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Stretch-Induced Map Kinase Activation in Cardiomyocytes of Angiotensinogen-Deficient Mice

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The renin–angiotensin system plays an important role in the hypertrophic responses in cardiac myocytes through the activation of signal transduction pathways and expression of oncogenes. In the present study, we examined mechanical stretch-induced activation of mitogen-activated protein kinases (MAP kinases) using cultured cardiac myocytes derived from neonatal angiotensinogen gene deficient mice (*Agt*−/−) and neonatal wild type mice (*Agt*+/+). Within 2 minutes of being added to cardiac myocytes, angiotensin II activated MAP kinases and the response was completely blocked by pretreatment of the cardiac myocytes with CV-11974, a selective antagonist of angiotensin II type 1 receptors. Interestingly, mechanical stretch resulted in significantly greater activation of MAP kinases in *Agt*−/− cardiac myocytes than in *Agt*+/+ cardiac myocytes. CV-11974 failed to suppress the stretch-induced activation of MAP kinases in *Agt*−/− cardiac myocytes while it inhibited the activation in *Agt*+/+ cardiac myocytes. BQ123, an endothelin type A receptor antagonist, had no effect on stretch-induced activation of MAP kinases in cardiac myocytes from either mouse strain. These results suggest that cardiac RAS is important for stretch-induced MAP kinase activation in *Agt*+/+ cardiac myocytes; however, angiotensin II is not indispensable for mechanical stretch-induced activation of MAP kinases in *Agt*−/− cardiac myocytes.

A number of studies suggest that the circulating and cardiac renin-angiotensin system (RAS) plays an important role in the development of cardiac hypertrophy (1,2,3). All components of the RAS (eg, renin, angiotensinogen, angiotensin-converting enzyme, and angiotensin II receptor) have been identified in the heart at both the mRNA and protein levels (4). In *in vitro* studies, mechanical stretch of cardiac myocytes induced the secretion of angiotensin II (Ang II) and treatment of cardiac myocytes with Ang II increased activities of signal transduction pathways, expression of immediate early genes, and synthesis of proteins, through its binding to Ang II type 1 (AT1) receptor (5,6,7). In addition, our previous studies showed that the expression of cardiac angiotensinogen mRNA was increased in hypertensive rats (8,9). These studies suggest that Ang II play a major role in the mechanical stretch-induced hypertrophic responses in cardiac myocytes.

Interestingly, the inhibition of the mechanical stretch-induced hypertrophic responses in rat cardiac myocytes by AT1 receptor antagonist is significant but not complete, thereby suggesting that factors other than Ang II are also involved in these hypertrophic responses induced by mechanical stretch (6,7). Several candidates are proposed to play a role. One is endothelin 1 (ET-1). A recent study showed that mechanical stretch induced the secretion of ET-1 from rat cardiac myocytes and that the secreted ET-1 induces mitogen-activated protein kinase (MAP kinase) activation and protein synthesis (10). MAP kinase is a key molecule in intracellular signal transduction and plays an essential role in cellular proliferation and differentiation (11,12,13). As described in the previous study, MAP kinase is activated by mechanical stretch in cardiac myocytes (14), and MAP kinase activity is a sensitive and quantitative marker for hypertrophic responses of rat cardiac myocytes (6,10). In the present study, to clarify the role of the cardiac RAS in mechanical stretch-induced cardiac hypertrophy, we used cardiac myocytes derived from neonatal angiotensinogen defi-

