Cyclic stretch-induced TGF-β1 and fibronectin expression is mediated by β1-integrin through c-Src- and STAT3-dependent pathways in renal epithelial cells

Mona T. Hamzeh, Rashmi Sridhara, and Larry D. Alexander

1Midwestern University, Arizona College of Osteopathic Medicine, Department of Physiology, Glendale, Arizona; and 2Department of Biology, Division of Natural Sciences, University of Michigan-Dearborn, Dearborn, Michigan

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Hamzeh MT, Sridhara R, Alexander LD. Cyclic stretch-induced TGF-β1 and fibronectin expression is mediated by β1-integrin through c-Src- and STAT3-dependent pathways in renal epithelial cells. Am J Physiol Renal Physiol 308: F425–F436, 2015. First published December 4, 2014; doi:10.1152/ajprenal.00589.2014.—Extracellular matrix (ECM) proteins, including fibronectin, may contribute to the early development and progression of renal interstitial fibrosis associated with chronic renal disease. Recent studies showed that β1-integrin is associated with the development of renal fibrosis in a murine model of unilateral ureteral obstruction (UUO). However, the molecular events responsible for β1-integrin-mediated signaling, following UUO, have yet to be determined. In this study, we investigated the mechanism by which mechanical stretch, an in vitro model for chronic obstructive nephropathy, regulates fibronectin and transforming growth factor-β1 (TGF-β1) expression in cultured human proximal tubular epithelium (HK-2) cells. Mechanical stretch upregulated fibronectin and TGF-β1 expression and activated signal transducer and transcription factor 3 (STAT3) in a time-dependent manner. Stretch-induced fibronectin and TGF-β1 were suppressed by a STAT3 inhibitor, S3I-201, and by small interfering RNA (siRNA) targeting human STAT3 (STAT3 siRNA). Similarly, fibronectin and TGF-β1 expression and STAT3 activation induced by mechanical stretch were suppressed by the Src family kinase inhibitor PP2 and by transfection of HK-2 cells with a dominant-negative mutant of c-Src (DN-Src). Furthermore, mechanical stretch resulted in increased β1-integrin mRNA and protein levels in HK-2 cells. Furthermore, neutralizing antibody against β1-integrin and silencing of β1-integrin expression with siRNAs resulted in decreased c-Src and STAT3 activation and TGF-β1 and fibronectin expression evoked by mechanical stretch. This work demonstrates, for the first time, a role for β1-integrin in stretch-induced renal fibrosis through the activation of c-Src and STAT3 signaling pathways.

A hallmark of obstructive nephropathy and a major factor in the progressive loss of renal function in patients is excessive extracellular matrix (ECM) accumulation, especially fibronectin, of the tubulointerstitial compartment, leading to tubulointerstitial fibrosis. Mechanical stretching of tubular epithelium, caused by retrograde pressure shifts and urinary polluting, is regarded as highly significant in the progression of obstructive nephropathy. In fact, cyclic stretch has been used in vitro to mimic the changes in intrarenal pressure in unilateral ureteral obstruction (UUO) (16, 17, 51, 52), a well-established experimental model of renal inflammation and fibrosis that encompasses many aspects of obstructive nephropathy. In this in vitro model, a number of signaling molecules, including cytosolic PLA2 (cPLA2), MAPK family members, ERK1/2, the EGF receptor (EGFR), and the nonreceptor tyrosine kinase c-Src, are activated by mechanical stretch (1). In addition, the activation of ERK1/2 is abrogated by the Src family kinase inhibitor 3-(4-chlorophenyl)-1-(1,1-dimethylylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) and by transfection of proximal tubule cells with a dominant negative mutant of c-Src (DN-Src), indicating that c-Src is critical for stretch-induced ERK1/2 activation in renal proximal tubular cells (1). Moreover, studies carried out in vitro have recently shown that mechanical stretch induces transforming growth factor (TGF)-β1 expression in renal proximal tubular cells (35, 41, 54, 57). However, the molecular mechanisms by which mechanical stretch contributes to TGF-β1 production leading to renal fibrosis are not yet completely understood.

