Integrins Play a Critical Role in Mechanical Stress–Induced p38 MAPK Activation

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Abstract—Mechanical stress activates various hypertrophic responses, including activation of mitogen-activated protein kinases (MAPKs) in cardiac myocytes. Stretch activated extracellular signal–regulated kinases partly through secreted humoral growth factors, including angiotensin II, whereas stretch-induced activation of c-Jun NH$_2$-terminal kinases and p38 MAPK was independent of angiotensin II. In this study, we examined the role of integrin signaling in stretch-induced activation of p38 MAPK in cardiomyocytes of neonatal rats. Overexpression of the tumor suppressor PTEN, which inhibits outside-in integrin signaling, strongly suppressed stretch-induced activation of p38 MAPK. Overexpression of focal adhesion kinase (FAK) antagonized the effects of PTEN, and both tyrosine residues at 397 and 925 of FAK were necessary for its effects. Stretch induced tyrosine phosphorylation and activation of FAK and Src. Stretch-induced activation of p38 MAPK was abolished by overexpression of FAT and CSK, which are inhibitors of the FAK and Src families, respectively, and was suppressed by overexpression of a dominant-negative mutant of Ras. Mechanical stretch–induced increase in protein synthesis was suppressed by SB202190, a p38 MAPK inhibitor. These results suggest that mechanical stress activates p38 MAPK and induces cardiac hypertrophy through the integrin-FAK-Src-Ras pathway in cardiac myocytes. (Hypertension. 2002;39:233-238.)

Key Words: hypertrophy, cardiac n myocytes n stress, mechanical n integrins n protein kinases

Mechanical stress plays a crucial role in tissue morphogenesis and remodeling. Changes of cell morphology, which rely on the organization of cytoskeletal components and the modulation of cell adhesion, evoke specific intracellular signals from cell adhesion receptors and induce the expression of specific genes.$^{1,2}$ We and others have developed an in vitro system by which cultured cardiac myocytes are subjected to mechanical stress, and have demonstrated that mechanical stress induces a variety of hypertrophic responses such as activation of mitogen-activated protein kinases (MAPKs),$^{3-5}$ reprogramming of gene expressions,$^{4,6}$ and an increase in protein synthesis.$^{5,7,8}$ MAPKs—including extracellular signal–regulated kinases (ERKS), c-Jun NH$_2$-terminal kinase (JNK), and p38 MAPK—play pivotal roles in a variety of cell functions in many cell types.$^{9,10}$ Although ERKs were activated by mechanical stretch partially through enhanced secretion of angiotensin (Ang) II and endothelin-1,$^{11-13}$ JNK was strongly activated by stretch independently of Ang II,$^{1}$ suggesting that JNK might be directly activated by mechanical stress. p38 MAPK—which consists of $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-isoforms—was first isolated as a mammalian homolog of yeast HOG1,$^{14}$ which is activated by mechanical stresses, including osmotic stress.$^{15}$ The $\alpha$- and $\beta$-isoforms of p38 MAPK induce apoptosis and hypertrophy, respectively, in cardiac myocytes.$^{16}$ In addition, it has recently been reported that cardiac hypertrophy is induced by p38 MAPK but is suppressed by JNK,$^{17}$ and that SB203580, a specific p38 MAPK inhibitor, inhibits the myofibrillar organization and hypertrophic cell profile in neonatal rats.$^{18}$ These results suggest that p38 MAPK might play a critical role in the development of cardiac hypertrophy in response to mechanical stress.

When cardiac myocytes cultured on collagen- or laminin-coated silicone membranes were stretched, a variety of hypertrophic responses were induced.$^{4,6-8}$ It remains unclear, however, how mechanical stress is received and converted into intracellular biochemical signals. Integrins, which are receptors of extracellular matrix (ECM), have been considered as one of the candidates for potential receptors of mechanical stress. Although we observed that stretch activates focal adhesion kinase (FAK), an integrin-associated kinase,$^{19}$ it has been difficult to determine if integrin plays a critical role in mechanical stress–induced hypertrophic responses because of the lack of good strategies to inhibit the
only outside-in signals of integrins. PTEN was isolated as a tumor suppressor gene product and encodes a 403-amino-acid polypeptide with a high degree of homology to protein tyrosine phosphatases and tensin, a protein associated with the actin cytoskeleton at focal adhesion. Both genetic and biochemical studies have indicated that PTEN is involved in the regulation of several different cellular processes such as cell growth, ECM interactions, and cell migration. PTEN has been shown to inhibit integrin-mediated cell migration and cell spreading by inhibiting only outside-in signals but not inside-out signals of integrins through dephosphorylation of FAK.