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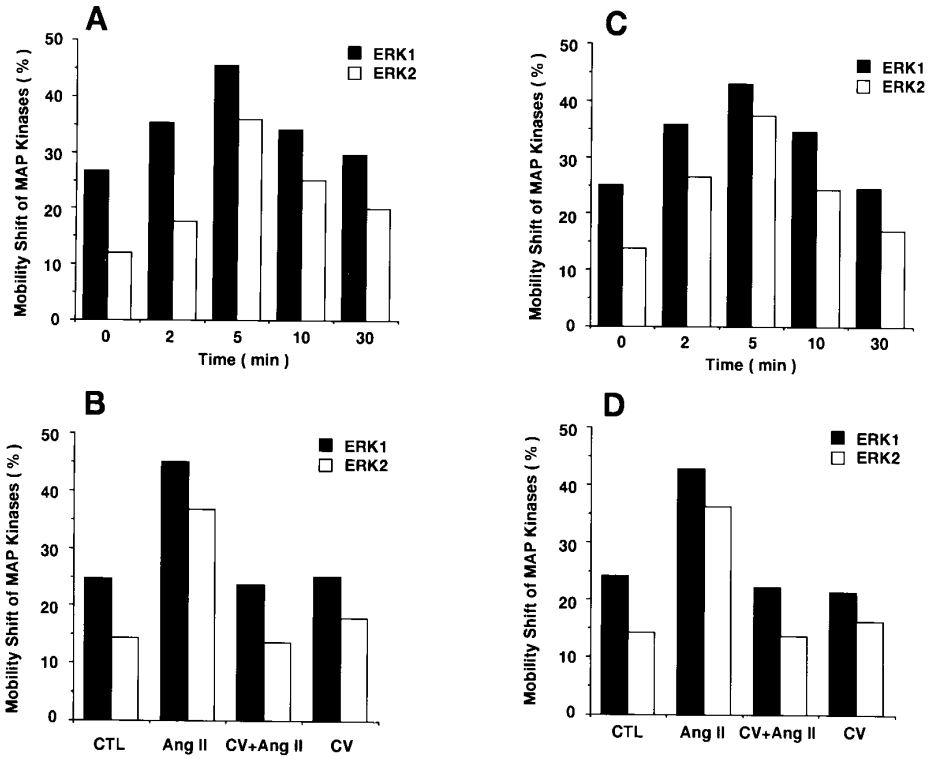

FIG. 1. Angiotensin II (Ang II)-induced mobility shift of MAP kinases ERK1 and ERK2 in *Agt*+/+ and *Agt*−/− cardiac myocytes. *Agt*+/+ (A) and *Agt*−/− (C) cardiac myocytes were exposed to 10^{-7} mol/L Ang II for the indicated periods of time. After pretreatment with CV-11974 (10^{-5} mol/L) for 30 min, *Agt*+/+ (B) and *Agt*−/− (D) cardiac myocytes were stimulated with Ang II (10^{-7} mol/L) for 5 min. Values represent the averages of two independent experiments.

cient mice and examined mechanical stretch-induced MAP kinase activation. Since angiotensinogen is the unique substrate of renin in the RAS *in vivo*, the effect of Ang II is completely absent in cardiac myocytes derived from angiotensinogen-deficient mice.

MATERIALS AND METHODS

Materials. Eagle's Minimum Essential Medium (E-MEM) was purchased from Nissui Pharmaceutical Co and fetal bovine serum (FBS) from Gibco BRL Co and trypsin from Difco Co and collagenase from Sigma Co. Ang II was purchased from Sigma Co, and ET-1 from Peptide Institute, Inc, and BQ123 from Phoenux Pharmaceuticals Inc. CV-11974 was generously provided by Takeda Chemical Industries, Ltd.

Animals. Angiotensinogen-deficient mice (*Agt*−/−) were established by gene targeting (15). They were maintained under controlled conditions of light, temperature, and humidity. All animals had free access to tap water and normal diet. ICR mice (*Agt*+/+) were used as controls.