A principal mechanism whereby UUO induces renal fibrosis may involve induction of integrins. Integrins are a broad family of cell surface adhesion and signaling molecules, consisting of an α-subunit and a β-subunit, which link the ECM to the cytoskeleton. Among the members of the integrin family, β1-integrin is the most critical one given that β1-integrin can pair with different α-subunits, making it become a receptor for many types of stimuli, and it is expressed in renal tubular cells (4, 18, 20, 38, 61). Integrins can also function as force sensors, transducing mechanical stimuli into biochemical signals. Under normal conditions, integrins are critical for maintaining cellular homeostasis, triggering a number of signaling pathways, some of which are primarily related to cell migration and cell adhesion, whereas others provide signaling to the cells that regulate cellular differentiation, proliferation, survival, and apoptosis. Conversely, under pathological conditions, integrins are associated with a wide variety of renal pathologies including, but not limited to, obstructive nephropathy (4, 9, 28, 38, 53, 70, 72). In this context, Yeh et al. (70) recently demonstrated that β1-integrin gene and protein expression was significantly upregulated in UUO mice. This was accompanied by correspondingly elevated tubular expression of TGF-β1. Along these lines, blocking of β1-integrin signals by treatment with an antibody to β1-integrin reduced TGF-β1 levels and ameliorated fibrosis, demonstrating strong correlations between the expression of β1-integrin within the tubulointerstitium and the presence of tubulointerstitial fibrosis (70). However, little is known about the role of β1-integrin in the pathogenesis of renal fibrosis induced by cyclic mechanical stretch. Moreover,
the molecular mechanism of β1-integrin-mediated renal fibrosis has not been studied. Because knockout of β1-integrin in mice is embryonically lethal (19, 58) and the limitation of a β1-integrin-blocking antibody in vivo, experiments on cultured proximal tubular cells will most likely provide more mechanistic information on the serial steps of β1-integrin-induced signal transduction. Given the increased expression of β1-integrin during tubulointerstitial fibrosis, and because it is the most prevalent β-chain of the heterodimers expressed in the kidney (32), we hypothesized that β1-integrin induction may contribute to fibrogenic renal disease. Using an in vitro model of UUO, we sought to determine: 1) whether cyclic mechanical stretch induced the expression of extracellular matrix (e.g., fibronectin) and profibrotic (e.g., TGF-β1) protein expression; 2) the effect of β1-integrin disruption and blockade on mechanical stretch-induced fibronectin and TGF-β1 protein expression; and 3) the potential mechanisms of β1-integrin-mediated renal fibrosis. In this study, we demonstrate for the first time that cyclic mechanical stretch induces the activation of signal transducer and transcription factor 3 (STAT3) and expression of TGF-β1 and fibronectin and that blocking or knockdown of STAT3 abrogated these effects. Similar results were obtained following pharmacological inhibition or overexpression of dominant-negative mutants of c-Src. Furthermore, we demonstrate for the first time that mechanical stretch upregulates β1-integrin gene and protein expression, and blocking or knockdown of β1-integrin signaling abrogated stretch-induced c-Src and STAT3 activation and TGF-β1 and fibronectin expression in HK-2 cells. These results suggest that β1-integrin is important for modulating renal fibrosis through c-Src/STAT3 signaling pathways and enhances our understanding of mechanotransduction pathways leading to fibrosis of proximal tubule epithelial cells.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human TGF-β1 and TGF-β1 ELISAs were purchased from R&D Systems (Minneapolis, MN). PP2 and 1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP3) were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against p-c-Src, c-Src, p-SAT3, and STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β1-integrin and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β1-integrin 4B4 was purchased from Coulter (Hialeah, FL). Anti-rabbit (goat) and anti-mouse (goat) horseradish peroxidase (HRP)-conjugated IgG antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and additives were purchased from Invitrogen (Grand Island, NY). All other chemicals were of best available quality, usually analytic grade.

Cell culture. Human renal proximal epithelial (HK-2) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F-12 medium containing insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (100 μg/ml), and streptomyacin (100 μg/ml) supplemented with 10% fetal calf serum. Cells were used for experiments at passages 8–15 and rendered quiescent in media containing 0.5% FCS for 24 h before treatment with cyclic mechanical stretch or TGF-β1.

