Mechanical Stretch Activates the JAK/STAT Pathway in Rat Cardiomyocytes

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Abstract—This study was designed to determine whether mechanical stretch activates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in cardiomyocytes and, if so, by what mechanism. Neonatal rat/murine cardiomyocytes were cultured on malleable silicone dishes and were stretched by 20%. Mechanical stretch induced rapid phosphorylation of JAK1, JAK2, Tyk2, STAT1, STAT3, and glycoprotein 130 as early as 2 minutes and peaked at 5 to 15 minutes. It also caused gel mobility shift of sis-inducing element, which was supershifted by preincubation with anti-STAT3 antibody. Preincubation with CV11974 (AT1 blocker) partially inhibited the phosphorylation of STAT1, but not that of STAT3. Preincubation with TAK044 (endothelin-1-type A/B-receptor blocker) did not attenuate this pathway. RX435 (anti–glycoprotein 130 blocking antibody) inhibited the phosphorylation of STAT3 and partially inhibited that of STAT1. Phosphorylation of STAT1 and STAT3 was strongly inhibited by HOE642 (Na+/H+ exchanger inhibitor) and BAPTA-AM (intracellular calcium chelator), but not by gadolinium (stretch-activated ion channel inhibitor), EGTA (extracellular Ca2+ chelator), or KN62 (Ca2+/calmodulin kinase II inhibitor). Chelerythrine (protein kinase C inhibitor) partially inhibited the phosphorylation of STAT1 and STAT3. Mechanical stretch also augmented the mRNA expression of cardiotrophin-1, interleukin-6, and leukemia inhibitory factor at 60 to 120 minutes. These results indicated that the JAK/STAT pathway was activated by mechanical stretch, and that this activation was partially dependent on autocrine/paracrine–secreted angiotensin II and was mainly dependent on the interleukin-6 family of cytokines but was independent of endothelin-1. Moreover, certain levels of intracellular Ca2+ were necessary for stretch-induced activation of this pathway, and protein kinase C was also partially involved in this activation. (Circ Res. 1999;84:1127-1136.)

Key Words: mechanical stretch ▪ angiotensin II ▪ cardiac hypertrophy ▪ JAK/STAT pathway ▪ glycoprotein 130

Cardiac hypertrophy is a compensatory response that allows the heart to cope with the pathogenic stimuli found with many cardiovascular diseases.1 Cardiac hypertrophy is induced by mechanical load and humoral factors, such as angiotensin II (Ang II),2 endothelin-1 (ET-1),3 and norepinephrine.4 Mechanical stretch is one of the most important stimuli of cardiac hypertrophy.5,6 Mechanical stretch-induced signal transduction is characterized by simultaneous activation of multiple second messenger systems. Many studies have demonstrated that mechanical stretch caused activation of multiple intracellular signal transduction pathways in cultured neonatal cardiomyocytes, such as phospholipases (C, D, and A2), tyrosine kinases, p21ras, Raf-1, mitogen-activated protein kinases, c-jun N-terminal protein kinases, and protein kinase C (PKC).7-9 Autocrine/paracrine–secreted growth factors such as Ang II and ET-1 play an important role in the stretch-induced hypertrophic response.10,11 Although mechanical stretch activates multiple second messenger systems, it remains unclear which molecules are directly activated by stretch and how mechanical stimuli are converted into intracellular signals to activate protein kinase cascades and stimulate the secretion of growth factors.

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway was initially discovered as a major signal transduction pathway of the cytokine superfamilies.12 It plays a crucial role in the growth and differentiation of a variety of cell types. Although the significance of the JAK/STAT pathway in cardiomyocytes has not been well characterized, increasing evidence suggests that this pathway might be involved in the development of myocardial hypertrophy. We and others have reported that the JAK/STAT pathway was activated by hypertrophic agonists (leukemia inhibitory factor [LIF], cardiotrophin-1 [CT-1], and Ang II) in vitro.13-16 LIF and
CT-1 activated JAK1, JAK2, Tyk2, STAT1, and STAT3, and Ang II activated JAK2, Tyk2, STAT1, and STAT2 in cardiomyocytes. Kunisada et al reported that overexpression of constitutive active STAT3 augmented the LIF-induced increase in [3H]leucine uptake and hypertrophy marker gene expression, whereas overexpression of a dominant-negative STAT3 decreased these events. We have also demonstrated that acute pressure overload in vivo activated the JAK/STAT pathway and that Ang II was partially involved in this activation. These results suggested that this pathway was critically involved in the hypertrophic response.

