Mechanical Stretch Induces Hypertrophic Responses in Cardiac Myocytes of Angiotensin II Type 1a Receptor Knockout Mice*

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Many lines of evidence have suggested that angiotensin II (AngII) plays an important role in the development of cardiac hypertrophy through AngII type 1 receptor (AT1). To determine whether AngII is indispensable for the development of mechanical stress-induced cardiac hypertrophy, we examined the activity of mitogen-activated protein kinase (MAPK) family and the expression of the c-fos gene as hypertrophic responses after stretching cultured cardiac myocytes of AT1a knockout (KO) mice. When cardiac myocytes were stretched by 20% for 10 min, extracellular signal-regulated protein kinases (ERKs) were strongly activated in KO cardiomyocytes as well as wild type (WT) myocytes. Both basal and stimulated levels of ERKs were higher in cardiomyocytes of KO mice than in those of WT mice. Activation of another member of the MAPK family, p38MAPK, and expression of the c-fos gene were also induced by stretching cardiac myocytes of both types of mice. An AT1 antagonist attenuated stretch-induced activation of ERKs in WT cardiomyocytes but not in KO cardiomyocytes. Down-regulation of protein kinase C inhibited stretch-induced ERK activation in WT cardiomyocytes, whereas a broad spectrum tyrosine kinase inhibitor (genistein) and selective inhibitors of epidermal growth factor receptor (tyrphostin, AG1478, and B42) suppressed stretch-induced activation of ERKs in KO cardiac myocytes. Epidermal growth factor receptor was phosphorylated at tyrosine residues by stretching cardiac myocytes of KO mice. These results suggest that mechanical stretch could evoke hypertrophic responses in cardiac myocytes that lack the AT1 signaling pathway possibly through tyrosine kinase activation.

Recent clinical studies have suggested that cardiac hypertrophy is an independent risk factor of cardiac morbidity and mortality (1). Therefore, it has become even more important to clarify the mechanism of how cardiac hypertrophy is developed.

Cardiac hypertrophy is induced by a variety of factors such as mechanical load (2–5) and neurohumoral factors (6–9). Among humoral factors, angiotensin II (AngII)† has recently attracted great attention, because of its established importance in vivo as well as in vitro (6, 10). AngII directly induces cardiomyocyte hypertrophy, independent of an increase in vascular resistance or cardiac afterload (6, 10). A growing body of evidence has suggested that locally produced AngII, more than circulating AngII, is a pivotal stimulator of cardiac hypertrophy and that hemodynamic overload induces cardiac hypertrophy by activating the local renin-angiotensin system (6, 10). Baker et al. (11) have reported that an increase in left ventricular mass induced by constricting the abdominal aorta is completely prevented by an angiotensin-converting enzyme inhibitor without decreasing the arterial pressure. Moreover, it has been reported that mechanical stress stimulates secretion of AngII from cardiac myocytes and that the AngII induces cardiomyocyte hypertrophy through AngII type 1 (AT1) receptor (12). However, because AT1-specific antagonists only partially inhibit stretch-induced hypertrophic responses (13), signaling pathways other than AngII might be involved in mechanical stress-induced hypertrophy. Indeed we have reported that another vasoactive peptide endothelin-1 (ET-1) is also involved in stretch-induced cardiomyocyte hypertrophy (14). More recently, it has been reported that passive load and AngII evoke differential responses in gene expressions and protein synthesis (15). Therefore, it remains to be determined whether AngII is really necessary for the development of cardiac hypertrophy induced by mechanical stress.