Cyclic mechanical stretch. For studies involving mechanical stretch, differentiated HK-2 cells were seeded onto commercially available silastic six-well collagen I-coated stretch plates (Flexcell, Hillsborough, NC) for 3 days. After being serum-starved for 24 h, culture medium was replaced with new serum-free medium. The culture plates were placed on vacuum-based loading docks of the Flexcell FX-4000T apparatus (Flexcell) in the incubator and subjected to pulsatile mechanical stretch (10–20% of equibiaxial elongation) at a frequency of 0.1 Hz. Previous reports have shown that these parameters induce a significant difference in TGF-β1 secretion between nonstretched renal proximal tubule cells and stretched cells (41, 42, 54, 57). Nonstretched cells (control) were exposed to identical experimental conditions but without mechanical stretch. To assess the effects of the indicated inhibitors, drugs were added to cells 30 min before stimulation with cyclic mechanical stretch.

![Fig. 1. Mechanical stretch increased the expression of fibronectin in HK-2 cells.](http://ajprenal.physiology.org/)

Fig. 1. Mechanical stretch increased the expression of fibronectin in HK-2 cells. A: HK-2 cells are treated with cyclic stretch (20% elongation, 6 cpm) for the indicated time period (A) or with indicated levels of stretch for 48 h (B). Expression of fibronectin was determined by Western blotting, and respective quantitation analysis for expression fibronectin was performed (A and B). β-Actin was used to verify equivalent loading. Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 6 independent experiments. *P < 0.05, **P < 0.01 vs. no stretch (control) cells.)
Transient transfections. Commercially available small interfering RNA (siRNA) of Smartpool siRNA for human β1-integrin, human STAT3, and negative control (scramble) siRNA were purchased from Santa Cruz Biotechnology. Briefly, HK-2 cells were transfected with either 100 nM of siRNA targeting human β1-integrin (siRNA β1), human STAT3 (siRNA STAT3), or with the same amount of control (scramble) siRNA (siRNA scramble), or with 10 μg of dominant-negative plasmid of c-Src or with the same amount of empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours posttransfection, cells were stimu-

Fig. 2. Effects of S3I-201 on mechanical stretch-induced signal transducer and transcription factor 3 (STAT3) activation and fibronectin expression. HK-2 cells serum-starved for 24 h were exposed to mechanical stretch (20% elongation, 6 cpm) for the indicated times (A) or pretreated without or with the STAT3 inhibitor S3I-201 (50 μmol/l) for 1 h before treatment with mechanical stretch (20% elongation, 6 cpm) for 15 min (B) or for 48 h (C). Immunoblotting with specific antibodies against p-STAT3 (Tyr705), STAT3, fibronectin, or β-actin and respective quantitation analysis for expression of STAT3 and fibronectin were performed. Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 5 independent experiments. **P < 0.01, ***P < 0.001 vs. no stretch (control) cells. **P < 0.01, ***P < 0.001 vs. S3I-201-treated cells with stretch.

Fig. 3. Ablation of STAT3 decreases cyclic stretch-induced fibronectin expression. HK-2 cells were transfected with 100 nM of small interfering (si) RNA specific for human STAT3 (siRNA STAT3) or with the same amount of scrambled siRNA (siRNA scramble), or with 10 μg of dominant-negative plasmid of c-Src or with the same amount of empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours posttransfection, cells were stimu-

A

B

C

Trans. f. S3I-201 on mechan. stretch-induced signal transducer and transcription factor 3 (STAT3) activ. and fibronectin expression. HK-2 cells serum-starved for 24 h were exposed to mechanical stretch (20% elongation, 6 cpm) for the indicated times (A) or pretreated without or with the STAT3 inhibitor S3I-201 (50 μmol/l) for 1 h before treatment with mechanical stretch (20% elongation, 6 cpm) for 15 min (B) or for 48 h (C). Immunoblotting with specific antibodies against p-STAT3 (Tyr705), STAT3, fibronectin, or β-actin and respective quantitation analysis for expression of STAT3 and fibronectin were performed. Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 5 independent experiments. **P < 0.01, ***P < 0.001 vs. no stretch (control) cells. **P < 0.01, ***P < 0.001 vs. S3I-201-treated cells with stretch.