In the present study, we investigated whether the JAK/STAT pathway was activated by mechanical stretch in cultured cardiomyocytes and, if so, how. We investigated (1) the involvement of autocrine/paracrine–secreted growth factors in stretch-induced activation of this pathway, (2) the role of ion channel and exchanger, and (3) the cross talk between the JAK/STAT pathway and other signaling pathways.

**Materials and Methods**

**Cell Culture and Cardiomyocyte Stretching**

Primary cultures of cardiomyocytes were prepared from the ventricles of 1-day-old Wistar rats or ICR mice (Japan Clea Co Ltd) by enzymatic dissociation in 0.03% trypsin, 0.03% collage-nase, and 20 μg/mL of DNase I as described previously. The cardiomyocytes were collected by differential adhesiveness. Cardiomyocyte-enriched suspensions were removed from the culture dishes and plated at a density of 1×10^5 cells/cm² on silicone rubber dishes (241 cm²) precoated with laminin (2 μg/cm²). Twelve hours after plating, bromodeoxyuridine was added to the medium at a concentration of 10^{-7} mol/L for 12 hours to deplete residual nonmyocytes. The experiment was performed on the second day from the primary culture, and the final density of the cells was 1.1×10^5 to 1.2×10^5 cells/cm². The nonmyocyte population amounted to ~5 to 10% of the total cell population as determined by immunofluorescence staining with monoclonal anti-sarcomeric myosin antibody (MF20). The cells were grown in M199/DMEM medium supplemented with 10% FBS, penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37°C in humid air with 5% CO₂. The culture medium was changed 24 hours after seeding to a medium containing 0.1% FBS. After 8 hours in the 0.1% FBS medium, the culture dishes were stretched by 20%. Stretching of cardiomyocytes was accomplished essentially according to the method of Komuro et al and Izumo. A 20% stretch was shown to be sufficient to activate various signal transduction pathways and not to damage the cells.

**Immunoprecipitation and Western Blot Analysis**

Polyclonal antibodies to JAK1, JAK2, Tyk2, STAT1α/β, STAT3, and glycoprotein (gp) 130, and monoclonal antibody to STAT1α, were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Polyclonal antibody to phosphospecific STAT3 was purchased from New England Biolabs, Inc.

To prepare cell extracts, cells were washed 3 times in PBS and then extracted in lysis buffer containing (in mmol/L) Tris-HCl (pH 7.5) 40, sodium acetate 40, and 10% glycerol, 0.1% SDS, 1.0% deoxycholic acid, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. The lysates were centrifuged at 10 000 × g for 15 minutes. Protein concentration was determined by the Bio-Rad protein assay.

Cell lysates were incubated with 1 μg/mL of the respective antibodies overnight at 4°C. Immunocomplexes were collected by incubating with 50 μL of protein A– or G–Sepharose for 2 hours. Immunoprecipitates were washed 4 times with ice-cold lysis buffer. The pellets were resuspended in 2× sample buffer containing 50 mmol/L Tris (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 2% glycerol, and bromphenol blue. The samples were subjected to SDS-PAGE and were transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% BSA in Tris-buffered saline–TWEEN solution (20 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, and 0.05% Tween 20) for 2 hours at room temperature. Blots were immunolabeled overnight at 4°C with anti-phosphotyrosine antibody or anti-gp130 antibody. Proteins were visualized by enhanced chemiluminescence (Amersham). The blots were stripped and reprobed with the same antibodies for their immunoprecipitation, to ensure equal loading of the proteins.

For Western blot analysis, cells were lysed in lysis buffer containing (in mmol/L) Tris (pH 7.6) 20, NaCl 250, EDTA 3, EGTA 3, and β-glycerophosphate 3, and 0.5% NP-40 and the protease inhibitors described above. Immunoblotting was conducted with anti-phospho-STAT3, and anti-STAT3 antibodies.

