Molecular Mechanism of Mechanical Stress-induced Cardiac Hypertrophy

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SUMMARY

Mechanical stress is a major cause of cardiac hypertrophy. Although the mechanisms by which mechanical load induces cardiomyocyte hypertrophy have long been a subject of great interest for cardiologists, the lack of a good *in vitro* system has hampered the understanding of the biochemical mechanisms. For these past several years, however, an *in vitro* neonatal cardiocyte culture system has made it possible to examine the biochemical basis for the signal transduction of mechanical stress. Passive stretch of cardiac myocytes cultured on silicone membranes activates phosphorylation cascades of many protein kinases including protein kinase C, Raf-1 kinase and extracellular signal regulated kinases, and induces the expression of specific genes as well as an increase in protein synthesis. During that process, the secretion and production of vasoactive peptides such as angiotensin II and endothelin, are increased and they play critical roles in the induction of these hypertrophic responses. Although the involvement of vasoactive peptides in the development of cardiac hypertrophy is clinically important, the “mechanoreceptor” which receives the mechanical stress and converts it into intracellular biochemical signals remained unknown. We have recently obtained evidence suggesting that ion channels and integrins may be the “mechanoreceptor”, the activation of which leads to cardiac hypertrophy. (Jpn Heart J 2000; 41: 117-129)

Key words: Cardiac hypertrophy, Mechanical stress, Angiotensin II, Protein kinase, Ion channel, Integrin

CARDIAC hypertrophy is a very important issue for cardiologists not only because it shows diastolic dysfunction and often leads to congestive heart failure but also because it is an independent risk factor for many cardiac diseases

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such as ischemic heart disease, arrhythmia and sudden death.¹⁾ Hemodynamic overload is a major cause of cardiac hypertrophy, however, the mechanism by which hemodynamic overload induces cardiomyocyte hypertrophy has long been elusive. An *in vitro* system by which mechanical stress is imposed on cultured neonatal cardiocytes has made it possible to examine the signal transduction of mechanical stress-induced cardiac hypertrophy.²⁾ Passive stretch of cardiac myocytes cultured on silicone membranes activates protein kinase cascades of phosphorylation, and induces the expression of specific genes as well as an increase in protein synthesis.²⁻⁴⁾ During this process, the secretion and production of vasoactive peptides such as angiotensin II (Ang II) and endothelin-1 (ET-1), are increased and they play critical roles in the induction of these hypertrophic responses.⁵⁻⁷⁾ These results are consistent with the observation that angiotensin converting enzyme inhibitors and angiotensin II type 1 receptor antagonists efficiently inhibit the development of cardiac hypertrophy in humans. Although both experimental results and clinical observations clearly indicate that vasoactive peptides are critically involved in the generation of cardiac hypertrophy, the "mechanoreceptor" which receives the mechanical stress and converts it into intracellular biochemical signals is still unknown. In this review, the possibility that ion channels and integrins are the "mechanoreceptor" will be discussed.

MECHANICAL STRESS INDUCES CARDIAC HYPERTROPHY

A growing body of evidence has suggested that humoral factors in the circulating blood may have marginal effects on the development of cardiac hypertrophy in response to hemodynamic overload and that mechanical stress induces cardiac hypertrophy via increasing the local production of humoral factors. How is mechanical stress perceived by a cardiomyocyte as a stimulus? With the use of Langendorff preparation, it has been shown that stretch of the ventricular wall as a consequence of increased aortic pressure is the mechanical parameter most closely related to an increase in protein synthesis.⁸⁾ This observation has been confirmed by experiments using cardiocytes cultured *in vitro* with serum-free media. When cardiocytes cultured on deformable silicone membranes were stretched, an increase in protein synthesis was observed.²⁾ Stretching cardiocytes also induced expression of specific genes, such as immediate early response genes (IEG) and fetal type genes.^{3, 4, 9, 10)} These observations suggest that mechanical stress (hemodynamic overload) induces cardiac hypertrophy by stretching cardiomyocytes.