Trans. f. Ablation of STAT3 decreases cyclic stretch-induced fibronectin expression. HK-2 cells were transfected with 100 nM of small interfering (si) RNA specific for human STAT3 (siRNA STAT3) or with the same amount of scrambled siRNA (siRNA scramble), or with 10 μg of dominant-negative plasmid of c-Src or with the same amount of empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours posttransfection, cells were stimu-

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lated with indicated stimuli or vehicle, lysed, and analyzed for β1-integrin expression by Western blotting with a rabbit anti-β1-integrin polyclonal antibody, and with phospho-specific anti-rabbit polyclonal antibodies for c-Src and STAT3. Blots were stripped and reprobed with a mouse monoclonal c-Src, STAT3, or β-actin antibody to control for protein loading and for silencing efficiency and specificity.

Western blot analysis. Western blot analysis was carried out as previously described (1, 2). Briefly, proteins were extracted with buffer containing 50 mM Tris, pH 7.2, 1% (vol/vol) Triton X-100, 1 mM Na3VO4, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 10 μg/ml aprotinin. Whole-cell lysate of treated cells was subjected to 4–20% SDS-PAGE. The fractionated proteins were transferred onto nitrocellulose membranes, which were then incubated with various primary antibodies, and target proteins were detected by enhanced chemiluminescence (ECL) and exposed to X-ray films. All experiments had at least one membrane reprobed with antibodies recognizing non-phosphorylated kinases to confirm equal protein loading. The exposure autoradiograph was analyzed by Un-Scan-It gel, version 5.1, to obtain densitometry data. Protein contents were determined by BCA assay (Pierce).

Real-time RT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen), treated with DNase I (Ambion) to remove potential genomic DNA contamination, and purified using an RNeasy Mini Kit (Qiagen). Total RNA concentration was measured, and the purity of the samples was estimated by the OD ratios (A260/A280, ranging within 1.8–2.2). cDNA was synthesized from 2 μg of DNA-free total RNA in a 25-μl reaction volume in duplicate, using SYBR Green and an ABI 7500 real-time PCR system (Applied Biosystems). The sense primer for human TGF-β1 (human-1), β-actin (human-2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (human-3) was synthesized by Sigma. The primers for real-time RT-PCR were as follows:

- TGF-β1 (human-1): 5′-CCAGCTTCTGATGTCGATTG-3′ (forward) and 5′-GGTTCTGCACACAAGATACG-3′ (reverse)
- β-actin (human-2): 5′-GGAATCATTGGGATGATGTC-3′ (forward) and 5′-CCTGTGTCGCTAGGCTCAG-3′ (reverse)
- GAPDH (human-3): 5′-GGAAGATGGTGATGGGATT-3′ (forward) and 5′-GGGTTGTCGTCCTCCACAA-3′ (reverse)

Gene-specific transcriptional levels were determined in a 20-μl reaction volume, in duplicate, using SYBR Green and an ABI 7500 real-time PCR system (Applied Biosystems). The sense primer for human TGF-β1 was analyzed by ELISA. Values are means ± SE of 6 independent experiments. ***P < 0.001 vs. no-stretch (control) cells. **P < 0.01 vs. no-stretch (control) cells. ***P < 0.001 vs. S3I-201-treated cells with stretch. C: siRNA scramble or siRNA STAT3 was transfected in HK-2 cells followed by mechanical stretch (20% elongation, 6 cpm) stimulation for 48 h, after which TGF-β1 was analyzed by ELISA. Values are means ± SE of 6 independent experiments. ***P < 0.001 vs. no-stretch (control) cells. **P < 0.01 vs. siRNA STAT3-treated cells with stretch. D: HK-2 cells were stimulated in the presence of blocking antibodies (10 μg/ml) to TGF-β1 for 48 h in serum-free medium. Immunoblotting with specific antibodies against fibronectin or β-actin and respective quantitation analysis for expression of fibronectin was performed. Fibronectin protein expression levels were normalized to β-actin. Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 5 different experiments. **P < 0.01 vs. no-stretch (control) cells. **P < 0.01 vs. anti-TGF-β1 antibody-treated cells with stretch.