Many reports have suggested that the mitogen-activated protein kinase (MAPK) family plays an important role in the differentiation and proliferation of many cells (16). One member of the MAPK family, extracellular signal-regulated protein kinase (ERK), has been reported to be required for phenylephrine-induced expression of specific genes such as atrial natriuretic factor, c-fos, and myosin light chain 2 gene (17, 18). Although activation of ERKs is not sufficient to fully promote cardiac hypertrophy (18, 19), recent evidence using an antisense oligodeoxynucleotide has shown that ERKs are necessary for phenylephrine-induced sarcomerogenesis and increase in cell size (20). We and others have reported that mechanical stress activates ERKs in cultured cardiac myocytes (13, 21–23). Moreover, we have recently reported that another member of

† The abbreviations used are: AngII, angiotensin II; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; ET, endothelin; KO, knockout; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PCR, polymerase chain reaction; PKC, protein kinase C; RT, reverse transcriptase; TPA, 12-O-tetradecanoylethano-13-acetate; WT, wild type.
the MAPK family, c-Jun N-terminal kinase, which has been reported to be activated by various cellular stresses and to play an important role in gene expression and apoptosis in many cell types (24, 25), is also activated in cardiac myocytes by mechanical stress (26) and AngII (27). The third member of the MAPK family is p38MAPK, a mammalian homolog of yeast Hog1 that is activated by osmotic stress (28). p38MAPK has been reported to be activated by a variety of stimuli similar to those for c-Jun N-terminal kinase (25, 28) and to be involved in secretion of cytokines and in development of cardiomyocyte hypertrophy and apoptosis (29).

In the present study, we elucidated the role of AngII in mechanical stress-induced hypertrophic responses by examining the activation of the MAPK family and the expression of the c-fos gene using cardiac myocytes of AT1a knockout (KO) neonatal mice (30). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that although AT2 was expressed at almost the same levels in both wild type (WT) and KO cardiomyocytes. AT1a and AT1b were not detected in KO cardiac myocytes by 40-cycle amplification. The addition of AngII to cultured media activated ERKs in cardiac myocytes of WT mice but not those of KO mice, suggesting that signals through AT1 were markedly reduced in KO cardiac myocytes. When cardiac myocytes were stretched, however, ERKs and p38MAPK were markedly activated in cardiomyocytes of KO mice as well as those of WT mice. Unexpectedly, basal and stimulated levels of ERKs, but not of p38MAPK, were higher in KO cardiomyocytes than in WT cardiomyocytes. Mechanical stretch also induced expression of the c-fos gene in cardiac myocytes of both mice. Unlike WT cardiac myocytes, an AT1 antagonist had no effects on stretch-induced activation of ERKs in KO cardiac myocytes. Down-regulation of protein kinase C (PKC) strongly attenuated stretch-induced ERK activation in WT cells, whereas in KO cells, tyrosine kinase inhibitors, but not PKC down-regulation, inhibited ERK activation by stretch. Moreover, EGF receptor was phosphorylated by stretching cardiac myocytes of KO mice. These results suggest that AngII is not indispensable for mechanical stretch-induced hypertrophic responses and that the tyrosine kinase pathway plays a critical role in cardiac myocytes where signals from AT1 are deleted.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stretching of Cardiac Myocytes**—Cardiac myocytes were cultured from 1-day-old neonatal mice of AT1a KO and WT as described previously (4). Stretching of cardiac myocytes by 20% was conducted as described previously (4). In brief, cardiac myocytes were plated at a density of 1 \times 10^4 cells/cm^2 on silicone rubber culture dishes. The culture medium was changed 24 h after seeding to a solution consisting of Dulbecco’s modified Eagle’s medium containing 0.1% fetal bovine serum. Mice were housed under climate-controlled conditions with a 12-h light/dark cycle and were provided with standard food and water ad libitum as described previously (30). All protocols were approved by local institutional guidelines.