**Gel Mobility Shift Assay**

Cardiomyocytes were rinsed with PBS at 0°C and scraped into the same buffer. Nuclear extracts were prepared according to standard methods described previously. Five micrograms of nuclear extracts were incubated with 1 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech) with or without competitor oligonucleotide in 20 μL of a solution containing (in mmol/L) HEPES (pH 7.9) 10, NaCl 50, and 10% glycerol, for 20 minutes at 25°C. The samples were incubated with 1 or 2 flmol of radiolabeled probes (~5000 cpm) for 10 minutes at 25°C. The probes were washed from Santa Cruz Biotechnology, and their sequences were described (six-inducing element [SIE]-DNA, 5′-CAGTCTCCCGTCAATC3′; mutant SIE-DNA, 5′-CAGCCACCGTCATACTC). Binding reactions were resolved on a 4% native polyacrylamide gel containing TAE buffer containing (in mmol/L) Tris (pH 7.5) 40, sodium acetate 40, and EDTA 1 for 3 hours at 150 V at 4°C. The gel was dried and exposed to x-ray film for 12 hours. For supershift assays, nuclear extracts were incubated with 2 μg of antibodies to STAT1, STAT3, or both and incubated at room temperature for 1 hour, and the complexes were resolved by gel electrophoresis.

**DNA Extraction and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was extracted from neonatal rat cardiomyocytes using TriZol reagent (GIBCO). The samples were treated with DNase I and then subjected to first-strand synthesis using oligo(dT) primer and reverse transcriptase (Superscript II). The PCR reaction was performed in a reaction volume of 20 μL containing 250 mmol/L of dNTP, 50 pmol of each specific primer, and 2.5 U of Taq polymerase. The primers used were as follows: CT-1 sense, CCCTCTTCACCTCAACAGTG, and antisense, GAGGGCAGAGCAGGAGAG-GA; interleukin (IL)-6 sense, GACCTTCAGAGGATACCACCC, and antisense, GACCTTACAGGATACCACCC; LIF sense, AGTCAACTGGGTCACTACCTAC; and antisense, CTGGGCA-AACACATITATGACT; and ciliary neurotrophic factor (CNTF) sense, GGATGGCTTTCGCAGAGCAAAC, and antisense, AGTGCTGGTCCTCGAGAGCAAA, and antisense, AG-GACCTCGACCCGTCATAGCT. Denaturing, annealing, and extension reactions were performed at 95°C for 30 seconds, 58 to 60°C for 1 minute, and 72°C for 1 minute, respectively. Before the quantitative analysis, the linear range of the PCR cycles was measured for each cytokine, and the appropriate number of PCR cycles was determined. α-Tubulin was used as an internal control for each sample.
Mechanical Stretch Activates the JAK Family Kinases

We initially determined whether mechanical stretch would result in tyrosine phosphorylation of the JAK kinases. After stretching, cardiomyocytes were lysed and immunoprecipitated with anti-JAK1, anti-JAK2, or anti-Tyk2 antibody and immunoblotted with anti-phosphotyrosine antibody (Figure 1). JAK1, JAK2, and Tyk2 were rapidly phosphorylated at 2 minutes, which peaked at 5 minutes and decreased thereafter. These findings indicated that mechanical stretch could directly or indirectly activate the JAK kinases in rat cardiomyocytes.

Mechanical Stretch Activates STAT1 and STAT3

To demonstrate that mechanical stretch would activate the STAT families, we first analyzed the tyrosine phosphorylation of STAT1 and STAT3 by immunoprecipitation-Western blot analysis (Figure 2A). After mechanical stretching, STAT1 and STAT3 were phosphorylated as early as 2 minutes, which peaked at 5 to 15 minutes and then gradually decreased, but remained elevated at 30 minutes. The dual bands in the upper panel indicate STAT1α and STAT1β.

The DNA binding activity of STATs depends primarily on tyrosine phosphorylation. To investigate whether mechanical stretch induces sis-inducing factor (SIF)-like activity in cardiomyocytes, we performed a gel mobility shift assay using 32P-labeled SIE oligonucleotide. As shown in Figure 2B, left, stimulation of cardiomyocytes by mechanical stretch resulted in the induction of protein complexes that bound to the SIE oligonucleotide. The specific DNA-protein complex increased from 5 minutes, peaked at 30 minutes, and decreased at 60 minutes. No mobility shift was observed with mutant SIE probe (lane 7), and the addition of higher concentrations of unlabeled SIE competed with the SIF complexes (lane 6), confirming the specificity of binding to SIE. A supershift assay revealed that incubation of the DNA-protein complex with anti-STAT1 antibody only mildly inhibited binding of the SIF band, but incubation with anti-STAT3 antibody or with both anti-STAT1 and anti-STAT3 antibodies significantly inhibited the binding of SIF complexes in stretch-stimulated cardiomyocyte extracts (Figure 2B, right). The antibody to STAT3 removed the SIF complexes rather than supershifting them. The anti-STAT3 antibody used is a polyclonal antibody that recognizes amino acids 626 to 640. This antibody does not directly recognize the DNA binding domain but seems to have inhibited the binding of STAT3 to DNA. These results indicated that mechanical stretch induced homodimerization or heterodimerization of STAT1 and STAT3 and formation of SIF complexes.