Cell culture and stretching of cardiomyocytes. Primary cultures of cardiac myocytes were prepared from ventricles of 1- to 3-day old *Atg*+/+ or *Atg*−/− essentially according to the method of Goshima et al (16,17). Briefly, trypsinization and collagenization were performed with 0.06% trypsin and 0.025% collagenase at 37°C for 10 min, respectively. Cells from the first treatment were discarded, and the sequence was repeated until all the tissue was dissociated (about 3 cycles). Cardiocytes were maintained at 37°C in humidified air with 5% CO_2 in order to achieve a final medium pH of 7.35-7.40. To reduce the number of contaminating nonmuscle cells, dissociated cells were preplated into 100-mm culture dishes in E-MEM with 10% fetal bovine serum for 1 h. We purchased elastic culture dishes (15 × 30 × 10 mm, Ikemoto Chemical, Co, Tokyo, Japan) which were vulcanized with liquid silicone rubber consisting of methylvinyl polysiloxane and dimethylhydrogen silicone resin using platinum as a catalyst. The bottoms of these dishes are 0.5-mm-thick, and cultured cells are easily observed by microscopic examination. The cardiac myocyte-rich fraction was plated onto laminine-coated (20 μg/ml) silicone dishes at a field density of 1×10^5 cells/cm^2. The culture medium was changed 24 h after seeding to a serum-free chemically defined solution consisting of E-MEM, 10 μg/ml insulin, 5.5 μg/ml transferrin, and 6.7 μg/ml selenium. At this point, more than 90% of the cells were beating in the cardic myocyte-rich fraction while fewer than 20% were beating in the non-myocyte-rich fraction. We removed hearts from 10 to 20 neonatal mice at one time and dissociated and plated cells into 15-30 dishes. Cardiac myocytes were stretched by 20% and lysed on ice with buffer containing 2% SDS, 2% 2-mercaptoethanol, 20 mmol/L Tris-HCl, 40% glycerol, and 0.012% Bromophenol blue. Stretch and control experiments were carried out simultaneously in each experiment with the same pool of cells.

Measurement of MAP kinase activation. Cardiac myocyte extracts were separated by SDS-PAGE (18). After electrophoresis, the proteins were electrotransferred onto a PVDF membrane using a Milli Blot-SDS system (Atto Co, Ltd, Tokyo, Japan). The unoccupied protein binding sites on the membrane were blocked with 5% skim milk in buffer containing 50 mmol/L Tris-HCl, 0.2 mol/L NaCl, and