MECHANICAL STRESS ACTIVATES A PROTEIN KINASE CASCADE OF PHOSPHORYLATION

Mitogen-activated protein kinases (MAPK) family: A number of intracellular signals are transduced into a nucleus through a protein kinase cascade of phosphorylation.¹¹⁾ Once a mechanical stimulus is converted into biochemical signals involving phosphorylation events, how do these signals then regulate protein synthesis and gene expression in cardiac myocytes? A growing body of evidence has suggested that the mitogen-activated protein kinase (MAPK) family plays a critical role in cell proliferation, differentiation and death.¹¹⁻¹³⁾ Several members of the MAPK family have been isolated to date and three-extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinase / stress-activated protein kinase (JNK / SAPK) and p38MAPK-have been particularly well characterized.

Extracellular signal regulated kinases (ERKs). ERKs have been reported to be activated by a variety of growth factors, hormones, cytokines and phorbol esters and to play critical roles in cell proliferation and differentiation of many cell types including cardiac myocytes.^{12,13)} ERKs have been reported to be important for the induction of hypertrophic responses including specific gene expression and an increase in protein synthesis.¹⁴⁾ Although the activation of ERKs is neither sufficient nor indispensable for the development of cardiomyocyte hypertrophy,¹⁵⁾ antisense experiments have showed that activated ERKs are critical to induce specific gene expression and sarcomere formation as well as an increase in protein synthesis.¹⁶⁾ Stretching cultured cardiac myocytes by 20% for 10 min activated ERKs.^{17,18)} Stretching also activated its upstream kinases such as Raf-1 kinase and MAPK kinase (MEK).¹⁹⁾ Activation of PKC is known to increase the activity of Raf-1 kinase and ERKs in various cell types.²⁰⁾ When PKC was depleted by preincubating myocytes with phorbol ester or its activity was blocked by PKC-inhibitors, stretch-induced ERK activity was decreased by more than 80%, suggesting that the stretch-induced increase in ERK activity occurs mainly through a PKC-dependent pathway. Although Ras exists upstream of ERKs in many cell types and it was reported that stretch activates Ras,¹⁷⁾ the inhibition of Ras had no effects on stretch-induced ERK activation.²¹⁾ These results suggest that although it is not clear why activated Ras does not lead to activation of ERKs, stretch-induced ERK activation is dependent on PKC but not on Ras. Stretching also activated p90 S6 kinase as a downstream kinase of ERKs.^{17,18)} Although the signaling pathway remains to be determined, p70 S6 kinase was also activated by stretching. Stretch-induced activation of kinases such as PKC, Raf-1, MEK, ERKs, and p90 and p70 S6 kinases were partly suppressed by antagonists for AngII and ET-1 receptors, suggesting the involvement of vasoactive peptides in stretch-induced activation of protein kinases (see section III).

JNK / SAPK. JNK was first reported to be activated in response to cellular stresses such as osmotic stress, ultra violet irradiation, oxidative stress and heat shock.²²⁾ JNK is not activated by authentic growth factors such as EGF and basic FGF but is activated by proinflammatory cytokines including tumor necrosis factor- α (TNF- α). Since there are some similarities between osmotic stress and stretch, we examined whether mechanical stretch activates JNK in cardiac myocytes. Stretching by 20% for 10 min strongly activated JNK in cultured cardiac myocytes.²³⁾ Of interest, unlike ERKs, stretch-induced JNK activation was not inhibited by AngII receptor antagonists. Although vasoactive peptides such as AngII and ET-1 could activate JNK in cultured cardiac myocytes,²⁴⁾ the degree of activation by stretch was several fold stronger than that by AngII. These results suggest that mechanical stress itself may activate JNK. JNK is activated by a dual protein kinase, JNK kinase (JNKK / SEK / MKK4 or 7). MEKK1 is a JNK kinase and is also activated by stretch in cardiac myocytes.¹⁹⁾ Recently Ask1 has been reported to be a JNK kinase.²⁵⁾ We have observed that stretch activates Ask1 and inhibition of Ask1 attenuates stretch-induced JNK activation (unpublished observation). Elucidating the molecular mechanism by which stretch activates JNK may pave the way to understanding how mechanical stress is converted into biochemical signals.

