchial, uterus, and bladder contraction. These cells are continuously responding to and generating tensional forces in their native environment. Perturbation of the force equilibrium within the tissue triggers major pathophysiological changes, i.e., hypertrophy/hyperplasia, fibrosis [Chaqour et al., 1999a; Chiquet, 1999]. The signals controlling this process are complex and may involve alteration of the expression of a number of peptide growth factor genes whose encoded proteins act locally to orchestrate the cellular and molecular changes.

Studies have shown that the insulin-like growth factor-I (IGF-I) is a mitogen, an anti-apoptotic agent, a strong chemoattractant, and a potent modulator of smooth muscle synthetic phenotype [Arnqvist et al., 1995; Stewart and Rotwein, 1996]. Interestingly, increased expression of IGF-I gene has been reported in several mechanical stress-related pathologies, i.e., pressure overload-induced cardiac hypertrophy, bladder obstructive disorders, formation of restenosis after angioplasty, and local trauma such as muscle injury and balloon catheter denudation [Hansson et al., 1987; Khorsandi et al., 1992; Chen et al., 1994]. However, the difficulty of isolating the influence of the mechanical input per se from other factors present in the tissue has prevented the identification of the

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actual variables that regulate IGF-I gene expression and the molecular mechanisms involved.

The molecular actions of IGF-I are mediated through the IGF receptor, a tyrosine kinase receptor that is linked to the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathways [Chen et al., 1994; Imai and Clemmons, 1999]. Six binding proteins (IGFBP-1–6) that bind to IGF-I with high affinity and specificity further modulate the bioactivity of IGF-I [Kalus et al., 1998; Hwa et al., 1999]. Each tissue environment appears to contain a particular set of these proteins that can be bound (1) to the extracellular matrix (e.g., IGFBP-5), (2) to the cellular membrane (e.g., IGFBP-3), and (3), in the case of IGFBP-4, to an unknown site in the extracellular space. Their biological actions include modulation of IGF-I action in a positive or a negative fashion by either competition with the receptor for ligand binding or by interacting directly with the receptor, storage and transport of IGF-I, or by interaction with other growth factor systems. In addition, IGFBPs appear to mediate IGF-independent actions, including either inhibition or enhancement of cell growth and induction of apoptosis. In turn, cleavage of IGFBPs by proteases plays a key role in modulating levels and actions of free IGFBPs and IGF-I [Rechler and Clemmons, 1998]. The actual purpose of this multiprotein system, apparently designed to modulate IGF access to the cells, remains puzzling and may underlay yet unknown functions of these proteins. In addition, the regulatory mechanisms that govern expression of the individual binding proteins are still poorly understood.

This study was designed to investigate the effects of mechanical forces on IGF-I and IGFBP gene expression. Mechanical stimulation of the cells was performed by utilization of a well-defined in vitro mechanical device that imparts a cyclic stretch to smooth muscle cells cultured on an elastomeric membrane. We used such a device to investigate a potential coordinate expression among IGF-I and its binding protein genes and to define the potential mechanisms involved.

MATERIALS AND METHODS

Materials

M199 tissue culture media, penicillin, streptomycin, and L-glutamine were obtained from Life Technologies. Fetal bovine serum was obtained from Atlanta Pharmaceuticals. Angiotensin II, saralasin, and molecular biology reagents were obtained from Sigma and Fisher Scientific. Recombinant IGF-I and antibodies to human IGF-I were obtained from Dr. Parlow of the National Institute of Diabetes, Digestive and Kidney Diseases, National Hormone and Pituitary Program. Merck kindly provided losartan. Nitrocellulose and Zeta-probe membranes were purchased from Biorad.

Methods

Cell culture. Primary cultures of smooth muscle cells were prepared from fetal bovine bladders obtained from mid-to-late gestational fetal calves as previously described [Baskin et al., 1993]. Briefly, under sterile conditions, the smooth muscle layer of each bladder was dissected from the urothelium–lamina propria layers, minced into small pieces and digested with bacterial collagenase (100 U/ml). After inactivation of the enzyme with fetal bovine serum, cells were allowed to attach to a plastic tissue culture flask overnight. Cells were maintained in modified medium M199 supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO_2 in air at 37°C. Freshly isolated smooth muscle cells were phenotypically characterized using muscle specific antibodies against smooth muscle actin. Our previous studies have shown that these cells maintain several differentiated properties in culture even after several passages. Cells between their third and tenth population doubling level, were used for stretch experiments.

Mechanical stretching of the cells. Mechanical deformation of the cells was performed using a device designed to apply precise and reproducible biaxial strains to a type I collagen-coated Tecoflex membrane (Thermedics, Inc.) on which the cells were grown. Design, calibration, and description of the equibiaxial strain system for cultured cells have been previously reported [Winston et al., 1989; Chaqour et al., 1999a].

Typical experiments were carried out with 13 control and 13 experimental wells in which the cells were seeded at a density of 250,000 cells/well and incubated for 24 h in serum-containing medium. The medium was then removed and replaced by serum-free medium. For experimental wells, biaxial strain was applied by

stretching the elastic membrane of the wells cyclically at a frequency of 0.3 Hz. For controls, cells were cultured in the apparatus wells under the same conditions but were not subjected to mechanical strain. The strain level used was sufficient to produce molecular changes without inducing apparent cell injury. After completion of the stretch regimen, control and stretched cells were pooled, divided into two samples, and processed for RNA analysis. Pooling of the content of at least six individual wells was necessary in order to extract a sufficient amount of RNA for analysis. Stretch and control experiments, using the same pool of cells, were carried out simultaneously and analyzed identically. All experiments were repeated twice using cells prepared from a different animal. When experiments were replicated three times or more, statistical comparisons were made according to the Student's *t* test for unpaired data.

RNase protection assay. Total RNA was extracted from either stretched or control unstretched cells as described [Chomczynski and Sacchi, 1987]. IGF-I mRNA levels in both stretched and unstretched cells were measured by RNase protection assay relative to those of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Accordingly, two bovine riboprobes were used. One probe is complementary to IGF-I mRNA and protects a 250-nucleotide fragment of exon 3 and a second probe is complementary to GAPDH RNA and protects a 181-nucleotide fragment. The riboprobes were generated by reverse transcription and PCR amplification using the following primers. For IGF-I: 5′GAATTCAAGCCCACGGGGTATGGCT3′ and 5′AAGCTTCTGGGTCTTGGGCATGT3′ [Fostis et al., 1990]; For GAPDH: 5′TCCAGTATGATTCCACCC3′ and 5′TACTCAGCACCAGCATCACC3′ (GenBank accession number U85042). The PCR products were cloned into the cloning vector pCRII from Invitrogen and further sequenced to verify their orientation and identity. For the RNase protection assay, the plasmids were linearized and serve as a template for in vitro transcription by either SP6 or T7 RNA polymerase to generate [^{32}P]-uridine triphosphate-radiolabeled RNA probes using an in vitro transcription kit (Promega). Total RNA (12 μg) from stretched or control unstretched cells was resuspended in hybridization buffer containing 80% formamide, 1 mM EDTA, 40 mM piperazine-*N*,*N*′-bis-(2-hexanesulfonic acid, pH 6.4, and 0.2 M sodium acetate, 1×10^6 cpm riboprobe and denatured at 85°C for 5 min. After 24 h of incubation at 45°C, nonhybridized RNAs were digested with 40 μg/ml ribonuclease A and 100 units/ml ribonuclease T1. The protected hybrids were then precipitated and separated on a 4% polyacrylamide/urea denaturing sequencing gel followed by autoradiography. The protected bands were quantitated by using a molecular dynamics phosphorimager.