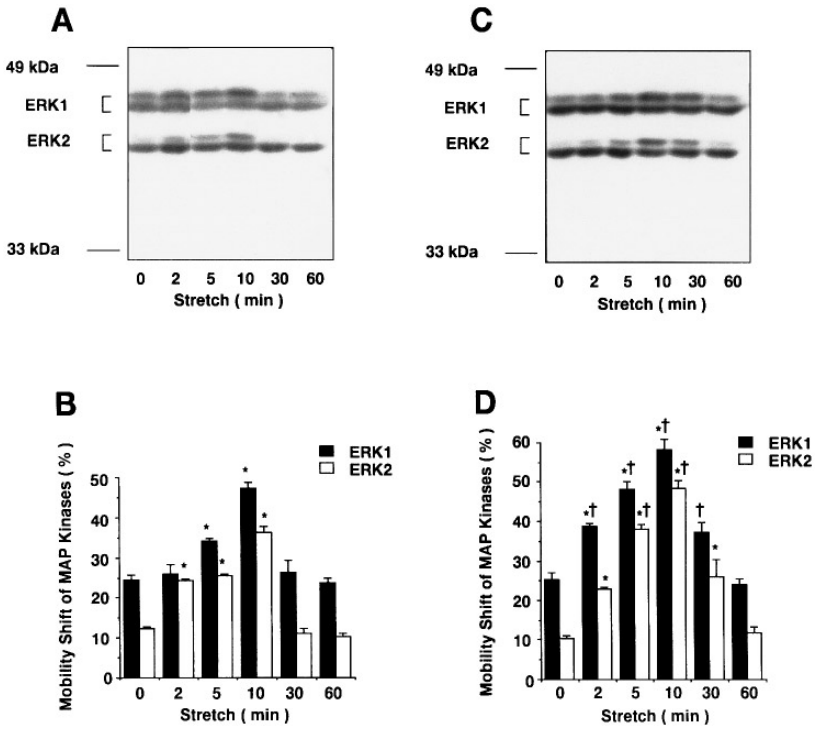

FIG. 2. Stretch-induced mobility shift of MAP kinases ERK1 and ERK2 in *Agt*+/+ and *Agt*−/− cardiac myocytes. *Agt*+/+ (A, B) and *Agt*−/− (C, D) cardiac myocytes were stretched by 20% for the indicated periods of time. (A) and (C) show typical blots while the values in (B) and (D) show the means±SE for four independent experiments. (*$p < .05$ vs control, †$p < .05$ vs *Ang* +/+)

0.05% Tween 20 for 1 h at room temperature. The membrane was incubated with 0.5 μg/ml of the anti-ERK1 antibody R2 (Upstrate Biotechnology Incorporated, New York, USA), for 1 h at 37°C. R2 is an affinity-purified rabbit polyclonal antibody raised against the 35 amino acid sequence deduced from rat ERK1 MAP kinase cDNA (19). After washing, the membrane was incubated for 1 h with anti-rabbit IgG-antibody conjugated with peroxidase as a second antibody, and developed using an Enhanced Chemiluminescence Western blotting analysis system (18). The intensities of the bands corresponding to ERK1 and ERK2 were quantified by densitometric scanning of the blots. The activation of MAP kinases was expressed as percentage mobility shift (shift band/non-shift band + shift band) × 100 for each MAP kinase (21).

Statistical analysis. The unpaired Student's *t*-test was used for the statistical analysis of differences among groups. All quantifiable data were expressed as means±SE.

RESULTS

MAP kinase is activated by phosphorylation that results in an upward shift in electrophoretic mobility (22). Since the mobility of MAP kinase was reported to be well correlated with kinase activity in our (21) and others previous studies (23), we used the mobility shift assay to monitor MAP kinase activation in cardiac myocytes.

As shown in Figure 1A, Ang II activated MAP kinases, ERK1 and ERK2, in *Agt*+/+ cardiac myocytes in a time dependent manner. The activation monitored as mobility shift on Western blots started within 2 min, reached a maximal level at 5 min, and returned to basal levels 30 min after stimulation. The concentration of Ang II required for maximal MAP kinase activation was 10^{-7} mol/L (data not shown). MAP kinase activation was completely blocked by pretreatment with CV-11974 (Fig 1B). Similar experiments using *Agt*−/− cardiac myocytes gave similar results (Figs 1C and 1D)

In addition to Ang II stimulation, MAP kinases ERK1 and ERK2 were activated in response to 20% mechanical stretch in *Agt*+/+ cardiac myocytes as shown in Figures 2A and 2B. The activation started within 2 min, reached a maximal level at 10 min, and returned to basal levels within 30 min after the beginning of stretch. In order to estimate the contribution of the Ang II signaling system in stretch-induced MAP kinase activation, we next examined whether *Agt*−/− cardiac myocytes responded to stretch stimulation. Unexpectedly, *Agt*−/− cardiac myocytes responded to stretch stimulation and the resulting MAP kinase activation followed a similar time course to that in *Agt*+/+ cardiac myocytes (Figs 2C and 2D). Interestingly, the magnitude of the response was significantly greater than that observed in *Agt*+/+ cardiac myocytes (Figs 2B and 2D). The basal band shifts of MAP kinases ERK1 and ERK2 were not significantly different between the two groups.