Inhibition of Phosphorylation of STATs by a JAK2 Kinase Inhibitor

Recent studies have reported that STAT1 and STAT3 can be phosphorylated by several tyrosine kinases other than JAK families. To determine whether the phosphorylation of STAT1 and STAT3 induced by stretching was mediated by the JAK kinase–dependent pathway, we investigated the effect of a specific JAK2 kinase inhibitor, AG490, on the phosphorylation of STATs. Cardiomyocytes were pretreated with 4 × 10^-5 mol/L of AG490 for 30 minutes before stretching. As shown in Figure 2C, AG490 significantly inhibited the phosphorylation of STAT1 and STAT3. We have observed that AG490 did not affect the LIF-induced tyrosine phosphorylation of JAK1 and Tyk2, but it inhibited LIF-induced phosphorylation of STAT3 by 88% and slightly inhibited that of STAT1 (H.K., K.F., unpublished data, 1998). These data suggested that JAK2 kinase activity was necessary for stretch-induced activation of STAT1 and STAT3 in cardiomyocytes.

Autocrine/Paracrine–Secreted Ang II but Not ET-1 Is Partially Involved in Phosphorylation of STATs

Previous studies have reported that mechanical stretch stimulates secretion of Ang II and ET-1 in cardiomyocytes and that both factors are involved in the development of mechanical stretch-induced cardiac hypertrophy.19,20 We have reported that pressure overload-induced activation of the JAK/STAT pathway was partially dependent on Ang II, mediated via the AT1.21 Thus, we investigated whether these 2 factors were involved in the mechanical stretch-induced phosphorylation of STATs. As shown in Figure 3A and 3B, CV11974 (AT1 antagonist) partially inhibited the phosphorylation of STAT1 and STAT1β by mechanical stretch, whereas it completely inhibited that of STAT1 induced by Ang II. CV11974 did not affect the phosphorylation of STAT3 induced by mechanical stretch. Pretreatment with TAK044 (ET-1 type A/B receptor antagonist) did not inhibit the phosphorylation of STAT1 and STAT3 induced by mechanical stretch (Figure 3C). As shown in Figure 3D, we have observed that ET-1 stimulation did not tyrosine phosphorylate STAT3 until 30 minutes. It induced tyrosine phosphorylation of STAT1 at 2 to 5 minutes, although only at a very low level. TAK044 completely inhibited the phosphorylation of STAT1 induced by ET-1. These findings indicated that autocrine/paracrine-secreted Ang II or ET-1 did not seem to be
involved in the activation of STAT3 induced by mechanical stretch, and Ang II was partially involved in the activation of STAT1, whereas ET-1 did not seem to be involved in this activation.

**Involvement of gp130-Related Signaling in Stretch-Induced Activation of STATs**

To demonstrate that gp130-related signaling was involved in the stretch-induced activation of this pathway, we preincubated cardiomyocytes with a JAK2 kinase inhibitor (AG490) for 30 minutes and stretched by 20% for 6 minutes. The phosphorylation of STATs was detected. The blot presented is representative of 3 independent experiments. IP indicates immunoprecipitation; pTyr, phosphotyrosine; +, incubation or stimulation (+); and −, incubation or stimulation (−).
bated the cells with anti-mouse gp130-specific blocking antibody RX435 for 30 minutes and observed the tyrosine phosphorylation of STAT1 and STAT3. RX435 specifically blocks the actions of LIF, IL-6, IL-11, and oncostatin M on mouse myeloid leukemic M1 cells and inhibits the binding of CT-1 to M1 cells.20 RX435 strongly reduced the stretch-induced phosphorylation of STAT3 and slightly inhibited the phosphorylation of STAT1 (Figure 4A and 4B). We then detected the phosphorylation of gp130 (Figure 4C), gp130 was rapidly phosphorylated at 2 minutes after stretching, gradually decreased, and returned to the control level at 30 minutes. These findings suggested that the IL-6 family of cytokines is critically involved in the stretch-induced activation of STATs, especially STAT3, in cardiomyocytes.