Northern blot analysis. Samples of total RNA (10 μg) were dissolved in 50% formamide, 2.2 M formaldehyde, 0.1 M 3-[*N*-morpholino]-propane sulfonic acid, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8, and denatured by heating at 65°C for 10 min. RNA was then fractionated by electrophoresis in 1% agarose/formaldehyde gel for 4 h at 70 V and transferred overnight by capillary blotting in 20× standard saline citrate (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) to a Z-probe nylon filter. The nylon filter was then prehybridized for 24 h at 42°C in 50% formamide, 7% SDS, 0.25 M Na_2HPO_4, pH 7.2, and 0.25 M NaCl. Specific rat DNA probes for IGFBP-2, IGFBP-4, and IGFBP-5, generously provided by Dr. Shimasaki [Shimasaki and Ling, 1991], were labeled with [^{32}P]-α dCTP using a random priming DNA labeling kit. After hybridization at 42°C for 24 h, the filter was washed twice in 2× SSC, 0.1% SDS at room temperature, twice in 0.1× SSC, 0.1% SDS at 50°C and exposed to a phosphorimager screen. The hybridization signals were quantitated and normalized to GAPDH mRNA used as a control gene.

Tritiated thymidine incorporation assay. To determine the rate of DNA synthesis, bladder smooth muscle cells were seeded in wells and subjected to cyclic mechanical stretch for 24 h as described above. The control cells were not exposed to mechanical stimulation but were seeded in wells and treated identically. Another group of cells were pretreated with either losartan (10 μM) or neutralizing antibodies to IGF-I (1:500 dilution) prior to the application of mechanical stretching. During the last 8 h of stretching, 1 μCi/ml of [methyl-^{3}H] thymidine (ICN Biochemicals, Inc.) was added to the culture medium in each well. After completion of the stretch regimen, cells were washed three times with PBS and twice with cold 5% trichloroacetic acid for 10 min at 4°C, dissolved in 200 ml of 0.1 M NaOH and 1% SDS at room temperature for 1 h and processed for

liquid scintillation counting. Stretch-conditioned medium was tested in a similar fashion and compared to conditioned medium from cells cultured under static conditions.

Statistical analysis. Student's t test was used to compare the differences between control and test groups. Values are means $\pm$ SE. $P < 0.5$ was considered significant.

RESULTS

Effects of Mechanical Stretch on IGF-I mRNA Levels

The IGF-I mRNA levels in smooth muscle cells, cultured either under static conditions or subjected to mechanical stretch, were determined using the solution hybridization/RNase protection assay. As shown in Figure 1, hybridization of total RNA to the IGF-I exon 3 fragment and GAPDH riboprobes yielded the predicted 250- and 181-bp protected RNA bands, respectively, following RNase digestion. No protected bands were present using tRNA for control hybridization (data not shown). First, we examined the time course of activation by applying a 5% strain to the cells, considered to be the level of strain that the cells may experience in vivo [Baskin et al., 1993; Chang et al., 1999]. There were no marked changes of IGF-I transcript levels after 1, 2 or 4 h of stretch (not shown). However, the increase in IGF-I mRNA levels was significant 8 h after application of mechanical strain and the mRNA levels were still rising after 24 h (Fig. 1A). Densitometric analysis of the hybridization signals from multiple experiments showed that these levels increased fourfold after 8 h and up to eightfold after 24 h of stretching. There were no significant changes in GAPDH mRNA levels throughout the time period of the application of

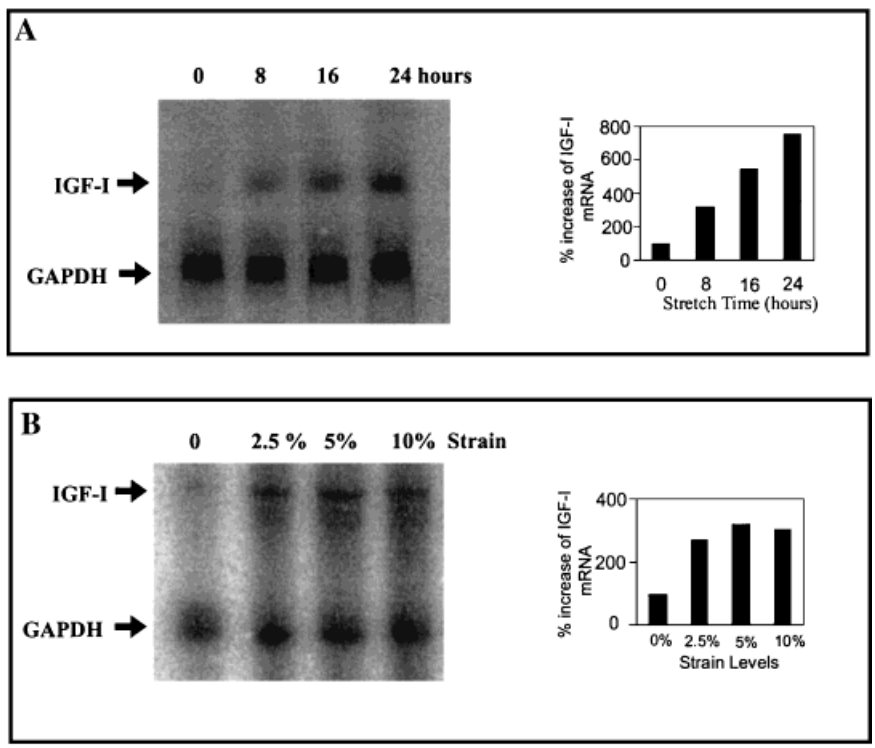

Fig. 1. Effects of mechanical strain on IGF-I mRNA levels as determined by hybridization/RNase protection assay in smooth muscle cells. Cells were seeded separately in 26 wells, half of which were used to apply cyclic stretch to the cells at either the indicated time periods (**A**) or the indicated strain levels for 8 h (**B**), whereas the other half were used as a control (no mechanical stimulation). The cell content of individual wells of either the stretch or the control group were then pooled and processed for RNA analysis. Total RNA (12 μg) was co-hybridized to IGF-I- and GAPDH [^{32}P]-labeled riboprobes and subjected to RNase digestion. The protected hybrids are 250 and 181 bases long for IGF-I and GAPDH, respectively. The formed hybrids were phenol/chloroform extracted, separated by electrophoresis in a polyacrylamide denaturing gel and visualized by autoradiography. The hybridization signals shown in the autoradiograms represent the equivalent of 13 wells. Left panels show percentage increase of IGF-I mRNA levels normalized to those of GAPDH. The values represent the average of determinations from duplicate cultures. Each experimental point was repeated twice with cells prepared from a different animal with similar results.