**RT-PCR Analysis**—Total RNA was prepared from cardiac myocytes using RNA STAT-60 (TEL-TEST B, Inc.) according to the manufacturer’s instructions. The prepared RNA was treated with DNase to eliminate any contamination of genomic DNA. Reverse transcribed AT1a, AT1b, and AT2 mRNA were amplified by PCR using AT1a-, AT1b-, and AT2-specific primers, respectively. Specific PCR primers for AT1a were designed from the murine cDNA sequences of the coding region (31): 5'-TCACCTGCTCATCATCTGCG-3' for sense and 5'-AGCTGGATAA-GAAGATGATTGAG-3' for antisense. A sense primer for AT1b was the same as that for AT1a. An antisense primer for AT1b was designed from the same region with different sequences (31): 5'-AGCTGGTG-TAGAATAGTACG-3'. The four underlined nucleotides are different between AT1a and AT1b. Each antisense primer specifically amplified AT1a and AT1b. Primers for AT2 were designed according to the coding region of the rat AT2 gene (32, 33): 5'-CTGACCTGGAGCATGTTTG-3' for sense and 5'-GGGTCTATTCTCTAAGAG-3' for antisense. Denaturing (94 °C for 45 s), annealing (58 °C for 1 min), and extension (72 °C for 1 min) reactions were repeated 40 times.

**Western Blot Analysis of Activated p38 MAPK**—Cell lysates were prepared and subjected to Western blot analysis using a polyclonal antibody against p38MAPK, aY91, in the presence of 0.15% SDS, and the immunoprecipitates were subjected to electrophoresis on an SDS-polyacrylamide gel containing 0.5 mg/ml MBP.

Phosphorylation of MBP was assayed by incubating the gel with [γ-32P]ATP. After incubation, the gel was washed extensively, dried, and then subjected to autoradiography.

**Northern Blot Analysis of the c-fos Gene**—20 μg of total RNA was fractionated on 1% formaldehyde-denatured agarose gels. Human c-fos cDNA (a kind gift from Dr. Tom Curran) was labeled by a random priming method, and Northern blot analysis was performed as previously reported (4).

**Phiion phosphorylation of EGF Receptor**—EGF receptor was immunoprecipitated from cell lysates using anti-EGF receptor polyclonal antibody (Santa Cruz). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was incubated with anti-phosphotyrosine polyclonal antibody (4G10), and phosphorylation of EGF receptor was visualized using ECL detection kit (Amersham Pharmacia Biotech) according to the manufacturer’s directions.

**RESULTS**

**RT-PCR Analysis of AT1 and AT2 mRNA in Cardiac Myocytes of Neonatal Mice**—AT1a gene is completely replaced by AT1b in cardiac myocytes of KO mice, and there are no transcripts of AT1a gene in KO tissues (30). Because AT1b and AT1a have been reported to be expressed in cardiac myocytes of neonatal rats (34), we first examined expressions of AT1a and AT1b in cultured cardiac myocytes of neonatal mouse by RT-PCR analysis using specific primers for AT1a and AT1b (Fig. 1). Although AT1a gene was expressed in WT cardiac myocytes, AT1b was not amplified by 40-cycle PCR in cardiac myocytes of KO mice (Fig. 1). AT1b mRNA was not detected in cultured cardiac myocytes of both types of neonatal mice (Fig. 1). We amplified AT1b from mRNA of WT adult heart by the same PCR primers and condition (data not shown). AT2 was expressed in both WT and KO cardiac myocytes at almost the same levels (Fig. 1).

**Effects of AngII on the Activity of ERKs in Cultured Cardiac Myocytes**—To determine whether there is no signal through AT1 in cardiac myocytes of KO mice, we added 10^{-6} M AngII to

![Fig. 1. AngII receptor expression in cardiac myocytes of WT and KO mice.](image)
cultured cardiac myocytes and examined activation of ERKs, which has been reported to be induced by AngII through AT1 (13, 35, 36). AngII activated ERKs in cardiac myocytes of WT mice as observed in cardiomyocytes of neonatal rats (Fig. 2A) (13, 35, 36). In cardiac myocytes of KO mice, some basal activities of ERKs were observed before stimulation (Fig. 2A). Although 10^{-7} M TPA (Fig. 2A) and 10^{-7} M endothelin-1 (Fig. 2B) strongly activated ERKs in KO cardiomyocytes as well as in WT cardiomycocytes, 10^{-6} M AngII did not enhance the ERK activity above the basal levels in KO cardiac myocytes (Fig. 2A). These results suggest that there is little functional AT1, if any, in cardiac myocytes of KO mice.

