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European Journal of Pharmacology 512 (2005) 215–222

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Inhibition of Rho-kinase by fasudil attenuated angiotensin II-induced cardiac hypertrophy in apolipoprotein E deficient mice

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Received 8 November 2004; received in revised form 7 February 2005; accepted 18 February 2005
Available online 7 April 2005

Abstract

Recent evidence indicates that the GTPase activated Rho/Rho-kinase pathway contributes angiotensin II-induced cardiac hypertrophy and vascular remodeling. We tested this hypothesis in vivo by determining the effects of fasudil, a Rho-kinase inhibitor, on angiotensin II-induced cardiac hypertrophy, coronary vascular remodeling, and ventricular dysfunction. Six-month-old apolipoprotein E deficient (apoE-KO) mice were subcutaneously infused with angiotensin II (1.44 mg/kg/day) using an osmotic mini-pump. Mice were randomly assigned to either vehicle or fasudil (136 or 213 mg/kg/day in drinking water) group. Infusion of angiotensin II for 4 weeks resulted in cardiac enlargement, myocyte hypertrophy, and myocardial interstitial and coronary artery perivascular fibrosis. These changes were accompanied by reduced aortic flow velocity and acceleration rate. Cardiac gene expression levels of atrial natriuretic peptide (ANP) and collagen type III detected by real-time reverse transcriptase polymerase chain reaction were significantly increased in angiotensin II-infused mice. Treatment with fasudil dose-dependently attenuated angiotensin II-induced cardiac hypertrophy, prevented perivascular fibrosis, blunted the increase in ANP and collagen type III expression, and improved cardiac function, without changing blood pressure. These data are consistent with a role for Rho-kinase activation in angiotensin II-induced cardiac remodeling and vascular wall fibrosis.

Keywords: Rho-kinase; Angiotensin II; Cardiac hypertrophy; Perivascular fibrosis; Cardiac dysfunction; Apolipoprotein E knockout mice

1. Introduction

Cardiac hypertrophy occurs in response to sustained increases in afterload, wall stress, and neurohumornal stimulation, eventually leading to ventricular dysfunction and heart failure. Angiotensin II induces cardiac hypertrophy, by directly stimulating cardiomyocyte growth and by increasing ventricular afterload. Rho-kinase, a target protein of the small GTP-binding protein Rho, can affect cell growth and motility, focal adhesions and cytokinesis (Amano et al., 1997), and has been implicated in cardiovascular disease (see review (Shimokawa, 2002)). Data suggest that some of the cardiac effects of angiotensin II be mediated by Rho/Rho-kinase signaling. Activation of angiotensin 1 (AT_1) receptors by angiotensin II has been shown to activate Rho, which, in turn, induces protein synthesis in cardiomyocytes, leading to hypertrophy (Aikawa et al., 2000; Aoki et al., 1998). Angiotensin II also promotes inflammation by up-regulating the expression monocyte chemotactic protein (MCP-1), macrophage colony-stimulating factor (M-CSF), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, and by promoting monocyte/macrophage migration (Tham et al., 2002b). Rho-kinase has been shown to mediate angiotensin II-induced MCP-1 expression, macrophage infiltration (Aikawa et al., 2000; Aoki et al., 1998; Funakoshi et al., 2001; Miyata et al., 2000), and connective tissue growth factor production, thus contributing to fibrosis (Iwanciw et al., 2003). Recent data

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doi:10.1016/j.ejphar.2005.02.024

indicate that inhibition of Rho-kinase can prevent angiotensin II-induced expression of plasminogen activator inhibitor-1, and attenuate cardiac remodeling in the rat (Kobayashi et al., 2002a,b). In the present study, we used a Rho-kinase inhibitor, fasudil, to test the hypothesis that Rho-kinase mediates angiotensin II-induced cardiac hypertrophy and coronary vascular remodeling in apolipoprotein E deficient (apoE-KO) mice.

2. Method

2.1. Animal preparation

The Institutional Animal Care and Use Committee approved all animal protocols. Osmotic mini-pumps (model 2004, Alzet, Palo Alto, CA) containing either phosphate buffered saline (PBS) or angiotensin II (1.44 mg/kg/day in PBS, Calbiochem, CA) were implanted subcutaneously in 6-month-old apoE-KO male mice (Jackson Lab, Bar Harbor, ME). Two days prior to pump implantation, mice were provided with either tap water (vehicle group) or tap water dissolved with fasudil at a concentration of 0.5 (L) or 1.0 mg/mL (H) as fasudil groups. The calculated average daily dose of fasudil based on measured daily water consumption was 136 ± 12 mg/kg for the low dose group and 213 ± 10 mg/kg for the high dose group. The treatment duration continued for 30 days. At the end of the experiment, blood was taken for determination of plasma drug levels with a LC-MS/MS based analytical method. In mice treated with high dose fasudil, the average plasma drug concentrations were 0.7 ± 0.4 μM for fasudil and 4.2 ± 0.7 μM for the active metabolite, hydroxyfasudil. Hattori et al. reported that administration of fasudil in mice via drinking water at a daily dose of 100 mg/kg that reached a plasma concentration of hydroxyfasudil at ~ 0.4 μM significantly inhibited tissue level of Rho-kinase (Hattori et al., 2004a,b). It is known that fasudil is metabolized to hydroxyfasudil after oral administration (Shimokawa, 2002). Hydroxyfasudil is a specific inhibitor for Rho-kinase, which has been shown approximately 100 and 1000 fold more potent than that for protein kinase C and for myosin light-chain kinase, respectively (Higashi et al., 2003). Thus, at the present concentration of hydroxyfasudil ~ 4 μM, the therapeutic effects following oral administration of fasudil is likely related to the inhibition of Rho-kinase.