Fig. 4. Role of STAT3 in stretch-induced TGF-β1 expression. A: HK-2 cells were treated with cyclic stretch (20% elongation, 6 cpm) for indicated time intervals, after which TGF-β1 was analyzed by ELISA. Values are means ± SE of 6 independent experiments. ***P < 0.001 vs. time-matched control. B: HK-2 cells serum-starved for 24 h were pretreated without or with the STAT3 inhibitor S3I-201 (50 μM) for 1 h before treatment with mechanical stretch (20% elongation, 6 cpm) for 48 h, after which TGF-β1 was analyzed by ELISA. Values are means ± SE of 5 independent experiments. ***P < 0.001 vs. no-stretch (control) cells. **P < 0.01 vs. S3I-201-treated cells with stretch. C: siRNA scramble or siRNA STAT3 was transfected in HK-2 cells followed by mechanical stretch (20% elongation, 6 cpm) stimulation for 48 h, after which TGF-β1 was analyzed by ELISA. Values are means ± SE of 6 independent experiments. ***P < 0.001 vs. no-stretch (control) cells. **P < 0.01 vs. siRNA STAT3-treated cells with stretch. D: HK-2 cells were stimulated in the presence of blocking antibodies (10 μg/ml) to TGF-β1 for 48 h in serum-free medium. Immunoblotting with specific antibodies against fibronectin or β-actin and respective quantitation analysis for expression of fibronectin was performed. Fibronectin protein expression levels were normalized to β-actin. Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 5 different experiments. **P < 0.01 vs. no-stretch (control) cells. ++P < 0.01 vs. anti-TGF-β1 antibody-treated cells with stretch.
β1-integrin was 5'-GCAAGTTGAGTTGTGGATCA-3'; and the antisense primer was 5'-TGCCACAAAGTTTCCCATCT-3'. The sense for the human GAPDH was 5'-GAAGGTGAGGTCGGAGTC-3'; and the antisense primer was 5'-GAAGATGTTGATGGGATTCT-3'. A quantitative analysis was performed to evaluate the expression of β1-integrin and normalized to GAPDH. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as 2^−ΔΔCt. Melting curve analysis was performed to check for any presence of nonspecific application products.

**Determination of TGF-β1.** HK-2 cells were passaged onto commercially available silastic membranes coated with collagen type I (Flexcell, McKeesport, PA) and grown to confluence. After an incubation period of 12–72 h with the treatment, the medium was removed and kept at −80°C for ELISA. TGF-β1 in the media of stretch and nonstretched HK-2 cells was measured using ELISA kits (Quantikine; R&D Systems). To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the immunoassay, samples were acidified with 1 N HCl, incubated at room temperature for 10 min, and neutralized with 1.2 N NaOH. The remainder of the assay was carried out according to the manufacturer’s instructions with absorbance read at 450 nm.

**Statistical analyses.** Values are means ± SE. For statistical significance, multiple comparisons among three or more groups were performed using one-way ANOVA followed by a Student-Newman-Keuls multiple comparison post hoc test when appropriate. For comparing two groups, an unpaired t-test was used. Differences with values of P < 0.05 were considered significant.

**RESULTS**

Fibronectin expression is upregulated in response to cyclic mechanical stretch in HK-2 cells. We first examined the time course of cyclic stretch-dependent fibronectin expression in HK-2 cells. Nonstretched cells were used as a control. We found that cyclic stretch at 20% elongation increased fibronectin expression, which was significant at 12 h, with a maximum 2.6-fold increase at 48 h, and then declining after 72 h to 2.5-fold (Fig. 1A). In addition, cyclic stretch-induced fibronectin protein expression was found to be intensity dependent. When exposed for 48 h to increasing intensities of cyclic stretch (10–20% stretch), fibronectin protein expression was significant at 15% stretch with a maximal effect at 20% stretch, compared with control nonstretched cells (Fig. 1B). For subsequent studies, cells were treated for 48 h at 20% stretch to detect maximal fibronectin protein expression, unless otherwise indicated.