**Activation of ERKs and p38MAPK by Mechanical Stress**—Our and other laboratories have reported that mechanical stress activates ERKs in cultured cardiac myocytes of neonatal rats (13, 21–23). When cultured cardiac myocytes of WT mice were stretched by 20% for 10 min, ERKs were activated as in cardiac myocytes of neonatal rats (Fig. 3A). In cardiac myocytes of KO mice, some basal activities of ERKs were detected before stretch and stretch by 20% for 10 min markedly activated both 42- and 44-kDa ERKs (Fig. 3A). The time course of ERK activation by stretch in KO cardiomycocytes was the same as that in WT cardiac myocytes. The activity of ERKs was increased from 2 min, peaked at 10 min, and returned to basal levels at 30 min in cells of both mice (data not shown). Both basal and stimulated levels of ERKs were higher in KO cardiomycocytes than in WT cells (Fig. 3A). These results suggest that mechanical stress could activate ERKs in cardiac myocytes that lack signals from AT1 and that loss of AT1 signaling somehow increases basal and stimulated levels of ERKs in cardiac myocytes.

We next examined the activity of another member of the MAPK family, p38MAPK, which has been reported to be activated through different pathways from ERKs (28, 37). When cardiac myocytes were stretched by 20% for 10 min, p38MAPK was also activated in cardiac myocytes of both WT and KO mice (Fig. 3B). Unlike ERKs, both basal and stimulated levels of p38MAPK in KO cardiac myocytes were almost the same as in WT cardiomycocytes.

**Expression of the c-fos Gene by Mechanical Stress**—Induction of immediate-early response gene expression is one of genetic responses to mechanical stress in cardiac myocytes (2). We thus examined whether stretch induces expression of c-fos gene in cardiac myocytes of WT and KO mice. Although 10^{-6} M AngII did not induce c-fos gene expression in KO myocytes (data not shown), stretch by 20% for 30 min strongly induced expression of c-fos gene in KO cardiomycocytes as well as in WT cardiomycocytes (Fig. 4). Expression levels of the c-fos gene after stretch were higher in KO cardiomycocytes than in WT cells as in the case of ERKs.

**Effects of AT1 Antagonist on Mechanical Stress-induced ERK Activation**—To confirm whether AngII is involved in mechanical stress-induced ERK activation in KO cardiomycocytes, cardiomycocytes were stretched in the presence of AngII receptor antagonists. When WT myocytes were stretched in the presence of an AT1 antagonist, CV11974 (10^{-6} M), stretch-induced ERK activation was markedly attenuated (Fig. 5) as reported before in rat cells (13, 14, 23). The pretreatment with an ETA receptor antagonist, BQ485 (10^{-6} M), slightly reduced the activation in WT cardiomycocytes (Fig. 5). In KO cardiac myocytes, however, although BQ485 attenuated stretch-induced ERK activation at the same degree as in WT cardiac myocytes, CV 11974 had no effects on stretch-induced activation of ERKs (Fig. 5). An AT2 receptor antagonist, PD123319 (10^{-6} M), had no effects in cardiac myocytes of both mice. These pharmacological results strongly suggest that in WT cardiac myocytes, vasoactive peptides such as AngII and ET-1 are involved in mechanical stress-induced activation of ERKs as in neonatal rat cardiomycocytes (12–14, 23) and that in AT1 KO myocytes, AngII is not involved in stretch-induced ERK activation.
Mechanical Stress-induced Signal Transduction Pathways—We next examined the stretch-induced signal transduction pathways leading to activation of ERKs using cultured cardiac myocytes of both mice. In WT cardiomyocytes, down-regulation of PKC by incubation of cardiac myocytes with 10^{-7} M TPA for 24 h strongly inhibited stretch-induced ERK activation (Fig. 6A). A calcium chelator, EGTA (5 × 10^{-5} M), or a tyrosine kinase inhibitor, genistein (2 × 10^{-5} M), had no significant effects on the activity of ERKs. In contrast, in KO cardiomyocytes, stretch-induced ERK activation was strongly inhibited by genistein but not affected by down-regulation of PKC or by chelation of calcium (Fig. 6B). To get insights into what tyrosine kinases are involved in stretch-induced activation of ERKs in KO cardiomyocytes, we preincubated KO cardiac myocytes with another type of tyrosine kinase inhibitors, tyrphostins (AG1478, AG1295, and B42) (38, 39). AG1478 (a very potent and selective inhibitor of EGF receptor) (38) and B42 (a potent inhibitor of EGF receptor and Jak2) (39) but not AG1295 (a selective inhibitor of platelet-derived growth factor receptor) (38) suppressed ERK activation by stretch (Fig. 6B).