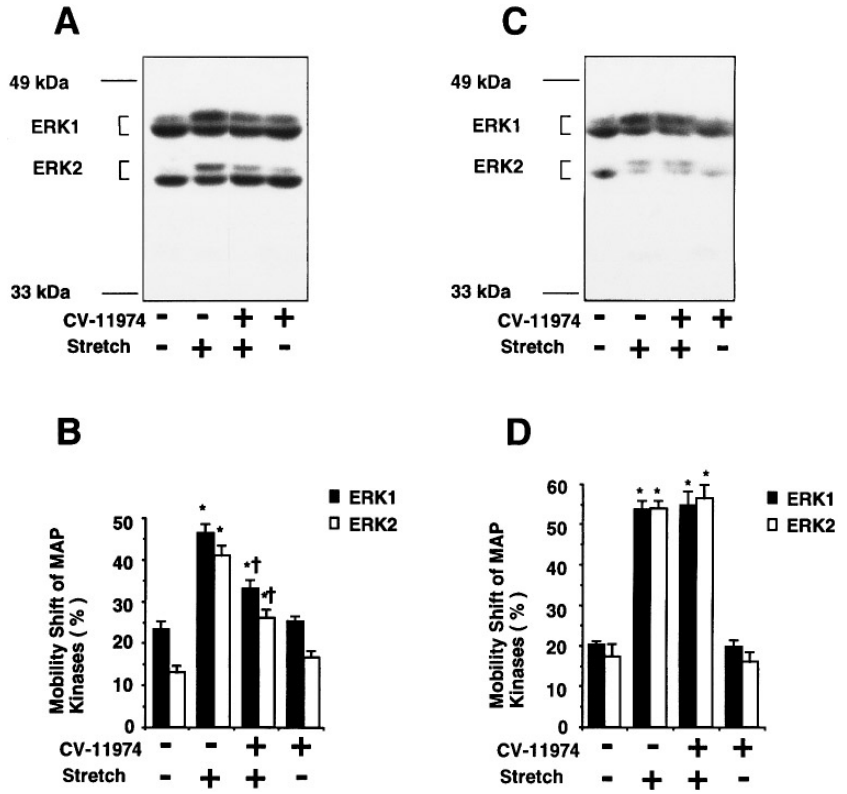

FIG. 3. The effect of CV-11974 on the stretch-induced mobility shift of MAP kinases ERK1 and ERK2 in *Agt*+/+ and *Agt*−/− cardiac myocytes. *Agt*+/+ (A, B) and *Agt*−/− (C, D) cardiac myocytes were stretched for 10 min with or without pretreatment with CV-11974 (10^{-5} mol/L) for 30 min. (A) and (C) show typical blots while the values for (B) and (D) show the means±SE for four independent experiments. (*$p < .05$ vs control, †$p < .05$ vs stretch without CV-11974)

CV-11974 significantly inhibited stretch-induced MAP kinase activation in *Agt*+/+ cardiac myocytes (Figs 3A and 3B). On the other hand, CV-11974 did not decrease stretch-induced activation of MAP kinases at all in *Agt*−/− cardiac myocytes (Figs 3C and 3D).Figure 5B showed that BQ123, an antagonist of endothelin type A receptor (ETA), at a concentration of 10^{-5} mol/L did not decrease stretch-induced MAP kinase activation, whereas the same treatment almost completely inhibited ET-1-induced MAP kinase activation (Fig 4B). The maximal MAP kinase activation was obtained at 10^{-6} to 10^{-7} mol/L ET-1 for 5 min (data not shown). Similar experiments using *Agt*+/+ cardiac myocytes gave very similar results as shown in Figures 4A and 5A.

DISCUSSION

In this study, we used cultured cardiac myocytes of *Agt*−/− to investigate the role of tissue RAS in mechanical stretch-induced activation of MAP kinases, ERK1 and ERK2. Since angiotensinogen is the unique substrate of renin in the RAS *in vivo,* Ang II is not produced at all in *Agt*−/−. Thus, *Agt*−/− cardiac myocytes are completely unable to produce and secrete Ang II in response to various stimuli including mechanical stretch. We examined MAP kinase acti-