Cyclic stretch upregulates fibronectin and TGF-β1 expression via STAT3 in HK-2 cells. In a recent study, Pang et al. (46) has shown that the STAT3 inhibitor S3I-201 alters fibronectin expression in a mouse model of renal interstitial fibrosis induced by UUO. Therefore, we examined the effect of S3I-201 in stretch-induced fibronectin expression. We first tested the effects of cyclic stretch on STAT3 phosphorylation by using an antibody recognizing the tyrosine 705 phosphorylated form of STAT3. As shown in Fig. 2A, cyclic stretch induces...
STAT3 phosphorylation in a time-dependent manner, reaching a maximum value after 15 min of stimulation. Pretreatment of proximal tubule cells with S3I-201 (50 µmol/l) significantly reduced the phosphorylation of STAT3 and expression of fibronectin in the presence of cyclic stretch (Fig. 2, B and C, respectively). In addition, transfecting proximal tubule cells with siRNA targeting human STAT3 (siRNA STAT3) abrogated stretch-induced STAT3 and fibronectin expression (Fig. 3, A and B, respectively). Moreover, stimulation of proximal tubule cells with cyclic mechanical stretch induced expression of TGF-β1 time dependently (Fig. 4A). In addition, application of S3I-201 or overexpression of STAT3 siRNA effectively suppressed stretch-induced TGF-β1 expression (Fig. 4, B and C, respectively). Furthermore, treatment of proximal tubule cells with an anti-TGF-β antibody attenuated stretch-induced fibronectin expression in HK-2 cells whereas nonspecific IgG had no effect (Fig. 4D). These results show that STAT3 is involved in stretch-induced fibronectin and TGF-β1 expression, and that stretch-stimulated fibronectin expression in HK-2 cells was mediated by TGF-β1.

Inhibition of c-Src reduces STAT3 activation and fibronectin and TGF-β1 expression in HK-2 cells. c-Src is a well-known regulator of STAT3 (11, 24, 50) and fibronectin (2, 59, 60). Moreover, previous studies from this laboratory have shown that mechanical stretch activates c-Src in renal tubular cells (1). To examine the role of c-Src in STAT3 activation and fibronectin and TGF-β1 expression by mechanical stretch, cells were pretreated either with the Src family kinase inhibitor PP2, its inactive analog PP3, or transiently transfected with a dominant-negative mutant of c-Src (DN-Src). Pretreatment of proximal tubule cells with PP2 (10 µmol/l) significantly decreased stretch-induced STAT3 activation and fibronectin and TGF-β1 expression, whereas its inactive analog PP3 (10 mmol/l) had no significant effect (Fig. 5, A, B, and C, respectively). Similarly, transfection of proximal tubule cells with DN-Src significantly reduced stretch-induced STAT3 activation and fibronectin and TGF-β1 expression (Fig. 6, A, B, and C, respectively). By analogy, pretreatment with the selective STAT3 inhibitor S3I-201 had no effect on stretch-induced c-Src activation (data not shown). These data strongly implicate c-Src in the activation of STAT3 and the expression of fibrogenic protein expression in human proximal tubular cells exposed to cyclic mechanical stretch.

Stretch-induced fibronectin and TGF-β1 expression is β1-integrin dependent. Although it had been shown that mechanical stretch induced β1-integrin protein expression in different tissues and that β1-integrin is highly expressed in renal tubular epithelial cells (22, 33, 66, 67, 70), the functions of β1-integrin in renal epithelial cells during mechanical stretch-induced fibrogenesis has never been reported. To address whether the fibrogenic effect of mechanical stretch is β1-integrin dependent, we first examined whether β1-integrin was upregulated by cyclic mechanical stretch in HK-2 cells. As shown in Fig. 7, A and B, respectively, mechanical stretch upregulated β1-integrin protein and mRNA levels in a time-dependent manner. Blockade of β1-integrin, with an anti-integrin β1-blocking antibody, 4B4, abrogated fibronectin and TGF-β1 expression after mechanical stretch (Fig. 8, A, B, and C, respectively). To further confirm the role of β1-integrin in stretch-induced fibronectin and TGF-β1 expression, β1-integrin was silenced by
**DISCUSSION**

Proximal tubular cells secrete a profibrotic mediators that may contribute to the pathophysiology of obstructive nephropathy-related disorders. Among these, the expressions of fibronectin and TGF-β1 are upregulated in the UUO model of obstructive nephropathy and may aid the progression to tubulointerstitial fibrosis (12, 25, 31, 34, 37, 55, 66, 68). Understanding the regulatory pathways that control their production may be paramount to developing effective therapeutics to treat these diseases.