JNK has been reported to be involved in gene regulation by phosphorylating transcription factors such as c-Jun and ATF-2.²²⁾ Recently JNK was reported to be involved in cardiomyocyte hypertrophy.²⁶⁾ Wang *et al.* have reported that specific activation of the JNK pathway in cardiac myocytes induced characteristic features of hypertrophy, including an increase in cell size, elevated expression of atrial natriuretic peptide (ANP) and induction of sarcomere organization and that co-activation of both JNK and p38MAPK in cardiomyocytes led to an induction of cytopathic responses and suppression of hypertrophic responses.²⁶⁾ In contrast, Nemoto *et al.* have reported that expression of constitutive active p38MAPK stimulated the expression of ANP and that activation of p38MAPK was required for ANP expression induced by the hypertrophic agonists.²⁷⁾ Activation of JNK led to inhibition of ANP expression induced by MEKK1 and the hypertrophic agonists. It remains unknown why contradictory results were obtained, however, the effects of JNK and p38MAPK may be dependent on how or how much they are activated or inhibited. Both papers demonstrated that activation of either JNK or p38MAPK promotes cardiac hypertrophy and that both kinases have opposing effects to each other on the development of hypertrophy. Since both JNK and p38MAPK are usually activated by many stimuli, it remains to be determined how JNK and p38MAPK are involved in the development of cardiac hypertrophy. Like ERKs, the precise role of JNK in the development of cardiac hypertrophy is still elusive.

p38MAPK. p38MAPK is usually activated by stimuli similar to those for JNK. Namely, cellular stresses such as osmotic stress, ultraviolet irradiation, oxidative stress and heat shock, and anticancer agents are good stimulators of p38MAPK.²⁸⁾ p38MAPK has been reported to play important roles in the induction of apoptosis and production of cytokines as well as gene regulation.²⁸⁾ Cytokines including interleukin-1 β (IL-1 β), IL-6 and TNF α attracted great attention in the cardiovascular field because they may cause cardiac dysfunction.²⁹⁾ It has been reported that p38MAPK is activated by hypertrophic agonists such as ET-1, phenylephrine (PHE) and leukemia inhibitory factor (LIF).³⁰⁾ As mentioned above, p38MAPK may have opposing effects to JNK on cardiomyocyte hypertrophy, but the precise role of p38MAPK in cardiac hypertrophy is still controversial. Recently Wang, *et al.* have reported interesting results with p38MAPK.³¹⁾ There are four different isoforms of p38MAPK.³²⁾ Activation of p38MAPK γ elicited apoptosis in cardiac myocytes, whereas overexpression of p38MAPK β induces characteristic hypertrophic responses. It remains to be determined how two very similar isoforms elicit completely different effects on cardiac myocytes. Precise mapping of the regions of p38MAPK α and β which are responsible for apoptosis and hypertrophy, respectively, may provide clues.

p70 S6 protein kinase: There are many pathways by which stretch induces cardiomyocyte hypertrophy (see section V). Among them, the phosphorylation of S6 protein in 40S ribosome is well correlated to the efficiency of protein synthesis and p70 S6 protein kinase (p70^{S6K}) is reported to phosphorylate S6 protein *in vivo*.³³⁾ Stretching by 20 % for 15 min activated p70^{S6K} in cardiac myocytes and the inhibition of p70^{S6K} by the specific inhibitor rapamycin strongly suppressed stretch-induced increase in protein synthesis without affecting the activity of ERKs and expression of c-fos.³⁴⁾ These results suggest that p70^{S6K} is deeply involved in stretch-induced cardiomyocyte hypertrophy and that the signaling pathway to p70^{S6K} activation is different from that of ERKs and c-fos. Phosphoinositide-3 kinase (PI3-K) activates p70^{S6K} in many cell types and indeed, the PI3-K specific inhibitor wortmannin abolished stretch-induced activation of p70^{S6K}. However, activation of authentic PI3-K could not be detected after stretch (unpublished observation), suggesting that atypical PI3-K including PI3-K γ may be activated by stretch or p70^{S6K} may be activated through PI3-K independent pathway in cardiac myocytes.