FAK, a nonreceptor protein tyrosine kinase, colocalizes with integrins at sites of cell attachment to ECM proteins and plays an important role in regulating cell migration and cell survival. FAK is activated by cell binding to ECM proteins, by overexpression of the β-integrin cytoplasmic domains, and by various growth factors. Thus, FAK may play a pivotal role in integrating the cellular responses to multiple extracellular stimuli. Recent studies have indicated that nonreceptor protein tyrosine kinases such as FAK and Src play a critical role in hypertrophic growth regulation by their association with cytoskeletal structures. When FAK is activated, Src is recruited and phosphorylates FAK on tyrosine residue 925, creating a binding site for a Grb2-Sos complex. Grb2 and Sos induce activation of Ras/ERK pathway. In the present study, we elucidated the mechanism of how mechanical stretch induces cardiac hypertrophy by examining the signal transduction pathway leading to activation of p38 MAPK in cardiac myocytes. Our results demonstrate that integrin-mediated outside-in signaling plays a critical role in stretch-induced cardiomyocyte hypertrophy through activation of p38 MAPK.

Methods

Anti-p38 MAPK and anti–phospho-specific p38 MAPK were purchased from New England Biolabs. Anti-Flag monoclonal antibody was from Kodak Co. Anti–c-Src, anti-Grb2, and anti-FAK antibodies were from Santa Cruz Biotech. Enolase, myelin basic protein, and other reagents were purchased from Sigma Chemical Co.

Cell Culture and Amino Acid Uptake

Primary cultures of cardiac myocytes were prepared from ventricles of 1-day-old Wistar rats as previously described, and the relative amount of protein synthesis was determined by assessing the incorporation of the radioactivity into a trichloroacetic acid–insoluble fraction as previously reported. Uniaxial single strain was applied by stretching the silicone dish as previously described.

Transfection

Flag-tagged p38 MAPK (Flag–p38 MAPK) was a gift from M. Karin. C-terminal Src kinase (CSK) and the dominant-negative mutant of Tec were provided by H. Sabe and H. Mano, respectively. Twenty-four hours after plating the cells on culture dishes, we transfected DNA by using the calcium phosphate method as previously described.

Results

Mechanical Stress Activates p38 MAPK

We first examined whether mechanical stress activated p38 MAPK in cultured cardiac myocytes of neonatal rats by Western blot analysis using a phosphorylated p38 MAPK–specific antibody as reported previously. When cardiac myocytes cultured on a deformable silicone dish were stretched by 20%, p38 MAPK activity was increased from 5 minutes, peaked at 15 minutes, and gradually returned to basal levels within 60 minutes (Figure 1A). The increase in p38 MAPK activity was proportional to the degree of stretching (10% to 30%) (Figure 1B). Although stretch-induced activation of ERKs depends in part on secreted humoral growth factors, including Ang II and endothelin-1, pretreatment with the Ang II type I receptor–specific antagonist CV11974 or endothelin-1 receptor antagonist had no inhibitory effects on stretch-induced activation of p38 MAPK.

Immuno precipitation and Western Blotting

To analyze p38 MAPK phosphorylation, we performed Western blot analysis using polyclonal anti-phospho p38 MAPK and anti-p38 MAPK antibodies (New England Biolabs). Activation of FAK and Src was examined as previously described.

Kinase Assay of Transfected Flag–p38 MAPK

After stimulation, Flag–p38 MAPK was immunoprecipitated with an anti-Flag monoclonal antibody, resuspended in 25 μL of the kinase buffer, and incubated with 25 μg myelin basic protein as a substrate at 25°C for 10 minutes. After incubation, the reaction was terminated by adding Laemmli’s sample buffer to the samples. The supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gel was dried and subjected to autoradiography.