Expression of the IL-6 Family of Cytokines in Stretched Cardiomyocytes

On the basis of the above results, we further tested the levels of expression of CT-1, IL-6, LIF, and CNTF mRNA by RT-PCR in stretched cardiomyocytes (Figure 4D). We used 23 cycles for IL-6 and α-tubulin, 28 cycles for CT-1 and CNTF, and 30 cycles for LIF. The expression of CNTF was unaffected by stretch stimulation. In contrast, expression of CT-1, IL-6, and LIF mRNA increased after stretching and peaked at 60 to 120 minutes. These results were reproducible in 3 separate experiments. These findings indicated that mechanical stretch was involved not only in rapid secretion but also in mRNA expression of the IL-6 family of cytokines in cardiomyocytes.

Role of ion channels and Na+/H+ exchanger (NHE) in Stretch-Induced Phosphorylation of STATs

To determine whether mechanosensitive ion channels and NHE are involved in stretch-induced activation of STATs, we pretreated the cells with either a specific inhibitor of the stretch-sensitive cation channel (10⁻⁵ mol/L gadolinium) or an NHE inhibitor (10⁻⁵ mol/L HOE642) and stretched the cells by 20% for 6 minutes. The results are shown in Figure 5. Stretch-induced phosphorylation of STAT1 and STAT3 was not affected by pretreatment with gadolinium. In contrast, phosphorylation of STAT1 and STAT3 was strongly inhibited by HOE642. These results suggested that stretch-induced phosphorylation of STATs was partially dependent on the activation of NHE.

Role of Ca²⁺ in Mediating Stretch-Induced Phosphorylation of STATs

A previous study reported that mechanical stretch increased intracellular Ca²⁺. It remains unclear whether these pathways are involved in the activation of STATs induced by mechan-
ical stretching. In this study, we observed that the addition of EGTA (4×10⁻³ mol/L) to the culture medium resulted in no significant reduction of the phosphorylation of STAT1 and STAT3. In contrast, the stretch-induced phosphorylation of STAT1 and STAT3 was completely attenuated by buffering the intracellular free Ca²⁺ with the pretreatment of membrane-permeable BAPTA-AM (1×10⁻⁴ mol/L). This inhibition was dose dependent (Figure 6A and 6B). Next, we pretreated the cells with the calmodulin kinase II antagonist KN62 for 30 minutes and found that KN62 had no significant effect on phosphorylation of STAT1 and STAT3 (Figure 6A). These results suggested that certain levels of intracellular Ca²⁺ play a pivotal role in stretch-induced activation of STATs, and that Ca²⁺ influx was not apparently necessary for stretch-induced activation of this pathway.

Role of PKC in Mediating Stretch-Induced Phosphorylation of STATs

Mechanical stretch activates PKC in cardiomyocytes, and PKC is critically involved in stretch-induced mitogen-activated protein kinase activation.⁷ To investigate the role of PKC in stretch-induced activation of STATs, we stimulated the cells with phorbol 12-myristate 13-acetate (PMA) (1×10⁻⁶ mol/L), a direct activator of PKC, and blocked the PKC activity with chelerythrine (1×10⁻⁵ mol/L) and observed the phosphorylation of STATs. As shown in Figure 7, PMA caused significant phosphorylation of STAT1 and STAT3. Pretreatment with chelerythrine for 30 minutes significantly attenuated the phosphorylation of STAT1 and STAT3. These results suggested that PKC is partially involved in stretch-induced activation of STATs.

Discussion

In the present study, we demonstrated that mechanical stretch induced rapid phosphorylation of JAK1, JAK2, Tyk2, STAT1, STAT3, and gp130 and induced formation of SIF complexes in rat cardiomyocytes. The stretch-induced activation of STAT1 and STAT3 was partially dependent on JAK2 kinase. We also found that autocrine/paracrine–secreted Ang II was partially involved in stretch-induced phosphorylation of STAT1 but that ET-1 was not involved in this activation. In contrast, the IL-6 cytokine family–related signaling pathway plays an important role in the stretch-induced activation of this pathway in cardiomyocytes. Moreover, mRNA expression of the IL-6 family of cytokines was upregulated by mechanical stretch. NHE activation was involved in the activation of STATs. In contrast, stretch-sensitive cation channels did not appear to be involved in this activation. We also demonstrated that intracellular Ca²⁺ was required for the stretch-induced activation of STATs and that the activation of PKC was partially involved in this activation in cardiomyocytes.