Tubular mechanical stretch represents a major insult to proximal tubular cells during obstructive nephropathy. Mechanical stretch represents a unique in vitro model to mimic tubular dilatation due to transient increase in intrarenal pressure accompanying obstructive nephropathy and a mechanism to stimulate profibrotic (TGF-β1) and extracellular matrix (fibronectin) gene and protein expression. However, the mechanisms responsible for mechanotransduction of this external strain to TGF-β1 and fibronectin expression are unknown. In this study, cyclic mechanical stretch induces fibronectin and TGF-β1 expression, which was attenuated by pretreatment of proximal tubular cells with a Src family kinase inhibitor, PP2, or by transfection of proximal tubular cells with DN-Src. The involvement of STAT3 in stretch-induced fibronectin and TGF-β1 expression was also confirmed by using a potent and selective STAT3 inhibitor, S3I-201 and by transfection of proximal tubular cells with siRNA of STAT3. Additionally, our results demonstrated for the first time that mechanical stretch is able to induce significant time-dependent increases in β1-integrin mRNA and protein expression in renal proximal tubule cells. Moreover, mechanical stretch-induced fibronectin and TGF-β1 expression was inhibited by blocking antibodies to β1-integrin and by knockdown of β1-integrin by transfection of siRNAs. Thus our results confirmed the notion that the mechanisms underlying the activation of c-Src and STAT3 lead to TGF-β1 synthesis by mechanical stretch and may be essential for the upregulation of fibronectin expression in HK-2 cells. To our knowledge, this is the first direct evidence that the β1-integrin/c-Src/STAT3 signaling pathway mediates fibronectin and TGF-β1 expression induced by mechanical stretch. Thus a novel pathway has been identified for mechanical stretch-initiated cellular events leading to integrin expression, kinase activation, and extracellular matrix synthesis culminating into renal fibrosis.

Integrins are heterodimeric cell surface adhesion proteins that link the ECM to the cytoskeleton and consist of different α- and β-subunits. While not possessing kinase domains, integrin receptors can transduce information from the ECM to the cell to activate various intracellular signaling pathways, thereby regulating cellular processes as diverse as cytoskeletal organization, proliferation, differentiation, apoptosis, and cell migration (8, 30, 47, 62, 65). Numerous studies have linked integrins in the pathogenesis of renal fibrosis. For example, deletion of α1-integrin causes the development of severe glomerulosclerosis in a model of glomerular injury by adriamycin (10). Similarly, anti-integrin-α1 antibodies reduced glomerular and tubulointerstitial scarring in rat models of glomerular injury (14). Obstructed kidneys from mice devoid of α5β6-integrin showed significant less tubulointerstitial fibrosis than observed in the wild-type (39). Recently, Amann et al. (3) showed that anti-αβ3-integrin-blocking antibodies interfered with glomerulosclerosis during experimental Habu glomerulo-
nephritis. In Alport mice, the expression of αvβ6 correlates with renal fibrosis, and blocking this integrin results in reduced deposition of collagen matrix (21). In several models of kidney diseases, ablation of β1-integrin abrogates profibrotic signaling and blocks accumulation of ECM and the development of tubulointerstitial fibrosis (15, 36, 70). However, the molecular mechanisms by which β1-integrin attenuates UUO-induced fibrosis are not well characterized. Although it had been shown that mechanical stretch induced β1-integrin protein expression in different tissues and that β1-integrin is highly expressed in renal tubular epithelial cells (22, 33, 66, 67, 70), the functions of β1-integrin in renal epithelial cells during mechanical stretch-induced fibrogenesis has never been reported.

Our data indicate that knockdown of β1-integrin attenuates fibronectin and TGF-β1 expression in mechanical stress-induced tubular cells. Furthermore, β1-integrin deficiency attenuated stretch-induced phosphorylation of c-Src and STAT3. The fact that both fibronectin and TGF-β1 expression and c-Src and STAT3 phosphorylation can be blocked by treatment with a β1-integrin-blocking antibody and β1-integrin siRNA strongly suggests that β1-integrin represents an important mechanoreceptor in renal tubular cells. Therefore, we present for the first time an alternative paradigm to αvβ6-induced regulation of TGF-β1 synthesis and renal fibrosis and propose the following model of mechanotransduction in HK-2 cells, as shown in Fig. 10: Conceivably, mechanical stretch, exerted by tubular distension, sensed by β1-integrin receptors, leads to increase β1-integrin mRNA and protein levels. β1-Integrin acts as a signaling molecule, activating c-Src. c-Src then phosphorylates STAT3 at Tyr705. This phosphorylation leads to increases synthesis of TGF-β1. Finally, TGF-β1 can modulate the expression of fibronectin, which may play a role in renal fibrosis.