mechanical stretch. The effects of strain magnitude were examined by varying the maximal elongation applied to the cells and assessing the mRNA levels of IGF-I. As shown in Figure 1B, mechanical stretch increases the IGF-I mRNA levels, to a relatively similar extent when the strain magnitude was varied between 2.5 and 10%. We were unable to detect significant and reproducible changes of IGF-I mRNA when the strain magnitude was set below 2.5% (data not shown). Strain levels higher than 10% were not assayed as this potentially leads to cell injury [Cheng et al., 1997].

Stretch-Mediated IGF-I mRNA Increase Is Partly Inhibited by Angiotensin Receptor Antagonists

Previous studies have suggested that angiotensin II secretion is increased in stretched cells and mediates mechanical stretch-induced cellular and molecular changes. To test whether increased angiotensin II secretion could account for stretch regulation of IGF-I mRNA levels, we pretreated the cells with either losartan (10 μM), an antagonist of angiotensin type I (AT1) receptor or saralasin (100 nM), an antagonist for both AT1 and AT2 receptors of angiotensin II, prior to the application of mechanical stretch. Incubation of our cells with losartan (10 μM) suppressed angiotensin-stimulation of IGF-I mRNA levels (data not shown). As shown in Figure 2A, the presence of either losartan or saralasin induced a 38–42% decrease of IGF-I mRNA levels but did not completely eliminate the stimulatory effect of mechanical strain. None of these antagonists alone affected the expression of IGF-I mRNA (not shown). We next tested if stretch-conditioned medium retains the ability to affect IGF-I mRNA levels. We transferred conditioned medium obtained from cells that were stretched for 8 h to cells cultured on regular culture flasks and measured the IGF-I mRNA levels after 8 h of incubation. Conditioned-medium from nonstretched cells was used as a control. As shown in Figure 2B, there was a 118% increase of IGF-I mRNA in cells treated with conditioned medium. Pretreatment of the recipient cells with losartan (10 μM) blocked the effect of the conditioned medium on IGF-I mRNA levels.

Effect of Mechanical Stretch on mRNA Levels of IGFBPs

Cultured smooth muscle cells from various tissues express mainly IGFBP-2, IGFBP-4, and

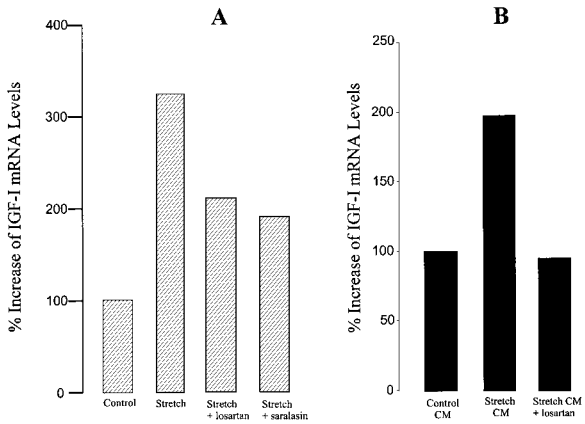

Fig. 2. Effects of angiotensin receptor antagonists on mechanical stretch-induced IGF-I mRNA levels in smooth muscle cells. **A**: Cultured cells were subjected to 5% cyclic strain for 8 h in the presence of either losartan (10 μM) or saralasin (100 nM). Total RNA was extracted and IGF-I mRNA levels were measured by RNase protection assay and normalized to those of GAPDH. **B**: Effect of stretch conditioned medium (CM) on IGF-I gene expression in the absence and in the presence of losartan (10 μM). Conditioned medium from nonstretched cells was used as control. IGF-I mRNA levels were expressed as average percentages of normalized values in controls from duplicate cultures. Each experimental point was repeated twice with cells prepared from a different animal with similar results.

IGFBP-5 [Clemmons, 1998]. To examine the effect of mechanical forces on the expression profile of these binding protein isoforms, we subjected the cells to cyclic strain for different periods of time and analyzed the mRNA levels of individual IGFBPs by Northern blot hybridization using specific probes. Cells cultured under static conditions were used as controls. The application of 5% cyclic strain induced a two-to-threefold decrease in IGFBP-2 and IGFBP-4 mRNA levels relative to those of the controls (Fig. 3A,B). Decreased mRNA levels were detected after 8 h and remained low for up to 24 h of mechanical stretching. In contrast, IGFBP-5 mRNA, while barely detectable in control cells and in cells stretched for up to 16 h, increased $250 \pm 61\%$ in 24 h-stretched cells ($P < 0.05$, $n = 4$ separate experiments). The same pattern was observed when strain magnitudes were varied from 2.5–10% (Fig. 3C).

Role of Angiotensin as a Mediator of Stretch-Induced Changes in IGFBP-2 and IGFBP-4 mRNA Levels

As previously shown for IGF-I, we sought to determine whether angiotensin II secretion in stretched cells mediates the changes in IGFBP gene expression. For this purpose, we measured

IGFBP mRNA levels (1) when the cells were pretreated with the AT1 receptor antagonist losartan prior to the application of cyclic stretch, and (2) when the cells were incubated with stretch-conditioned medium in the absence and in the presence of the AT1 receptor antagonist losartan. As shown in Figure 4A,B, in the presence of AT1 receptor antagonist, mechanical strain no longer affected either IGFBP-2 or IGFBP-4 mRNA levels. Conversely, the mRNA levels for IGFBP-5 remained comparable either in the presence or in the absence of the antagonist (Fig. 4C). The effect of stretch-conditioned medium on IGFBP mRNA levels was compared to that of conditioned-medium from nonstretched cells. Stretch-conditioned medium, when used to treat cells cultured under static conditions, mimicked mechanical stretch-induced changes on IGFBP-2 and IGFBP-4 mRNA levels (Fig. 4D,E). Their steady state mRNA levels were markedly decreased in the presence of the conditioned medium and this effect was eliminated when losartan was added to the stretch-conditioned medium. In contrast, expression of IGFBP-5 transcripts was not affected in the presence of the stretch-conditioned medium (Fig. 4F).