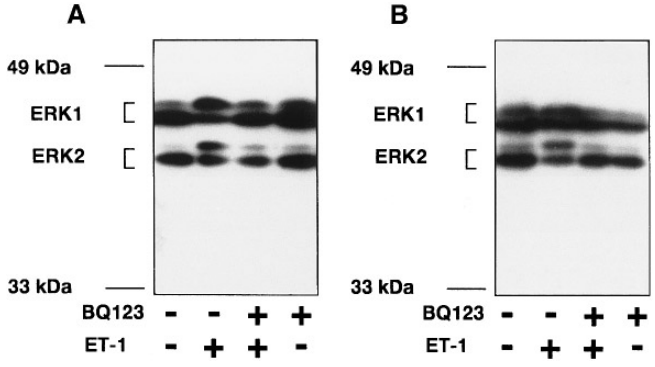

FIG. 4. The effect of BQ123 on endothelin-1 (ET-1) stimulated mobility shift of MAP kinases ERK1 and ERK2 in *Agt*+/+ and *Agt*−/− cardiac myocytes. *Agt*+/+ (A) and *Agt*−/− (B) cardiac myocytes were stimulated with endothelin-1 (ET-1) (10^{-7} mol/L) for 5 min with or without pretreatment with BQ123 (10^{-5} mol/L) for 30 min. Typical blots are shown. Similar results were obtained in two independent experiments.

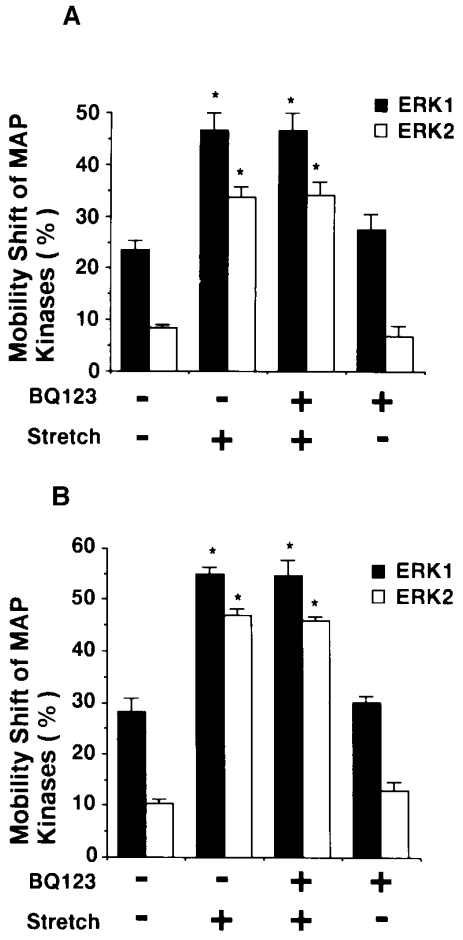

FIG. 5. The effect of BQ123 on the stretch-induced mobility shift of MAP kinases ERK1 and ERK2 in *Agt*+/+ and *Agt*−/− cardiac myocytes. *Agt*+/+ (A) and *Agt*−/− (B) cardiac myocytes were stretched for 10 min with or without pretreatment with BQ123 (10^{-5} mol/L) for 30 min. Values represent the means±SE of four independent experiments. (*$p < .01$ vs control)

vation as a marker of cardiac hypertrophy since the MAP kinase pathway is sensitive and, more importantly, is one of the signaling systems most often suggested to be involved in the pathogenesis of cardiac hypertrophy (6,10).