**Mechanical Stress-induced Signal Transduction Pathways**—We next examined the stretch-induced signal transduction pathways leading to activation of ERKs using cultured cardiac myocytes of both mice. In WT cardiomyocytes, down-regulation of PKC by incubation of cardiac myocytes with 10^{-7} M TPA for 24 h strongly inhibited stretch-induced ERK activation (Fig. 6A). A calcium chelator, EGTA (5 × 10^{-5} M), or a tyrosine kinase inhibitor, genistein (2 × 10^{-5} M), had no significant effects on the activity of ERKs. In contrast, in KO cardiomyocytes, stretch-induced ERK activation was strongly inhibited by genistein but not affected by down-regulation of PKC or by chelation of calcium (Fig. 6B). To get insights into what tyrosine kinases are involved in stretch-induced activation of ERKs in KO cardiomyocytes, we preincubated KO cardiac myocytes with another type of tyrosine kinase inhibitors, tyrphostins (AG1478, AG1295, and B42) (38, 39). AG1478 (a very potent and selective inhibitor of EGF receptor) (38) and B42 (a potent inhibitor of EGF receptor and Jak2) (39) but not AG1295 (a selective inhibitor of platelet-derived growth factor receptor) (38) suppressed ERK activation by stretch (Fig. 6B). These results suggest that in WT cardiomyocytes, PKC is critical for stretch-induced ERK activation, whereas in AT1a KO cardiomyocytes, tyrosine kinases, including EGF receptor tyrosine kinase, may be important. We next examined whether mechanical stress modulates EGF receptor. Mechanical stretch
ERK was measured by densitometric scanning. The band intensity of 42-kDa ERKs described in the legend to Fig. 2. A representative autoradiogram from three independent experiments is shown. The band intensity of ERK activity was measured as the averages ± S.D. (n = 3). ∗p < 0.05. N.S., not significant.

by 20% for 8 min significantly elevated tyrosine phosphorylation levels of EGF receptor in cardiac myocytes of KO mice (Fig. 7). Significant increase in phosphorylation levels of EGF receptor was not observed in cardiac myocytes of WT mice (data not shown).

**DISCUSSION**

Recent evidence has suggested that mechanical stress and neurohumoral factors induce cardiac hypertrophy in concert (2, 12–14). Although previous in vitro studies clearly showed that mechanical stress elicits hypertrophic responses in cultured cardiac myocytes (3–5, 12, 14, 21), it is still largely unknown how mechanical stress is converted into biochemical signals leading to cardiac hypertrophy. Our and other laboratories have reported that mechanical stress induces a variety of hypertrophic responses including activation of ERKs, expression of specific genes such as immediate-early response gene and fetal type genes, and an increase in protein synthesis in cardiac myocytes through the AngII/AT1 pathway (12–14, 21–23). However, AT1 antagonists only partially inhibited these stretch-induced cardiac events (13, 23), suggesting that factors other than AngII may be involved. We have recently reported that secretion of ET-1 is also stimulated by mechanical stress and that the ET-1 is involved in stretch-induced hypertrophic responses as well as AngII (14). Thus, there is a possibility that vasoactive peptides generally mediate mechanical stress-induced cardiac hypertrophy. However, stretch-induced various events were not completely inhibited even in the presence of both AngII and ET-1 antagonists (14). In addition, mechanical stress must evoke some signals first to induce secretion of AngII from secretory granules in cardiac myocytes (12). Because previous results were mainly obtained from pharmacological experiments (11–14, 23), we examined the role of AngII in mechanical stress-induced cardiac hypertrophy using genetically AT1a-deleted mice in the present study. Although RT-PCR analysis and response to direct addition of AngII indicated that there was no functional AT1 in cardiac myocytes of KO mice, mechanical stress strongly activated MAPKs such as ERKs and p38MAPK and induced c-fos gene expression in cardiac myocytes of KO mice as well as in those of WT mice. Surprisingly, basal and stimulated levels of ERKs, but not of p38MAPK, were higher in KO cardiomyocytes than in WT cardiomyocytes. AT1 and PKC play an important role in activation of ERKs in WT cells, whereas in KO cells, tyrosine kinases play a critical role in mechanical stress-induced ERK activation.