Figure 1. Mechanical stretch activates p38 MAPK in cardiac myocytes. A, Time course of p38 MAPK activation. Cardiac myocytes were stretched by 20% for the indicated periods of time or for 15 minutes after pretreatment with 10 μM CV11974 for 30 minutes. B, Stretch-extent dependence of p38 MAPK activation. Cardiac myocytes were stretched for 30 minutes by the indicated lengths expressed in percentage. A and B, Lower panels show relative quantities of total p38 MAPK as determined by anti-p38 MAPK blotting. Cells were lysed in 100 μL of lysis buffer, and 20 μL of lysates was subjected to Western blot analysis using a total p38 MAPK–specific antibody and a polyclonal phospho p38 MAPK–specific antibody sequentially. Total p38 MAPK and phosphorylated p38 MAPK was visualized by the ECL detection system.
PTEN Suppressed Stretch-Induced Activation of p38 MAPK by Inactivating FAK

To elucidate the role of integrin in stretch-induced hypertrophic responses, we overexpressed PTEN with Flag–p38 MAPK in cardiac myocytes. PTEN, a protein tyrosine phosphatase associated with the actin cytoskeleton at focal adhesion, has been reported to inhibit outside-in signals but not inside-out signals of integrins.20–23 Although a dominant-negative mutant of PTEN had no effects, overexpression of PTEN suppressed stretch-induced activation of p38 MAPK (Figure 2A). PTEN has been reported to interact with and inactivate FAK in some cell types.23 To elucidate whether FAK was involved in PTEN-induced inactivation of p38 MAPK, we overexpressed FAK and PTEN in cardiac myocytes. Overexpression of wild-type FAK activated p38 MAPK and partially antagonized the inhibitory effects of PTEN on stretch-induced activation of p38 MAPK (Figure 2B). Recent evidence indicates that PTEN downregulates phosphatidylinositol 3-kinase (PI 3-K) activities and phosphatidylinositol 3,4,5-triphosphates levels.25 Pretreatment with wortmannin, a specific inhibitor of PI 3-K, however, did not affect stretch-induced activation of p38 MAPK (data not shown). These results suggest that PTEN suppressed stretch-induced activation of p38 MAPK by inactivating FAK but not by inhibiting PI 3-K.

Tyrosine Residues at 397, 454, and 925 of FAK Were Crucial for Stretch-Induced p38 MAPK Activation

We next examined whether mechanical stretch activated FAK in cardiac myocytes. Stretch of cells by 20% rapidly activated p38 MAPK (Figure 3A). The levels of p38 MAPK phosphorylation were increased from as early as 2 minutes after stretching. This increase peaked at 10 minutes and declined thereafter. To get insights into how activation of FAK led to stretch-induced p38 MAPK activation, FAK mutants—including F397, F407, F454, F861, and F925 (the tyrosine residue at each position is phosphorylated by stretch)—and FAT (dominant-negative type of FAK) were transiently transfected with Flag–p38 MAPK into cardiac myocytes. Although overexpression of F407 or F861 had no effects on stretch-induced activation of p38 MAPK, overexpression of F397, F454, F925, and FAT strongly suppressed the activation in cardiac myocytes (Figure 3B). These results suggest that the tyrosine residues at 397, 454, and 925 were important for stretch-induced signal transduction, leading to p38 MAPK activation. It has been reported that phosphorylation of the tyrosine residue at 454 is essential for activation of FAK and that activation of integrin induces phosphorylation of Tyr-397 of FAK, resulting in recruitment of c-Src to FAK.28 The formation of an FAK–c-Src complex phosphorylates FAK at Tyr-925, thus enhancing FAK activity and recruiting Grb2 to the complex.26

Src and Ras Are Necessary for Mechanical Stretch-Induced Activation of p38 MAPK

Studies using FAK mutants have suggested involvement of Src in stretch-induced p38 MAPK activation. It has been reported that Src family kinases are important signaling molecules from integrin to Ras and that c-Src and Ras play a critical role in the development of cardiac hypertrophy.4,27 To elucidate the role of Src family kinases, we cotransfected CSK+ with Flag–p38 MAPK.32 Overexpression of CSK+ strongly inhibited stretch-induced p38 MAPK activation, whereas overexpression of kinase-negative CSK (CSK−) or dominant-negative mutant of Tec, non-src type cytoplasmic protein tyrosine kinases31 had no effect (Figure 4A).