To further verify this pattern, cells cultured in regular flasks (i.e., not in apparatus wells) were stimulated with exogenous angiotensin II and IGFBP mRNA levels along with those for IGF-I were measured after different incubation time periods. As shown in Figure 5, exogenous angiotensin II induced a 135% increase of IGF-I mRNA above the base level. In contrast, both IGFBP-2 and IGFBP-4 mRNA levels were markedly decreased in a time-dependent manner in angiotensin II-stimulated cells. The mRNA levels for IGFBP-5 was not affected in angiotensin-treated cells.

Role of IGF-I as a Mediator of Stretch-Enhanced IGFBP-5 Gene Expression

The IGF-I is known to modulate the gene expression of IGFBP-5 at the gene transcription level. This regulatory effect is mediated through the IGF receptor and requires the activation of

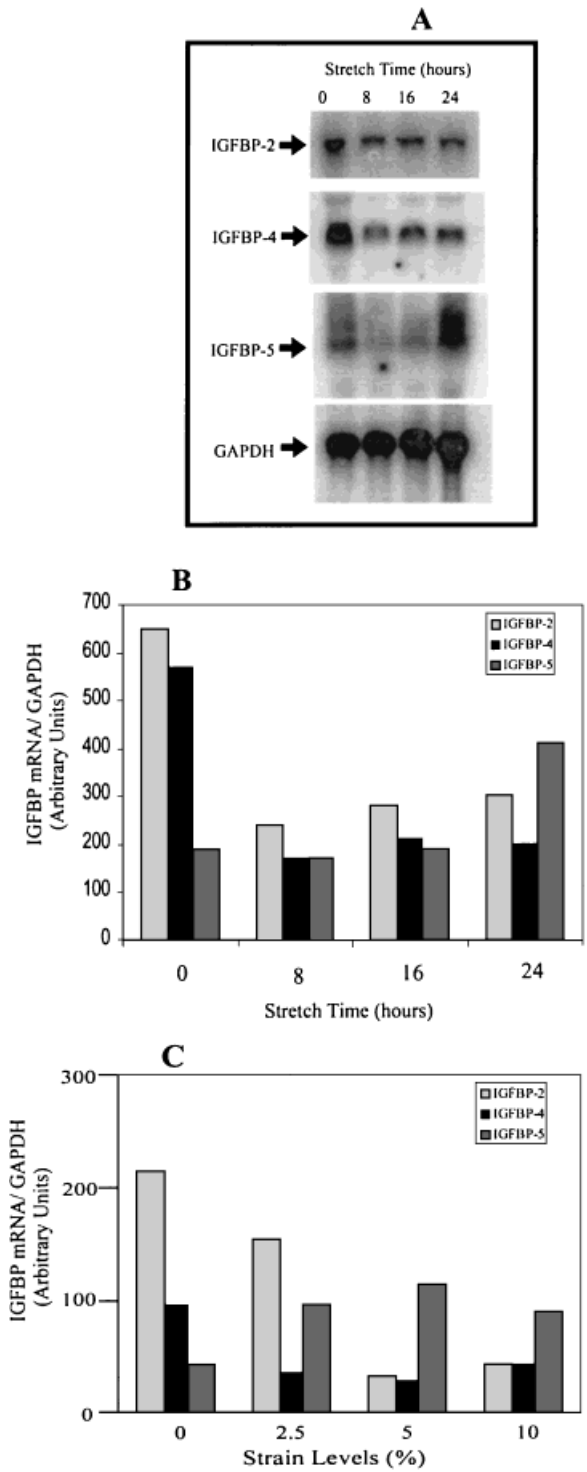

Fig. 3. Expression profile of IGFBP-2, IGFBP-4, and IGFBP-5 in smooth muscle cells subjected to cyclic stretch. **A**: Cells were subjected to 5% cyclic strain at 0.3 Hz for the indicated time periods. After completion of the stretch regimen, total RNA was isolated and analyzed by Northern blot hybridization using specific probes for IGFBP-2, IGFBP-4, and IGFBP-5. Each time point shown in the autoradiograms represents the equivalent of 13 wells. **B**: IGFBP transcript hybridization signals were scanned by densitometry and normalized to those of GAPDH. **C**: Different strain levels were applied to the cells for 24 h and the expression profile for each IGFBP was determined as described in Materials and Methods. These experiments were repeated twice with similar results.