NEUROHUMORAL FACTORS

Protein kinase cascade of phosphorylation activated by mechanical stress on cardiac myocytes is very similar to those evoked by the addition of growth factors and cytokines on many cell types. It is possible that cardiac myocytes and

non-cardiomyocytes such as fibroblasts, endothelial cells and smooth muscle cells secrete some hypertrophy-promoting factors following stretch and that they induce cardiac hypertrophy by an autocrine or paracrine mechanism. Mechanical stress on myocardial cells has been reported to increase the synthesis of some growth-promoting factors.³⁵⁾

Angiotensin II: A growing body of evidence has indicated that the local renin-angiotensin system plays a critical role in the development of cardiac hypertrophy.³⁶⁾ All components of the renin-angiotensin system such as angiotensinogen, renin and angiotensin converting enzyme (ACE) have been identified at both the mRNA and protein levels in the heart. AngII stimulates protein synthesis in cultured cardiomyocytes.³⁶⁾ Increases in angiotensinogen and ACE mRNAs have been reported in hypertrophied left ventricle of rats.³⁷⁾ Subpressor doses of ACE inhibitors can prevent or cause regression of cardiac hypertrophy with no change in systemic systolic blood pressure.³⁸⁾ An increase in left ventricular mass which was produced by abdominal aortic constriction, without a significant increase in plasma renin activity, was completely prevented with an ACE inhibitor without any change in afterload.³⁹⁾ These results strongly suggest that the local renin-angiotensin system but not the circulating one may play a critical role in pressure overload-induced cardiac hypertrophy.

AngII induces activation of ERKs and expression of the *c-fos* gene as well as an increase in protein synthesis in neonatal rat cardiocytes through AngII type 1 receptor (AT1).⁴⁰⁾ Activation of ERKs and up-regulation of *c-fos* gene expression by AngII are blocked by PKC inhibitors, and AngII actually increases production of inositol phosphates and activates PKC.^{40,41)} These signals elicited by AngII in cardiac myocytes are very similar to those evoked by mechanical stress as mentioned above. The involvement of AngII in the stretch-induced hypertrophic responses was examined using AT1-specific inhibitors. AT1 antagonists partially but significantly suppressed all stretch-induced hypertrophic responses such as an increase in amino acid incorporation, the induction of *c-fos* gene expression and the activation of ERKs.^{5,6)} It was reported that AngII is stored in secretory granules in neonatal ventricular myocytes and that mechanical stretch causes a release of AngII from cultured cardiocytes.⁵⁾ Although it remains to be determined how stretch induces release of AngII from the secretory granules in cardiac myocytes, these results suggest that AngII is responsible at least in part for hypertrophic responses evoked by mechanical stress on cardiac myocytes.

Intracellular signaling pathways evoked by AngII are different among cell types. AngII markedly activated ERKs in cardiac myocytes. Although AngII activates Fyn, a Src family tyrosine kinase and Ras,⁴²⁾ inhibition of these molecules had no effects on AngII-induced ERK activation. Instead, down-regulation of PKC or the PKC inhibitor calphostin C abolished AngII-induced

ERK activation.⁴³⁾ AngII also activated Raf-1 kinase and MEK, and the activation of these kinases are also suppressed by inhibition of PKC. Since PKC has been reported to directly activate Raf-1 kinase, AngII activates ERKs possibly through PKC, Raf-1 and MEK. Signaling pathways from AT1 to ERKs are divergent among cell types. In smooth muscle cells, pertussis toxin had no effects but PLC, Ca^{2+} and Src family are critical for AngII-induced activation of ERKs. AngII activates ERKs possibly through $\text{Gq-PLC}\gamma\text{-Ca}^{2+}\text{-Ca}^{2+}$ -dependent tyrosine kinase Pyk2 and Src-Shc-Grb2-Sos-Ras---ERKs.⁴³⁾ Cardiac fibroblasts produce and secrete extracellular matrix proteins such as collagen and fibronectin in response to AngII, which induces fibrosis of the heart. In cardiac fibroblasts, AngII activates ERKs through pertussis toxin sensitive G protein, possibly Gi protein. $\beta\gamma$ subunit of Gi activates Src and then ERKs through Shc-Grb2-Sos-Ras.⁴⁴⁾ It remains to be determined why signaling pathways are so divergent among cell types.