We next examined whether mechanical stretch activated Src in cardiac myocytes. Western blot analysis using anti-phosphotyrosine antibody revealed that auto-phosphorylation of Src was observed from as early as 2 minutes after mechanical stretch (Figure 4B). The Src kinase activity measured using enolase as a substrate was also transiently increased by stretch in cardiac myocytes (Figure 4B). Because the formation of an FAK–c-Src complex recruits Grb2 to the complex and activates Ras protein,28 we next examined whether stretch-induced binding of Grb2 to FAK and whether

Figure 2. PTEN suppressed stretch-induced activation of p38 MAPK by inactivating FAK. Flag-p38 MAPK was immunoprecipitated from cell lysates using a monoclonal antibody against Flag, and the kinase activity was measured using myelin basic protein as the substrate. A, After transfection of Flag-p38 MAPK with either PTEN or dominant-negative PTEN (D.N.PTEN), cardiac myocytes were stretched by 20% for 15 minutes. B, After transfection of Flag–p38 MAPK with PTEN and/or FAK, cardiac myocytes were stretched by 20% for 15 minutes.

Figure 3. Tyrosine residues at 397, 454, and 925 on FAK were crucial for stretch-induced p38 MAPK activation. A, After the cells were stretched, FAK was immunoprecipitated with the antibody against FAK and then immunoblotted with anti-phosphotyrosine antibody (4G10) or with anti-FAK antibody. B, After transfection of Flag–p38 MAPK with various FAK mutants, cardiac myocytes were stretched by 20% for 15 minutes. The activity of p38 MAPK was measured as described in the legend for Figure 2.
Ras was involved in stretch-induced activation of p38 MAPK. Western blot analysis using anti-Grb2 antibody after immunoprecipitation with anti-FAK antibody showed that Grb2 binding to FAK was induced by stretch within 2 minutes (Figure 4C). Overexpression of dominant-negative mutant of Ras abolished stretch-induced activation of p38 MAPK (Figure 4D), suggesting that stretch activated Ras by recruiting Grb2 to FAK.

**p38 MAPK Plays a Critical Role in Stretch-Induced Cardiomyocyte Hypertrophy**

We finally examined the role of p38 MAPK in stretch-induced cardiomyocyte hypertrophy. Mechanical stretch increased phenylalanine incorporation into cardiac myocytes by \( \approx 1.5 \)-fold compared with that of unstretched cardiomyocytes (Figure 5). This increase was significantly suppressed by pretreatment with SB202190, an inhibitor of p38 MAPK (Figure 5), suggesting that p38 MAPK plays a critical role in protein synthesis induced by stretch.

**Discussion**

A growing body of evidence has suggested that MAPKs play vital roles in many cell functions, including proliferation and differentiation. In particular, 3 subfamilies of MAPKs—ERK, p38 MAPK, and JNK—have been extensively characterized. They are regulated by distinctive signal transduction pathways and have different functions.

Vasoactive peptides, including Ang II and endothelin-1, partly mediate mechanical stretch–induced activation of ERKs in cardiomyocytes. On the other hand, p38 MAPK and JNK are preferentially activated by distinct stimuli such as the proinflammatory cytokine tumor necrosis factor-\( \alpha \), ultraviolet irradiation, reactive oxygen species, and cellular environmental stresses, including heat shock and osmotic stress. Mechanical stretch increased phenylalanine incorporation into cardiac myocytes, and this increase was significantly suppressed by pretreatment with SB202190, an inhibitor of p38 MAPK (Figure 5), suggesting that p38 MAPK plays a critical role in the cardiac hypertrophic response induced by stretch. Recent studies have shown that activation of p38 MAPK also involved in the signal for anthracycline-induced or ischemia-induced apoptosis.

In contrast, a separate study demonstrated that activation of p38 MAPK can prevent apoptosis of neonatal cardiac myocytes by inducing interleukin-6 expression. 

**Figure 5.** Amino acid uptake by cardiac myocytes. After treatment with SB203580, cardiac myocytes were stretched by 20% for 24 hours. The relative amount of protein synthesis was determined by assessing incorporation of the radioactivity into a trichloroacetic acid–insoluble fraction as previously reported.
by mechanical stress itself without the participation of humoral factors and that p38 MAPK might be a good marker to elucidate the molecular mechanism by which mechanical stress is received and converted into biochemical signals by cardiomyocytes.