Figure 4. Involvement of gp130-mediated signaling in stretch-induced tyrosine phosphorylation of STATs. A, Effect of RX435 on tyrosine phosphorylation of STAT3. Mouse cardiomyocytes were stretched by 20% for 6 minutes with or without pretreatment with RX435 (10 µg/mL), anti-gp130 blocking antibody, for 30 minutes. Cell lysates were electrophoresed and blotted with anti-phospho-STAT3 antibody, and the membrane was reprobed with anti-STAT3 antibody. Cells were stimulated with LIF (1000 U/mL, lanes 5 and 6) for 6 minutes as a positive control. Note that RX435 inhibited the LIF-induced phosphorylation of STAT3 (lane 6). Stretch-induced phosphorylation of STAT3 was inhibited by RX435. B, Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with antibodies to STAT1α/β or STAT3. Stretch-induced tyrosine phosphorylation of STAT3 was strongly inhibited by RX435, whereas that of STAT1 was only partially inhibited. C, Mechanical stretch-induced phosphorylation of gp130. Rat cardiomyocytes were stretched for the indicated times. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immonoblotted with anti-gp130 antibody. The data presented are representative of 3 independent experiments. D, Mechanical stretch induces CT-1, IL-6, and LIF mRNA expression. RT-PCR revealed that mechanical stretch induced mRNA expression of IL-6 family of cytokines in cardiomyocytes. Abbreviations as in Figure 2.
Ang II and ET-1 have been shown to play an important role in mediating stretch-induced cardiac hypertrophy in cardiomyocytes. Thus, we investigated whether the autocrine/paracrine–secreted Ang II and/or ET-1 was involved in the activation of STATs. We have previously reported that Ang II phosphorylated JAK2, Tyk2, STAT1, and STAT2, but not JAK1 or STAT3, at up to 30 minutes and that CV11974 significantly inhibited the Ang II–induced phosphorylation of STAT1.16 In this study, we observed that ET-1 did not cause phosphorylation of STAT3 at least up to 30 minutes and that it slightly tyrosine phosphorylated STAT1 at 2 to 5 minutes. Moreover, we observed that the phosphorylation of STAT1 was completely inhibited by TAK044. These findings indicated that autocrine/paracrine–secreted Ang II or ET-1 might be involved in the stretch-induced phosphorylation of STAT3 but would not be involved in that of STAT3. The present study revealed that autocrine/paracrine–secreted Ang II was partially involved in the stretch-induced phosphorylation of STAT1; however, we could not confirm that ET-1 was involved in this activation. Moreover, the present findings on STAT3 indicated that factors other than Ang II and ET-1 were involved in the activation of STATs induced by mechanical stretch.

We and others have previously demonstrated that LIF causes cardiac hypertrophy and activated the JAK/STAT pathway.13,14 The other IL-6–related cytokines, including CNTF, oncostatin M, and CT-1, also activate the JAK/STAT pathway via gp130.15,21,22 gp130 functions as a common cytokine signal transducer for the IL-6 family of cytokines, and targeting of the gp130 gene leads to the failure of myocardium to mature.23 Moreover, transgenic mice expressing both IL-6 and IL-6 receptor displayed constitutive tyrosine phosphorylation of gp130 in the myocardium and led to cardiac hypertrophy.24 These findings suggested that a gp130-dependent signaling pathway might be critically involved in the hypertrophic response of cardiomyocytes. In this study, we demonstrated that the gp130-dependent pathway plays an important role in mechanical stretch-induced activation of the JAK/STAT pathway. Pretreatment with RX435 significantly reduced the phosphorylation of STAT1 and STAT3, especially that of STAT3. We also found that gp130 receptor was rapidly phosphorylated by stretching. The finding that the phosphorylation of STAT3 was almost
completely inhibited by RX435 suggested that this family of cytokines plays a pivotal role in stretch-induced activation of STAT3. In contrast, given that the phosphorylation of STAT1 was partially inhibited by both RX435 and CV11974, it seems that both Ang II and the IL-6 family of cytokines might be involved in the activation of STAT1 in cardiomyocytes.