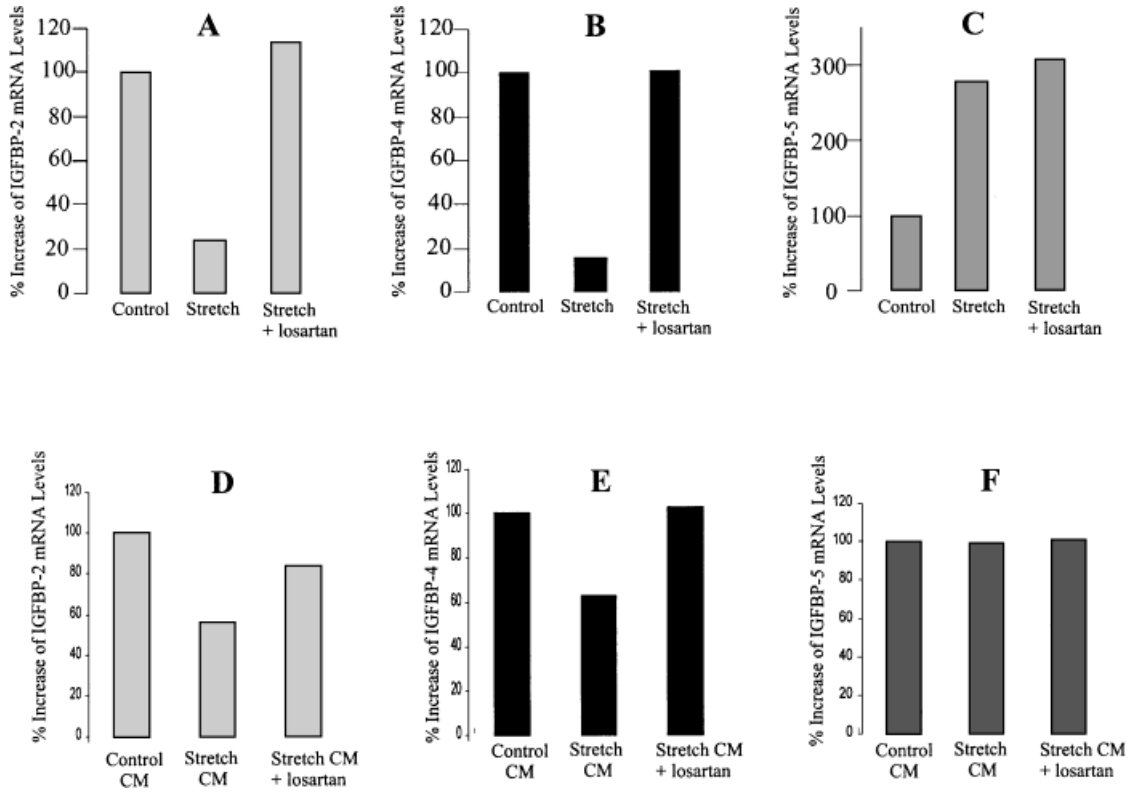

Fig. 4. Role of angiotensin II secretion in mechanical stretch regulation of IGFBP mRNA levels. The mRNA levels for IGFBP-2, IGFBP-4, and IGFBP-5 were determined either in cells subjected to 5% strain in the absence and in the presence of losartan (10 μM) (Fig. 4A–C) or in cells cultured in the presence of conditioned medium (CM) from stretched cells (Fig. 4D–F). Cells cultured under static conditions and conditioned medium from nonstretched cells were used as controls. IGFBP mRNA levels were evaluated by scanning densitometry of the Northern blot hybridization signals and expressed as average percentages of normalized values in controls from duplicate cultures. These experiments were repeated twice with cells prepared from a different animal with similar results.

the phosphatidylinositol 3-kinase-pKB/Akt-p70s6k pathway [Bo et al., 1998; Duan et al., 1999]. In addition, the expression pattern of IGFBP-5 in stretched cells is typical of genes whose expression is not induced as a result of the primary stimulus [Chien et al., 1998]. As the inhibition of angiotensin II transduction pathway was not involved in stretch-induced IGFBP-5 mRNA levels, we tested whether this action is mediated through the IGF-I receptor signaling pathway. For these experiments, smooth muscle cells were stretched for 24 h in the presence of increasing concentrations of IGF-I neutralizing antibodies or in the presence of specific inhibitors for the PI 3-kinase in order to inhibit pharmacologically the IGF-I receptor signaling events. As shown in Figure 6, IGFBP-5 transcript levels were substantially reduced in the presence of IGF-I antibodies in a concentration-dependent manner. The presence of nonrelevant antibodies (mouse IgG) did not affect stretch regulation of IGFBP-5 transcription levels. In similar experiments, PI 3-kinase inhibitors, wortmannin (20 μM) or quercetin (20 μM), were added to the medium prior to application of mechanical stretch. The presence of any one of these inhibitors completely eliminated the effects of stretch on IGFBP-5 gene expression indicating that stretch activation of IGFBP-5 gene is mediated through the IGF-I activation of the transducing signals via the PI 3-kinase pathway. For comparison purposes, incubation of cultured cells with exogenous IGF-I (10 ng/ml) induced a 2.5-fold increase of IGFBP-5 mRNA levels.

Effect of Mechanical Stretch and Stretch-Conditioned Medium on Bladder Smooth Muscle Cell Proliferation

We determined the effects of mechanical stretch on DNA synthesis by performing a tritiated thymidine incorporation assay. Cells were subjected to 5% strain for 24 h in the presence of tritiated thymidine that was added

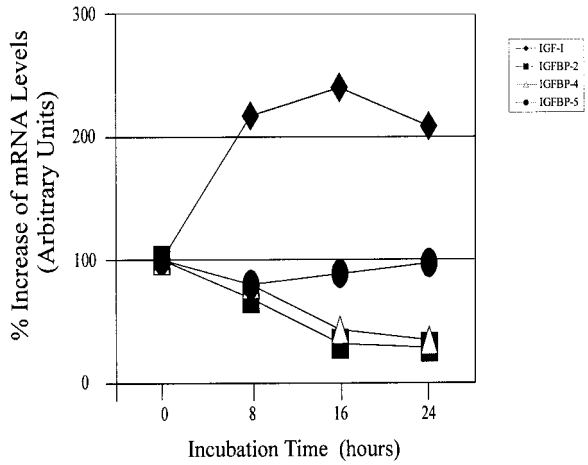

Fig. 5. Effects of exogenous angiotensin II on mRNA levels of IGF-I, IGFBP-2, IGFBP-4, and IGFBP-5. Smooth muscle cells were incubated in serum-free medium with angiotensin II (100 ng/ml) for the indicated time periods. Total RNA was extracted and assayed for mRNA levels of either IGF-I by RNase protection assay or IGFBP-2, IGFBP-4, and IGFBP-5 by Northern blot hybridization. Shown is the percentage of mRNA increase or decrease after normalization of the hybridization signals to those of GAPDH. The values represent the average of determinations from duplicate cultures. These experiments were repeated twice using different cell preparations with similar results.

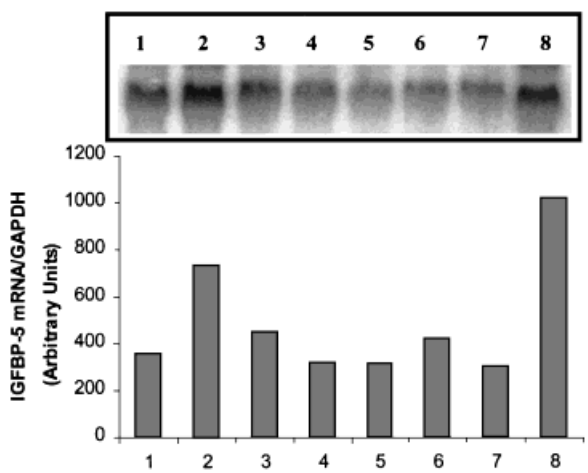

Fig. 6. Role of IGF-I secretion in stretch regulation of IGFBP-5 mRNA levels. The IGFBP-5 mRNA levels were measured in cells that were subjected to cyclic strain for 24 h in the presence of either anti-IGF-I antibodies or specific inhibitors of PI 3-kinase, wortmannin, or quercetin. **Lane 1**: control. **Lane 2**: stretched cells. **Lanes 3, 4,** and **5**: stretched cells in medium containing anti-IGF-I antibodies at 1/500, 1/1000, and 1/2000 dilutions, respectively. **Lanes 6** and **7**: stretched cells in medium containing wortmannin (20 μM) or quercetin (20 μM), respectively. **Lane 8**: cells cultured in regular flasks in serum-free medium containing 10 ng/ml recombinant IGF-I. The values represent the average of determinations from duplicate cultures. These experiments were repeated twice using different cell preparations with similar results.

during the last 8 h. Cells cultured under static conditions were used as control. As shown in Figure 7A, mechanical stretch induced a $85 \pm 23\%$ increase in thymidine incorporation ($P < 0.05$, $n = 6$). Pretreatment of the cells with either neutralizing antibodies to IGF-I (1:500 dilution) or losartan (10 μM), did not significantly affect the level of thymidine incorporation. We further tested the mitogenic activity of stretch-conditioned medium by testing conditioned-medium either obtained from cells that had been stretched for 24 h or from non-stretched control cells. As shown in Figure 7B, incubation with stretch-conditioned medium for 24 h did not change the thymidine incorporation levels. Pretreatment of the cells with either anti-IGF-I antibodies or losartan (10 μM) did not significantly affect thymidine incorporation levels. For comparison, cells cultured in regular dishes were treated for 24 h with either recombinant IGF-I or exogenous angiotensin II in serum-free medium and the new DNA synthesis was assessed by thymidine incorporation (Figure 7C). Cells cultured in serum-free medium were used as control. While IGF-I alone induced a $241 \pm 71\%$ increase in thymidine incorporation ($P < 0.05$), exogenous angiotensin II had no effect.