Endothelin-1 (ET-1): ET-1 has been reported to be a strong inducer of cardiac hypertrophy.⁴⁵⁾ Stretch-induced activation of ERKs are also suppressed by type A ET-1 (ETA) receptor antagonist,⁷⁾ suggesting that ET-1 is also involved in mechanical stress-induced hypertrophic responses. Since ET-1-induced activation of ERKs is also inhibited by down-regulation of PKC, ET-1 activates ERKs possibly through the same pathway as AngII. Recently it was reported that AngII does not have direct potent hypertrophy-promoting effects on cardiac myocytes and that AngII may evoke hypertrophic responses in cardiac myocytes by inducing ET-1 secretion from cardiac fibroblasts.⁴⁶⁾ Cross-talk among different cell types and among many molecules might be important not only for maintaining its highly differentiated phenotype but also for inducing cardiac hypertrophy. The beneficial effects of ET-1 receptor antagonists on myocardial infarction highlighted the importance of ET-1 on cardiovascular remodeling.⁴⁷⁾ Recently the importance of Gq protein in the development of pressure overload-induced cardiac hypertrophy was demonstrated using transgenic mice.⁴⁸⁾ Class-specific inhibition of Gq-mediated signaling was produced in the heart of transgenic mice by targeted expression of a carboxyl-terminal peptide of the α subunit of Gq. When pressure overload was surgically produced, the transgenic mice developed significantly less ventricular hypertrophy than control mice. Although the prevention of cardiac hypertrophy was not complete in the transgenic mice, this study suggests that Gq-coupled receptors such as AT1, ETA receptor and seven other transmembrane receptors are important in the development of pressure overload-induced cardiac hypertrophy.

MECHANISMS BY WHICH MECHANICAL STRESS IS CONVERTED INTO BIOCHEMICAL SIGNALS

Stretch-Sensitive Ion Channels and Exchangers: Many cells respond to a variety of environmental stimuli by ion channels in the plasma membrane. Mechanosensitive ion channels have been observed with single-channel recordings in more than 30 cell types of prokaryotes, plants, fungi and all animals so far examined.⁴⁹⁾ The activation of stretch-sensitive channels has been proposed as the transduction mechanism between load and protein synthesis in cardiac hypertrophy.⁵⁰⁾ The stretch-sensitive channels allow the passage of the major monovalent physiological cations, Na⁺ and K⁺, and the divalent cation, Ca²⁺. With the use of a Ca²⁺-binding fluorescent dye (fluo3) and the patch clamp technique, mechanically induced Ca²⁺ influx through stretch channels was shown to lead to waves of calcium-induced calcium release.⁵¹⁾ Therefore, stretch-sensitive ion channels are one good candidate as an initial responder for mechanical stress.

The importance of Ca²⁺ in the development of cardiac hypertrophy was recently highlighted.⁵²⁾ Overexpression of constitutively active mutants of a Ca²⁺-dependent phosphatase calcineurin and of its downstream transcription factor NFAT3 induced marked cardiac hypertrophy in transgenic mice and phenylephrine- and Ang II-induced cardiomyocyte hypertrophy in vitro was inhibited by calcineurin inhibitors.⁵²⁾ These observations suggest that an increase in intracellular Ca²⁺ levels could induce cardiac hypertrophy by activating calcineurin. We examined this possibility using the in vitro stretch device. When cultured cardiac myocytes were stretched by 20 % for 30 min, brain natriuretic peptide (BNP) gene was upregulated. The upregulation of the BNP gene was suppressed by Gadolinium (an inhibitor of stretch-sensitive ion channels) and attenuated by FK506 (a calcineurin inhibitor). These results suggest that Ca²⁺, which enters cardiomyocytes through stretch-sensitive ion channels, plays a critical role in stretch-induced gene expression.