Initially, we supposed that the activation of MAP kinase in *Agt*−/− cardiac myocytes in response to mechanical stretch would be almost absent or considerably weaker than that in *Agt*+/+ cardiac myocytes. However, in our experiments, mechanical stretch unexpectedly induced the activation of MAP kinases in *Agt*−/− cardiac myocytes, the degree of activation was significantly higher in *Agt*−/− cardiac myocytes than *Agt*+/+ cardiac myocytes (Figs 2A and 2B), and mechanical stretch-induced activation of MAP kinases in *Agt*−/− cardiac myocytes was not inhibited by CV-11974 (Fig 3C). In contrast to *Agt*−/− cardiac myocytes, CV-11974 significantly suppressed stretch-induced MAP kinase activation in *Agt*+/+ cardiac myocytes (Fig 3A and 3B). This result was consistent with previous studies in rat cardiac myocytes (5,6,7), and indicated that endogenous Ang II produced by mechanical stretch contributed to the stretch-induced activation of MAP kinases in *Agt*+/+ cardiac myocytes. Therefore, cardiac RAS plays an important role in the activation of MAP kinase in response to mechanical stretch in *Agt*+/+ cardiac myocytes. In this study, Ang II activated MAP kinases through the AT1 receptor in both *Agt*+/+ and *Agt*−/− cardiac myocytes in a similar pattern (Fig 1), thereby showing that the AT1 receptor-mediated pathway itself was intact in *Agt*−/− cardiac myocytes.

It is quite interesting that the degree of stretch-induced MAP kinase activation in *Agt*−/− cardiac myocytes is rather greater than in *Agt*+/+ cardiac myocytes (Fig 2). This result indicates that cardiac RAS is not necessary for the activation of MAP kinase in response to mechanical stretch in *Agt*−/− cardiac myocytes, and further suggests that unknown compensatory factors other than cardiac RAS induced by the chronic deficiency of angiotensin II are able to sufficiently mediate the activation of MAP kinase by mechanical stretch in Agt−/− cardiac myocytes. Thus, we investigated factors other than cardiac RAS which worked to activate MAP kinase in response to mechanical stretch in *Agt*−/− cardiac myocytes. One possible candidate is ET-1. There is a report that mechanical stretching enhances the secretion of ET-1 from rat cardiac myocytes (10). However, another study reports that the stretching of cardiac myocytes does not enhance ET-1 secretion from rat cardiac myocytes (5). In this study, BQ123 pretreatment had no effect on stretch-induced MAP kinase activation in either *Agt*+/+ or *Agt*−/− cardiac myocytes (Figs 5A and 5B), whereas it completely suppressed ET-1-induced activation of MAP kinases in both cardiac myocytes (Figs 4A and 4B). Thus, our results suggest that ET-1 is not involved in the mechanical stretch-induced activation of MAP kinase both in *Agt*+/+ and *Agt*−/− cardiac myocytes. The lack of any impairment in stretch-induced MAP kinase activation in *Agt*−/− cardiac myocytes suggests the presence of factors other than the cardiac RAS and the ET-1/ETA system.

It should be noted that MAP kinase activation does not provide a direct measurement of hypertrophy, although it is one of the most important signaling pathways leading to cardiac hypertrophy (6,10). Recent studies have shown that rapamycin, a 70- or 85-kD form of S6 kinase (p70S6 kinase) inhibitor, suppresses the Ang II-induced increase in protein synthesis, although rapamycin does not affect the Ang II-induced activation of MAP kinases (24). Recently, Ang II was reported to activate Jak/STAT pathway, a cytokine-

mediated signal transduction system, in rat aortic smooth muscle cells (25), suggesting a crosstalk between serine/threonine kinases (MAP kinase, protein kinase C, etc) activated by Ang II and tyrosine kinases including Jak activated by cytokines and growth factors. Therefore, to clarify further the role of cardiac RAS in stretch-induced cardiac hypertrophy, an evaluation of the Jak-STAT and the p70S6 kinase pathway as well as protein synthesis is also required.

In conclusion, we have found interesting finding. The activity of MAP kinase can be induced significantly by mechanical stretch in "RAS-deficient" state in cardiac myocytes, and the degree of stretch-induced activation of MAP kinase in "RAS-deficient" cardiac myocytes is significantly greater than that in "RAS-operating" cardiac myocytes, thereby suggesting that an unknown compensatory factor may be induced to take place of the RAS to activate MAP kinase in "RAS-deficient" cardiac myocytes.