Recent studies have revealed that ion channels and ion exchangers in the cell membrane can rapidly respond to extracellular stimuli and play a pivotal role in fundamental cell functions. Therefore, there is the possibility that the JAK/STAT pathway is directly activated by mechanical stretch or indirectly activated by these upstream modulators. By using a nonselective cation channel blocker, gadolinium, we demonstrated that the stretch-sensitive cation channels nonselective for Na\(^+\) and K\(^+\) were not involved in stretch-induced phosphorylation of STATs. In contrast, the NHE inhibitor HOE642 significantly reduced the phosphorylation of STAT1 and STAT3. Accumulating evidence has demonstrated that NHE can be activated by mechanical stretch, and the activation of NHE can increase H\(^+\) efflux and Na\(^+\) influx. Na\(^+\) entering the cell via NHE activation may be exchanged for Ca\(^{2+}\) via an Na\(^+\)/Ca\(^{2+}\) exchanger, leading to an increase in intracellular Ca\(^{2+}\) concentrations. Dose-dependent effects of BAPTA-AM demonstrated that certain levels of intracellular Ca\(^{2+}\) were required for the stretch-induced phosphorylation of STAT1 and STAT3. This suggests that the NHE might be involved in the stretch-induced phosphorylation of STATs through increasing Ca\(^{2+}\) levels. To our knowledge, this is the first report to show that intracellular calcium is required for the

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**Figure 7.** Role of PKC in stretch-induced activation of STATs. Cardiomyocytes were stretched by 20% for 6 minutes with or without pretreatment with chelerythrine (10 \(^{-5}\) mol/L) for 30 minutes. Cells were also stimulated with PMA (10 \(^{-6}\) mol/L) for 120 minutes (right lanes). Phosphorylation of STAT1 and STAT3 was partially inhibited by chelerythrine. Abbreviations as in Figure 2.

**Figure 8.** Proposed mechanism of stretch-induced activation of the JAK/STAT pathway in cardiomyocytes. IP \(_3\) indicates inositol triphosphate.
tyrosine phosphorylation of STATs. The precise mechanism needs to be clarified in the future (see proposed mechanism in Figure 8).

The JAK/STAT pathway was previously identified as a direct signaling pathway, linking cytokine and growth factor receptors directly to the nucleus. Recent studies have demonstrated interference between the JAK/STAT pathway and other known signaling pathways. Initially, tyrosine phosphorylation of STAT was thought to be the sole function of JAKs, but many lines of evidence have demonstrated that tyrosine phosphorylation of STAT can occur even in the absence of JAK kinase activity. Intrinsically receptor kinase activity of the growth factor receptors or intracellular kinases such as v-src and v-abl were demonstrated to induce phosphorylation of STAT directly or through the activation of JAK kinases. These results suggested that a cross talk existed between the JAK/STAT pathway and other different signaling pathways. In the present study, we have shown that the stretch-induced phosphorylation of STAT1 and STAT3 was partially dependent on PKC activity. A previous report showed that PKC was activated by mechanical stretch. The precise mechanism by which PKC modulates the mechanical stretch-induced phosphorylation of STATs remains unknown. There is the possibility of cross talk between the JAK/STAT pathway and PKC or the existence of other molecules upstream of the STATs. Further studies are needed to clarify this mechanism.

The mechanical stretch of cultured cardiomyocytes in vitro is not a perfect model of in vivo pressure overload and has some drawbacks when used as a surrogate for hypertrophic signaling, given that pressure overload-induced hypertrophy takes several days in animals and years in humans. We reported that acute pressure overload activated the JAK/STAT pathway via the Ang II–dependent and –independent pathway in rat heart. It should be clarified whether chronic pressure overload causes constitutive activation of this pathway in vivo.

In conclusion, the JAK/STAT pathway was activated by mechanical stretch, and this activation was partially dependent on autocrine/paracrine–secreted Ang II and was mainly dependent on the IL-6 family of cytokines but was independent of ET-1. Moreover, certain levels of intracellular Ca2+ were necessary for stretch-induced activation of this pathway, and PKC was also partially involved in this activation (Figure 8).

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