Extracellular Matrix and Cytoskeleton: A lot of data indicating that mechanical stress is transduced into the cell from sites at which cells attach to the extracellular matrix (ECM) have been accumulated.⁵³⁾ Therefore, transmembrane ECM receptors, such as the integrin family, are good candidates for mechanoreceptors. The large extracellular domain of the integrin receptor complex binds various ECM proteins, while the short cytoplasmic domain has been shown to interact with the cytoskeleton in the cell.⁵⁴⁾ Since cytoskeleton proteins can potentially regulate plasma membrane proteins such as enzymes, ion channels and antiporters, mechanical stress could modulate these membrane associated proteins and stimulate second messenger systems through the cytoskeleton. Integrins can

transmit signals not only by organizing the cytoskeleton but also by altering biochemical properties such as the extent of tyrosine phosphorylation of a complex of proteins including pp125^{FAK}.⁵³⁾ Stretching cultured mesangial cells actually enhanced phosphorylation of pp125^{FAK}.⁵⁵⁾ We have recently obtained results indicating that integrin plays a critical role in stretch-induced hypertrophic signals. When outside-in signal of integrin was inhibited, stretch-induced activation of p38MAPK was strongly suppressed. Stretching induced tyrosine phosphorylation of both FAK and Src. Stretch-induced activation of p38MAPK was abolished by overexpression of FAT and CSK, which are inhibitors of FAK and the Src family, respectively. These results suggest that mechanical stress activates p38MAPK through the integrin-FAK-Src pathway in cardiac myocytes.

The Rho small G protein family, consisting of Rho, Rac and Cdc42, is important for the function of integrins through regulating actin cytoskeleton.⁵⁶⁾ When the functions of the Rho family were inhibited by C3 exoenzyme or by overexpression of Rho GDP dissociation inhibitor and dominant negative mutants, stretch-induced activation of ERKs and expression of IEG were strongly suppressed.²¹⁾ Recently it has been reported that C3 exoenzyme inhibits MEKK- and PHE-induced ANP expression but not actin organization, indicating that Rho regulates ANP gene expression but not sarcomere assembly.⁵⁷⁾ Sah *et al.* also reported that Rho is involved in PHE- and G α_q -induced ANP expression but not in Ras- induced ANP expression.⁵⁸⁾ Activated RhoA and Ras produced synergistic effects on ANP gene expression, suggesting that Rho functions in a pathway separate from but complementary to Ras. Treatment of myocytes with AngII caused the formation of nonstriated actin fiber (premyofibrils), which was abolished by the C3 treatment.⁵⁹⁾ Expression of constitutively active RhoA caused the formation of premyofibrils. These results suggest that RhoA is involved in premyofibril formation but not in mature myofibril formation.

FUTURE DIRECTIONS

Once mechanical stress is received and converted into biochemical signals, the signal transduction pathway leading to an increase in protein synthesis is similar to the pathway which is activated by various humoral factors such as growth factors, hormones and cytokines in many other cells. Recently, yeast genes encoding members of MAP kinase have been isolated by complementation of yeast mutations as an essential protein for restoring the osmotic gradient across the cell membrane in response to increased external osmolality.⁶⁰⁾ This result suggests that mechanical stress-induced intracellular signal transduction pathways are highly conserved in evolution.

Although many biochemical events which occur in cardiac myocytes

subsequent to mechanical stretch have been clarified, an intriguing question remains unanswered. How is mechanical stress converted into biochemical signals? In other words, what is the mechanoreceptor or the transducer for mechanical stress in cardiac myocytes? It is conceivable that by stretching the plasma membrane, mechanical stress directly changes conformations of the functional proteins in the membrane such as ion channels, enzymes and G-proteins, or directly activates enzymes such as phospholipases by physically placing the enzymes close to their phospholipid substances in the plasma membrane. As mentioned above, the integrin-cytoskeleton complex seems to be an alternate candidate structure for a mechanoreceptor and a transducer. Integrin-cytoskeleton proteins not only play a "passive" role such as maintaining the cell structure, but also may play "active" roles in the regulation of cellular functions such as protein phosphorylation and activation of an antiporter.⁶¹⁾ The cytoskeleton has been also shown to play an important role in secretion. Mechanical stress may stimulate secretion of some cytokines, which generate multiple intracellular signals as a secondary event. Further studies are necessary to identify specific signaling molecules, including mechanoreceptors and mechanotransducers, and characterization of these molecules may pave the way to an understanding of the mechanism of mechanical stress-induced cardiac hypertrophy and to finally clarify the mechanisms by which adaptive cardiac hypertrophy deteriorates into congestive heart failure.

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