DISCUSSION

The in vivo mechanical cues in distensible tissues like the bladder wall are defined by a broad spectrum of coupled physical factors that can act on the cells and modulate their activities. These factors include hydrostatic pressure, fluid flow, muscle contraction, tissue stretch or distension, electrical currents, and the physicochemical environment. While it would be optimal to study the mechanical effects on cells in their native in vivo environment, interpretation of such studies is limited by the inability to either directly control or accurately measure these mechanical parameters. Studies like ours in which mechanical strain is imposed on cultured cells provide insights not only on important aspects of the physical factors involved in vivo but also on the molecular mechanisms involved in the transduction of the initial force signal. Conceptually, mechanical strain acts on the cellular membrane components to activate putative mechanoreceptors such as integrins, ion channels, G-proteins and receptor tyrosine kinases

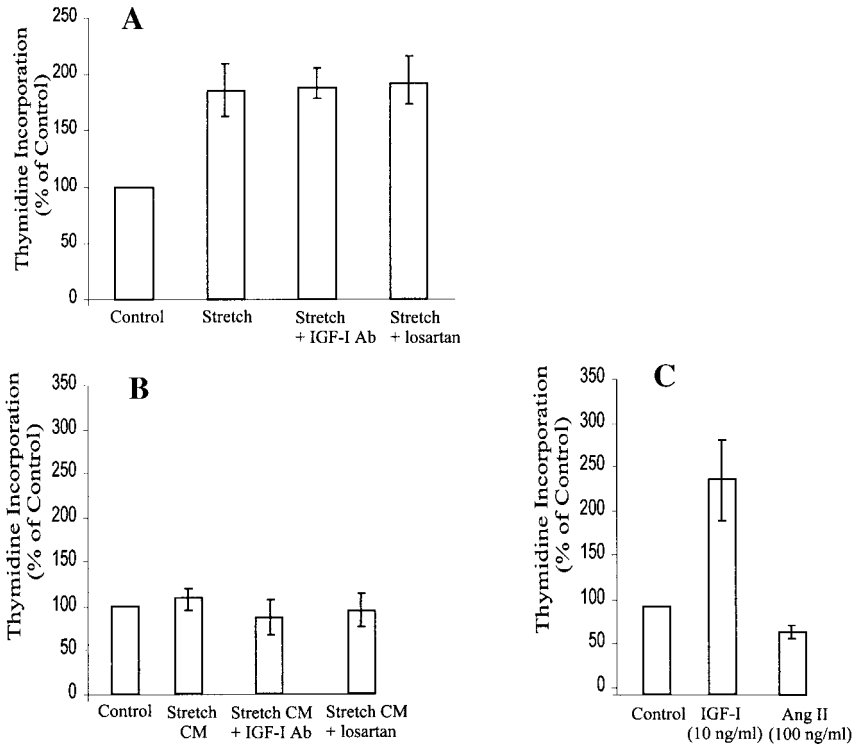

Fig. 7. Effects of mechanical stretch and stretch-conditioned medium on DNA synthesis in bladder smooth muscle cells. **A**: Cells were subjected to cyclic strain for 24 h either in the absence or in the presence of either anti-IGF-I antibodies (IGF-I Ab) at 1/500 dilution or losartan (10 μM). The culture medium in all wells was supplied with 1 μCi/ml of [methyl-^{3}H] thymidine during the last 8 h. Thymidine incorporation was determined as described in Materials and Methods. **B**: Effects of stretch conditioned medium (CM) on DNA synthesis was assessed by transferring the medium obtained from cells stretched for 24 h to cells cultured in regular culture dishes with [methyl-^{3}H] thymidine added during the last 8 h. Groups of recipient cells were treated with either anti-IGF-I antibodies at 1/500 dilution or losartan (10 μM) and thymidine incorporation was assessed as previously described. **C**: For comparison purposes, the effects of recombinant IGF-I or exogenous angiotensin II (Ang II) on DNA synthesis was assessed as well. The results are expressed as the percentage change from the controls. Values are means ± SE (n = 6 for each group). These experiments were repeated twice using different cell preparations with similar results.

[Banes et al., 1995; Chaqour et al., 1999b; Chiquet, 1999]. This mechanochemical transduction involves the activation of multiple signaling pathways and the production of second messenger molecules. The latter have been linked to the regulation of the expression of specific genes whose encoded proteins affect cell structure and function.

Our present study is the first to show a direct link between mechanical strain and IGF-I gene expression in cultured bladder smooth muscle cells. Previous studies have demonstrated such an effect mainly in skeletal muscle either in vitro or ex-vivo where increased IGF-I peptide secretion was reported [Perrone et al., 1995; McKoy et al., 1999]. This increase, however, may result from the release of already synthesized IGF-I, rather than newly synthesized IGF-I.

In bladder smooth muscle cells, the application of 5% strain induced a gradual increase of IGF-I mRNA after 8 h of cyclic stretch that was six-to-eightfold higher in comparison to control levels after 24 h. This expression pattern is similar to that of heparin-binding epidermal growth factor (HB-EGF) in rat bladder smooth muscle cells upon mechanical stretch stimulation [Park et al., 1998]. Our data also indicate that there is a strain threshold of 2–2.5% strain above which substantial changes in IGF-I mRNA levels can be detected. This result parallels those of Yang et al. [1998], which showed that vascular smooth muscle cells do not induce platelet-derived growth factor (PDGF) gene expression in response to small magnitudes of mechanical strain. Similarly, previous data from our laboratory have shown that bladder smooth muscle cells alter their synthetic phenotype only when subjected to 2.5% strain level or higher [Baskin et al., 1993]. Taken together, these observations support the hypothesis that the cells most likely respond to

changes in the force equilibrium rather than to the magnitude of an externally applied strain [Galbraith and Sheetz, 1998]. Therefore, it appears that IGF-I gene is highly responsive to changes in the mechanical demands in both smooth and skeletal muscle in which sustained and active mechanical stimulation leads invariably to muscle hypertrophy/hyperplasia while decreased load force generation or immobilization leads to atrophy. It is likely that this growth factor—by virtue of its regulatory role in controlling cell growth, survival and cell synthetic phenotype—plays an important role in mediating the biological effects of mechanical forces.