It is well established that STAT3 inhibition plays an important role in protecting the kidney during several types of renal injury. Yang et al. (69) reported that JAK2/STAT1/3 signals are activated in the kidneys after ischemia-reperfusion (I/R) and that blockage of JAK2 by AG490 attenuates I/R-induced...
renal injury. Others have reported STAT3 activation in renal tubular cells in response to experimental UUO (40, 46) and have demonstrated that S3I-201, a STAT3 inhibitor, decreased the expression of profibrotic markers following obstructive injury (46). In addition, in vitro, S3I-201 attenuated tubular cell profibrotic cellular changes and apoptosis in response to IL-18 stimulation (40). Broadbelt et al. (6) recently demonstrated that STAT3 is required for TGF-β1 and fibronectin expression in HK-2 cells. A: HK-2 cells were transfected with control (scramble) siRNA or β1-integrin siRNA followed by cyclic stretch treatment for 15 min (c-Src and p-STAT3) or 48 h (fibronectin and TGF-β1) in serum-free medium. The phosphorylation of c-Src and STAT3 and expression of fibronectin were determined by Western blotting. Fibronectin protein expression levels were normalized to β-actin. B: bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 5 different experiments. C: TGF-β1 was analyzed by ELISA. **P < 0.01, ***P < 0.001 vs. no stretch (control) cells. + + P < 0.01, + + + P < 0.001 vs. scramble β1-integrin-treated cells with stretch.

Src proteins consist of at least 14 related alternatively spliced gene products which are expressed in a tissue-specific manner (5). Src is activated by dephosphorylation of tyrosine residue at the C terminal to unfold the protein followed by tyrosine residue phosphorylation. The Src family members Fyn, Lyn, and c-Src play a key role in the signaling in response to mechanical stretch (1, 43, 44, 49, 64). For example, in vascular smooth muscle cells (VSMC), mechanical stretch-induced phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt), p21ras, and ERK1/2 activation has been suggested to be mediated at least in part by Src since pharmacological inhibition or overexpression of a kinase-dead c-Src mutant blocked these effects (26, 56). In vascular endothelial cells deficient of the Src family kinase Fyn, platelet endothelial cell adhesion molecule-1 (PECAM-1) activation by both stretch and flow was blocked, whereas knockdown of c-Src and Yes was ineffective (13). In contrast, treatment with c-Src pharmacological inhibitors or dominant-negative mutants of c-Src blocked stretch-induced focal adhesion kinase (FAK) activation in cardiac myocytes (63). These findings provide compelling evidence that Src family-related proteins play a critical role in the mechanical signaling of renal tubular cells.
role in cyclic mechanical strain-related signaling. Of the members of the Src kinase family, we have reported that mechanical stretch activates c-Src and enhances the activation of ERK1/2 in renal proximal tubular cells (1). Moreover, a variety of experimental studies implicate c-Src in the pathogenesis of progressive renal fibrosis (27, 29, 45). Additionally, numerous studies indicate that c-Src is critical for TGF-β1-mediated renal fibrosis (7, 23, 48). In line with the previously described role for c-Src in mediating renal fibrosis, we recently demonstrated that blockade of c-Src in proximal tubular cells is associated with a decrease in fibronectin expression induced by angiotensin II and G protein βγ-subunits (2). We now provide evidence that c-Src plays a critical role in regulating renal fibrosis induced by cyclic mechanical stretch. Using both pharmacological inhibitors and dominant-negative mutants of c-Src, we have reported that mechanical stretch activates c-Src and enhances the activation of ERK1/2.

In addition, the activation of c-Src results in an increase in the phosphorylation of STAT3, which promotes TGF-β1 production. TGF-β1 increases fibronectin synthesis.


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Author Contributions
Author contributions: M.T.H., R.S., and L.D.A. performed experiments; M.T.H., R.S., and L.D.A. analyzed data; M.T.H., R.S., and L.D.A. interpreted results of experiments; M.T.H., R.S., and L.D.A. edited and revised manuscript; M.T.H., R.S., and L.D.A. approved final version of manuscript; L.D.A. provided conception and design of research; L.D.A. prepared figures; L.D.A. drafted manuscript.

References
Consequences of lack of beta 1 integrin gene regulation through beta1-integrin and focal adhesion kinase.


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