A variety of extracellular stimuli induce clustering of integrins and formation of focal adhesions and stress fibers (outside-in signal), and signals generated by newly formed focal adhesions downregulate Rho to prevent excessive formation of focal adhesions (inside-out signal). Integrin is an integrated link between outside and inside of the cell and evokes signals leading to activation of FAK and specific members of the MAPKs. To clarify the role of integrin in mechanical stretch–induced hypertrophic responses, we used PTEN because PTEN is a protein tyrosine phosphatase associated with the actin cytoskeleton at focal adhesion and inhibits outside-in signals but not inside-out signals of integrins. PTEN is a new tumor suppressor gene product, which functions as a dual-specificity phosphatase and lipid phosphatase. It can inhibit cell growth, invasion, migration, and focal adhesion formation by dephosphorylating FAK in many types of cells. Stretch-induced activation of p38 MAPK was markedly suppressed by overexpression of PTEN, and overexpression of FAK partially rescued the PTEN-induced suppression of p38 MAPK activity. In addition, mechanical stretch activated FAK in cardiac myocytes. Stretch-induced FAK activation was not inhibited by CV19974 and BQ123 (data not shown). PTEN has been reported to inhibit PI 3-K activity and suppress the PI 3-K/Akt cell survival pathway. Pretreatment with wortmannin did not have any effect on stretch-induced activation of p38 MAPK, indicating that PTEN suppresses stretch-induced activation of p38 MAPK by inhibiting FAK but not PI 3-K. Although integrins and growth factors may crosstalk or share signaling pathways, leading to stretch-induced p38 MAPK activation, our results suggest that mechanical stress is directly received by integrin and is converted into activation of FAK and that the activation of integrin-FAK might be necessary for stretch-induced hypertrophic responses.

Tyr-397 of FAK was a critical phosphorylation site for stretch-induced p38 MAPK activation. It has been reported that activation of integrin induces auto-phosphorylation at Tyr-397 of FAK, which generates a high-affinity binding site for the SH2 domain of c-Src to FAK. The recruited c-Src phosphorylates Tyr-925 of FAK, which creates a Grb2-associated protein adapter and activated ERKs. Overexpression of F925, a dominant-negative type of FAK, completely suppressed stretch-induced p38 MAPK activation (Figure 3B), suggesting that p38 MAPK activation via integrin also requires phosphorylation of Tyr-925 of FAK.

Recent biochemical and genetic studies on signal transduction of tyrosine kinase receptors have demonstrated that p21Ras serves as a nodal point for transmitting signals from receptor tyrosine kinases to downstream serine/threonine kinase cascades. In addition, it has recently been reported that mechanical stretch induces activation of Ras in cardiac myocytes. We thus examined the role of a small G protein Ras in stretch-induced activation of p38 MAPK. Although Ras is not required for mechanical stretch–induced ERK activation in cardiac myocytes, it is critical for p38 MAPK activation by stretch (Figure 4D). These results may be related to the observation that stretch-induced activation of ERKs, but not of p38 MAPK, depends partly on the Ang II/PKC pathway. Further study is necessary to identify the pathways that are involved in p38 MAPK activation from Ras.

Mechanical stress is one of pivotal stimuli for cells and evokes a wide variety of intracellular signals. It has long been of great interest to know how cells receive mechanical stress and convert the stress into intracellular biochemical signals. Although many molecules such as ion channels and membrane enzymes have been considered as potential stretch receptors, it had been unclear whether they were involved in the development of stretch-induced cardiac hypertrophy. Integrins, the major family of cell surface receptors that mediate cell attachment to the ECM, have also been considered as possible stretch receptors. It was difficult to prove this notion, however, because of lack of good inhibitors. When basal functions of integrins are suppressed by inhibiting inside-out signals, cell attachment to the bottom of culture dishes is impaired. In the present study, we used PTEN, which suppresses only outside-in signaling, and were able to demonstrate for the first time that integrins play a critical role in stretch-induced hypertrophic responses and that integrins are not only static receptors of ECM but also dynamic mecano-receptors.

Acknowledgments

We wish to thank Kaoru Kuwabara and Chika Masuo for their excellent technical assistance. This work was supported by a Grant-in-Aid for Scientific Research, Developmental Scientific Research and Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan (I.K.).

References


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Hypertension. 2002;39:233-238
doi: 10.1161/hy0202.102699

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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