Accumulating evidence suggests that secretion of humoral and/or neuronal factors, possibly angiotensin II, by stretched cells plays an important role in stretch regulation of cellular and molecular changes [Sadoshima et al., 1993]. Tamura et al. [1998] have reported 20-fold-increase of angiotensinogen gene expression and angiotensin II secretion in cardiomyocytes subjected to mechanical stretch. In smooth muscle cells, angiotensin II secretion is increased in response to mechanical stretch and in vivo studies showed that increased bladder wall stretch/strain, in response to outlet obstruction induces the local production of angiotensin II in the smooth muscle layer [Cheng et al., 1999]. Angiotensin II is known as a trophic agent inducing smooth muscle cell hypertrophy/hyperplasia and increasing extracellular matrix protein production via an autocrine and/or paracrine pathway. In smooth muscle cells, angiotensin II acts through the AT1 subtype receptors and modulates the expression of an array of more than 20 genes including that of IGF-I [Delafontaine and Lou, 1993; Pratt, 1999].

Evidence of angiotensin II secretion by stretched smooth muscle cells and its potential role in stretch regulation of IGF-I mRNA levels is shown in our experiments. Incubation of cells cultured under static conditions with conditioned medium from stretched cells induced an almost twofold increase in IGF-I mRNA. This increase was eliminated in the presence of the AT1 receptor antagonist losartan. When cells were subjected to 5% strain in the presence of losartan, the AT1 receptor antagonist or saralasin, the antagonist of both AT1 and AT2 receptors, IGF-I mRNA accumulation was consistently decreased (30–40%) but was not completely suppressed in stretched cells. The concentrations of the angiotensin II-receptor antagonists used in these experiments were shown to be sufficient to completely block angiotensin receptor-transduced signals in cardiomyocytes and in rat bladder smooth muscle cells [Yamazaki et al., 1995; Park et al., 1998]. This indicates that strain-induced IGF-I mRNA is partially mediated through an AT1-receptor pathway. However, it is noteworthy that preincubation of the cells with either losartan or saralasin, affects mainly the receptors localized on the plasma membrane, whereas those located within the cell (e.g., nuclear) remains accessible to angiotensin II produced intracellularly. However, it is unlikely that the latter process alone accounts for the substantial increase of IGF-I mRNA in stretched cells.

The role of other humoral or growth factors in stretch-induced IGF-I gene expression should also be considered. Application of mechanical stretch was shown to increase the expression of PDGF, bFGF, and TGF-β in different cell types [Banes et al., 1995]. However, results on the effects of these factors on IGF-I mRNA levels are very inconsistent and controversial. PDGF and bFGF have been reported to induce either an increase or a decrease of IGF-I mRNA levels in rat aortic smooth muscle cells [Arnqvist et al., 1995]. The discrepancy among different reports may result either from differences in cultured cell density, the concentration of the growth factor used or the ability of those growth factors to activate specific transduction events.

As the biological activity of IGF-I can be modulated by IGF-binding proteins, we also examined their expression at the mRNA levels. Our results showed that mechanical strain is a novel major variable that regulates the gene expression levels of IGFBPs and that secreted angiotensin II plays a key role in the regulatory mechanism. First, we found that IGFBP-2 and IGFBP-4 transcript levels were significantly decreased in stretched cells. In the presence of the AT1 receptor inhibitor, losartan mechanical regulation of IGFBP-2 and IGFBP-4 was inhibited, thus suggesting that mechanical strain regulates IGFBP-2 and IGFBP-4 mRNA levels through an angiotensin-dependent pathway. Similar results were obtained when the cells were pretreated with saralasin (data not shown). Interestingly, incubation of cells with exogenous angiotensin II mimicked mechanical

strain effects on IGFBP-2 and IGFBP-4 transcript levels. In agreement with these results, studies by Arnqvist et al. [1995] and Delafontaine et al. [1993] recently reported a negative effect of angiotensin II on IGFBP-2 and IGFBP-4 gene expression in aortic and vascular and smooth muscle cells respectively [Gustafsson et al., 1999; Anwar et al., 2000]. Another study showed a trend toward a decrease of IGFBP-4 gene expression in normotensive aortas in coarcted animals characterized by high levels of angiotensin II as compared to normotensive aortas with normal levels of angiotensin II [Anwar and Delafontaine, 1994]. Thus, our data, as well as those from other groups, suggest that humoral factors (angiotensin II) and/or mechanical factors (increased pressure) regulate IGFBP-4 mRNA levels in vivo and in vitro.

In our study, stimulation of the cells by either cyclic stretch or exogenous angiotensin II affected IGFBP-2 and IGFBP-4 transcripts levels only after 6–8 h. This time course is consistent with a delayed type of gene expression pattern and may coincide with upregulation of the angiotensinogen gene that peaks after 6 h of exposure of the cells to either mechanical stretch or angiotensin II [Tamura et al., 1998]. However, an indirect mode of action of these stimuli cannot be excluded. Both angiotensin and mechanical strain are known to modulate the expression of several humoral and growth factor genes whose encoded proteins potentially regulate the transcript levels of IGFBPs (Chiquet, 1999; Pratt, 1999]. At present, the negative regulators of IGFBP-2 and IGFBP-4 are largely unknown. Only retinoic acid has been reported to definitely reduce IGFBP-2 gene expression in human neuroblastoma cells [Chambery et al., 1998]. A potential negative feedback loop where the endogenously produced IGF-I regulates IGFBP-2 and IGFBP-4 transcript is unlikely as exogenous IGF-I has no such an effect (data not shown). IGF-I has been reported to produce no effects or a small decrease in IGFBP-2 and IGFBP-4 mRNA levels in several cell lines [Mohan et al., 1992; Gustafsson et al., 1999]. In agreement with these in vitro observations, in vivo studies demonstrated that targeted overexpression of IGF-I in specific smooth muscle tissues was not associated with any particular changes in tissue IGFBP-2 or IGFBP-4 transcript levels [Wang et al., 1997].