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REFERENCES

1. Baker, K. M., Booz, G. W., and Dostal, D. E. (1992) *Annu. Rev. Physiol.* **54,** 227–241.
2. Linz, W., Scholkens, B. A., and Ganten, D. (1989) *Clin. Exp. Hypertens.* **11,** 1325–1350.
3. Baker, K. M., Chernin, M., Wixson, S. K., and Aceto, J. F. (1990) *Am. J. Physiol.* **259,** H324–H332.
4. Lindpaintner, K., and Ganten, D. (1991) *Circ. Res.* **68,** 905–921.
5. Sadoshima, J., Xu, Y., Slayter, H. S., and Izumo, S. (1993) *Cell* **75,** 977–984.
6. Kojima, M., Shiojima, I., Yamazaki, T., Komuro, I., Yunzeng, Z., Ying, W., Mizuno, T., Ueki, K., Tobe, K., Kadowaki, T., Nagai, R., and Yazaki, Y. (1994) *Circulation* **89,** 2204–2211.
7. Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Mizuno, T., Takano, H., Hiroi, Y., Ueki, K., Tobe, K., Kadowaki, T., Nagai, R., and Yazaki, Y. (1995) *Circ. Res.* **77,** 258–265.
8. Tamura, K., Umemura, S., Nyui, N., Yamakawa, T., Yamaguchi, S., Ishigami, T., Tanaka, S., Tanimoto, K., Takagi, N., Sekihara, H., Murakami, K., and Ishii, M. (1996) *Hypertension* **27,** 1216–1223.
9. Tamura, K., Umemura, S., Yamakawa, T., Nyui, N., Hibi, K., Watanabe, Y., Ishigami, T., Yabana, M., Tanaka, S., Sekihara, H., Murakami, K., and Ishii, M. *Am. J. Physiol.* in press.
10. Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Hiroi, Y., Mizuno, T., Maemura, K., Kurihara, H., Aikawa, R., Takano, H., and Yazaki, Y. (1996) *J. Biol. Chem.* **271,** 3221–3228.
11. Nishida, E., and Gotoh, Y. (1993) *Trends Biochem. Sci.* **18,** 128–131.
12. Pages, G., Lenormand, P. L., Allemain, G., Chambard, J.-C., Meloche, S., and Pouyssegur, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8319–8323.
13. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) *Cell* **77,** 841–852.
14. Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R., and Yazaki, Y. (1993) *J. Biol. Chem.* **268,** 12069–12076.
15. Tanimoto, K., Sugiyama, F., Goto, Y., Ishida, J., Takimoto, E., Yagami, K., Fukamizu, A., and Murakami, K. (1994) *J. Biol. Chem.* **269,** 31334–31337.
16. Nakamura, T. Y., Goda, K., Okamoto, T., Kishi, T., Nakamura, T., and Goshima, K. (1993) *Circ. Res.* **73,** 758–770.
17. Okamoto, T., Isoda, H., Kubota, N., Takahata, K., Takahata, T., Kishi, T., Nakamura, T. Y., Muromachi, Y., Matsui, Y., and Goshima, K. (1995) *Toxicol. Appl. Pharmacol.* **133,** 150–163.
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
19. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, N. H. (1990) *Science* **249,** 64.
20. Whitherhead, T. P., Kricka, L. J., Carter Timothy, J. N., and Thorpe Gary, H. G. (1979) *Clin. Chem.* **25,** 1531–1546.
21. Nishioka, N., Hirai, S., Mizuno, K., Osada, S., Suzuki, A., Kosaka, K., and Ohno, S. (1995) *FEBS Lett.* **377,** 393–398.
22. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Norgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos (1991) *Cell* **65,** 663–675.
23. Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992) *Cell* **71,** 335–342.
24. Sadoshima, J., and Izumo, S. (1995) *Circ. Res.* **77,** 1040–1052.
25. Marrero, M. B., Schieffer, B., Paxton, W. G., Heerdt, L., Berk, B. C., Delafontaine, P., and Bernstein, K. E. (1995) *Nature* **375,** 249–250.