There are two subtypes in AT1, AT1a and AT1b (31, 34). Both subtypes have been reported to be expressed in cardiac myocytes of neonatal rats (34). The expression ratio of AT1a and AT1b is different among tissues, species, and developmental stages (31, 34), and it has been unknown in cardiac myocytes of neonatal mice. RT-PCR analysis revealed that AT1a but not AT1b was expressed in cultured cardiac myocytes of WT neonatal mice. PCR using specific primers amplified neither AT1a nor AT1b from RNA prepared from AT1a KO cardiomyocytes. These results suggest that AT1a is predominantly expressed in cultured cardiac myocytes of WT neonatal mice and that there is very little expression of AT1 (AT1a + AT1b), if any, in cultured cardiac myocytes of KO neonatal mice. Moreover, the addition of maximum dose of AngII did not activate ERKs or induce c-fos gene expression in KO cardiac myocytes. All these results suggest that there is no functional AT1 in cardiac myocytes of KO mice.

Many lines of evidence have suggested that ERKs function as an integrator for mitogenic and differentiation signals in many cell types (16). In cardiac myocytes, activation of ERKs is also required for phenylephrine-induced expressions of specific genes (17, 18), sarcomerogenesis, and increase in cell size (20).
Mechanical stress strongly activated ERKs in cardiac myocytes of KO mice as well as WT mice. It is difficult to completely rule out the possibility of the involvement of AT1b in mechanical-stress-induced hypertrophic responses in KO mice; however, no amplification of AT1b by PCR and no response to exogenous AngII strongly suggest that signals from AT1b do not play a major role in mechanical-stress-induced hypertrophic responses in KO cardiac myocytes. It is noteworthy that basal and activated levels of ERKs were higher in KO cardiac myocytes than in WT cardiac myocytes. Although the reason is not clear at present, the AT1 signaling pathway may inhibit the tyrosine-ERK pathway by suppressing production of some factors that activate tyrosine kinases or by inhibiting some intracellular molecules that are involved in the tyrosine kinase signaling. It has been reported that AngII phosphorylates insulin receptor substrates 1 and 2 and reduces phosphorylidyinositol 3-kinase activity in the heart (40). Because ERKs are initial signaling molecules critical for promoting cardiac hypertrophy (20), these observations suggest that AngII may not be required for establishment as well as initiation of pressure trophy (20), these observations suggest that AngII may not be initial signaling molecules critical for promoting cardiac hyper-

signaling. It has been reported that AngII phosphorylates intracellular molecules that are involved in the tyrosine kinase cascade (16, 37), this result is consistent with the assumption that a certain tyrosine kinase cascade may be activated in KO cardiac myocytes. Although some of the observed differences between KO mice and WT mice could result from differences in the genetic backgrounds of these two mice, the removal of AT1a may change the intracellular signaling pathway in cardiac myocytes. This study indicates that mechanical stress could induce cardiac hypertrophy without AngII signaling. To elucidate how stretch induces cardiac hypertrophy in AT1-deficient cardiomyocytes would pave the way to understand the mechanism of conversion from mechanical stress to biochemical signals.

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