In contrast to the negative regulation of IGFBP-2 and IGFBP-4 by mechanical strain, the transcript levels of IGFBP-5 were increased in stretched cells. These changes were not affected by angiotensin receptor antagonism. Previous studies have shown that the gene expression of IGFBP-5 is mainly regulated by IGF-I in smooth muscle cells [Duan and Clemmons, 1998; Bo et al., 1998]. In stretched smooth muscle cells, the fact that increased IGFBP-5 mRNA levels occur consistently after 16–24 h of cell stretching prompted us to investigate the role of the endogenously produced IGF-I in stretch regulation of IGFBP-5 mRNA levels. By using neutralizing IGF-I antibodies, we found that IGFBP-5 mRNA levels in stretched cells decreased below those of the control cells indicating that IGFBP-5 mRNA is regulated by the neo-synthesized IGF-I. Surprisingly, neither conditioned medium from stretched cells, that would presumably be enriched in IGF-I, nor exogenous angiotensin II, that modestly increases IGF-I gene expression, affected IGFBP-5 mRNA levels. Therefore, if the stretch-induced autocrine production of IGF-I is involved in the regulation of IGFBP-5 mRNA levels, it must be either more biologically active or more accessible to the IGF-I receptor than the IGF-I present in the conditioned medium. We cannot, however, fully exclude the possibility that the IGF-I peptide, once secreted by stretched cells, was either rapidly degraded or immediately shunted to the cell membrane or the extracellular matrix.

Studies using different cell models have shown that a complex network of signaling events are activated upon IGF-I binding to the IGF receptor [Stewart et al., 1996; Imai and Clemmons, 1999]. In smooth muscle cells, IGF-I receptor signaling pathway involves an autophosphorylation of the receptor itself which, in turn, tyrosine phophorylates the insulin receptor substrate-1 (IRS-1). The latter acts then as a docking protein providing binding sites for diverse groups of cytosoloic proteins containing SH2 domains including the p85 subunit of PI 3-kinase. Thus, activation of IRS-1 leads invariably to the activation of PI 3-kinase also known for its important role in transmitting the anti-apoptotic and differentiation signals of IGF-I. Acting further downstream in this pathway is the activation of PKB/Akt and p70s6k that mainly activates several nuclear proteins in-

volved in the transcriptional regulation of specific genes.

Using the porcine vascular smooth muscle cell model, Duan et al. [1999] have shown that activation of PI 3-kinase is required for the action of IGF-I on IGFBP-5 gene expression. We tested this hypothesis in our cellular model by stretching the cells in the presence of specific inhibitors of the PI 3-kinase, i.e., wortmannin, quercetin. The latter were used at a concentration that effectively inhibits IGF-I-stimulated PI 3-kinase activity. We found that these inhibitors blocked stretch stimulation of IGFBP-5 gene expression suggesting that activation of PI 3-kinase may be required for the stretch regulation of IGFBP-5. This effect is specific to the PI 3-kinase inhibitors as the utilization of other inhibitors, that interfere either with the cytoskeleton machinery or the MAP kinase pathway, did not affect IGFBP-5 mRNA levels in response to stretch (data not shown).

Overall, our study showed that upregulation of IGF-I by mechanical stretch is accompanied by discordant alteration in gene expression of its binding proteins. IGFBP-2 and IGFBP-4 are decreased while IGFBP-5 is increased. While stretch effects on IGFBP-2 and IGFBP-4 are mediated via an angiotensin-dependent pathway, those for IGFBP-5 are secondary to the putative autocrine secretion of IGF-I. At the functional level, these changes could drastically affect the bioactivity of IGF-I for binding to its receptor. In several cultured cell systems, IGFBP-2 and IGFBP-4 were invariably found to be growth inhibitory, presumably because they are present exclusively as freely soluble proteins that sequesters IGF-I [Ferry et al., 1999]. On the other hand, secreted IGFBP-5 is predominantly bound to the extracellular matrix and to heparin-sulfate proteoglycan [Jones et al., 1993: Kalus et al., 1998]. This leads to a substantial decrease of IGFBP-5 affinity for IGF-I, thereby, increasing the local concentration of IGF-I immediately adjacent to the cell. Thus, decreased expression levels of IGFBP-2 and IGFBP-4 and increased IGFBP-5 expression levels should lead ultimately to an enhancement of the biological actions of IGF-I in mechanically stimulated cells.

Mechanical stretch induced a 155% increase in tritiated thymidine incorporation as compared to nonstretched cells which correlates well with previous reports [Park et al., 1998; Perrone et al., 1995]. However, neither pretreatment of the cells with an angiotensin receptor antagonist nor neutralizing antibodies to IGF-I (1:500 dilution) significantly alter this response. When angiotensin II and IGF-I were tested individually on cells cultured in regular flasks, IGF-I induced a potent stimulation of DNA synthesis while angiotensin II did not. It has been suggested that angiotensin by itself is a weak mitogenic factor but its effects become more pronounced when combined with other factors such as different extracellular matrices [Sudhir et al., 1993]. Meanwhile, stretch-conditioned medium failed to have a significant stimulatory effect of DNA synthesis in cells cultured in regular flasks and the presence of angiotensin antagonist in the conditioned-medium did not affect the cell response.

There are several potential reasons that explain the latter observations: (1) It is uncertain whether growth factors like IGF-I, once secreted, are present in an intact and active form in stretch-conditioned medium or whether they are immediately shunted to the extracellular matrix or the plasma membrane and eventually internalized and degraded. (2) There are likely multiple cellular factors implicated in cell growth in response to stretch and angiotensin II, and IGF-I may only be one of these regulatory factors. Mitogenic factors like parathyroid hormone-related-peptide and HB-EGF are induced solely by the application of cyclic stretch to rat bladder smooth muscle cells [Yamamoto et al., 1993; Park et al., 1998]. The work of Wilson et al. [1993] emphasized the role of platelet-derived growth factor in mechanical stretch-induced growth of rat neonatal aortic smooth muscle cells. The recent work of Iwasaki et al. [2000] indicates that epidermal growth factor receptor (EGFR) activation by stretch triggers vascular smooth muscle cell growth and suggests that the PDGF receptor is activated only when the targeted cells lack EGFR. Overall, these studies indicate that a vast array of potent growth factors are induced in response to mechanical stimulation and that IGF-I may have only a minor role in the overall growth response of the cells. (3) There is certainly a temporal regulation of these growth factors in response to mechanical forces. While the expression of genes like PDGF-A and HB-EGF is transient and returns to baseline within hours (1–4 h), the expression of other genes like TGF-β and IGF-I as shown here is delayed and long-lasting (6–24 h) and thus, may affect

the phenotypic features of the cells in the long term. This is also compatible with a model of progressive changes in cellular processes in which different growth factors act at discrete stages [Chien et al., 1998]. Therefore, additional studies are needed to analyze the potential cellular processes that are regulated by IGF-I in stretched cells including the changes in the cell synthetic phenotype, differentiation state, and survival. Our data are significant in that they emphasize the potential regulatory role of mechanical strain on IGF-I and IGFBP gene expression in bladder smooth muscle cells albeit along with many other factors. Additional studies are needed to further analyze the cellular processes that are regulated by IGF-I in stretched cells including the changes in the cell synthetic phenotype, differentiation state, and survival.

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