Mice were then euthanized, and the hearts perfused with Diethyl pyrocarbonate (DEPC) in saline at a physiological pressure. Hearts were excised, wet weight recorded, fixed in 10% formalin and embedded in paraffin for histology or snap-frozen in liquid nitrogen for quantification of gene expression.

2.2. Histology

Formalin-fixed hearts were encased in agarose and sectioned at 2.5 mm. The resulting tissue blocks were embedded in paraffin. Paraffin sections were cut at 5 μm thickness and stained with H&E, trichrome and Van Gieson's elastin. Using a stereology software (Computer Assisted Stereology Toolbar, CAST, Olympus Danmark, Albertslund, Denmark), average myocyte cross-sectional area was quantified by tracing the outer margin of 40 randomly chosen myocytes in cross section located in the middle part of the left ventricle. Interstitial fibrosis was evaluated in 2–3 sections from each heart by measuring the area of collagen-positive staining. Vascular wall remodeling was assessed in 3 large branches of the left anterior descending artery in the mid-section of the heart. The areas of vascular wall and perivascular fibrosis were quantified by tracing the adventitial, medial and luminal margins. Vessel and luminal diameters and vascular wall thickness were calculated by CAST software. An investigator blinded to the treatment regimens performed all measurements.

2.3. Gene expression analysis by real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from snap frozen heart tissues following standard laboratory protocols and purified using RNeasy columns (Qiagen Inc., Valencia, CA). PCR primers were designed using Primer Express software (Applied Biosystems Inc., Foster City, CA) based on sequence from GenBank. Gene expression data were normalized to rodent GAPDH. The primer sequences used were as follows:

Atrial natriuretic peptide (GenBank Accession # K02781)
5′ AGGAGAAGATGCCGGTAGAAGA 3′ (forward)
5′ GCTTCCTCAGTCTGCTCACTCA 3′ (reverse)
Collagen III (GenBank Accession # M18933)
5′ CCCAACCCAGAGATCCCATT 3′ (forward)
5′ GAAGCACAGGAGCAGGTGTAGA 3′ (reverse)
Control gene GAPDH (GenBank Accession # M32599)
5′ TGCACCACCAACTGCTTAGC 3′ (forward)
5′GTGGTCATGAGCCCTTCCA 3′ (reverse)

Real-time RT-PCR was performed in a two-step process. In the first step, sample RNA was reverse transcribed in a volume of 100 μl containing Taqman RT buffer, 5.5 mM $MgCl_2$, 500 uM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor and 1.25 U/μl MultiScribe reverse transcriptase at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min. In the second step, PCR was carried out in a MicroAmp Optical 96-well plate using SYBR® Green PCR Core Reagents (Applied Biosystems, Inc.). Each well contained 5 μl of a 1–100 fold dilution of cDNA template, SYBR green PCR buffer (5.5 mM $MgCl_2$, 200 μM each of dATP/dCTP/dGTP, 1 mM of dUTP, 2 mM each of forward and reverse primers, 0.01 U/μl of AmpErase UNG, and 0.025 U/μl

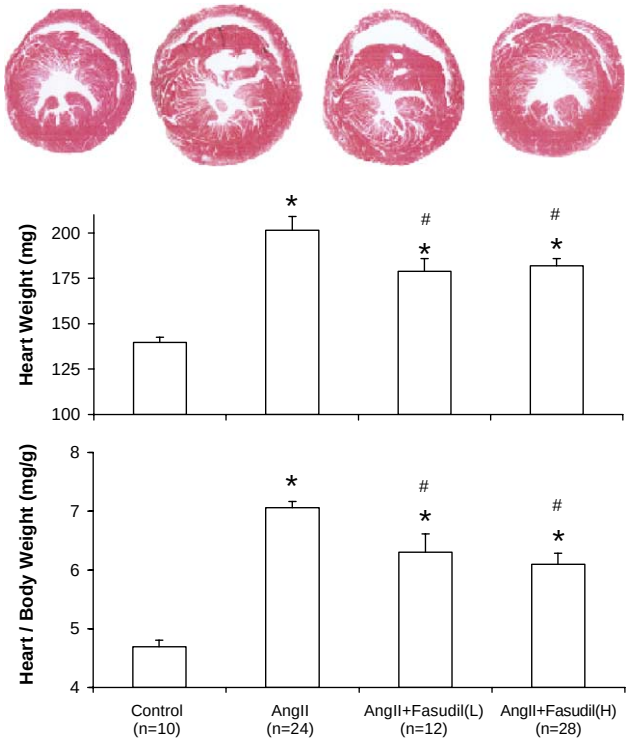

Fig. 1. Fasudil decreased angiotensin II-induced cardiac hypertrophy (top) measured by the heart weight (middle) and the ratio of heart over body weight (bottom) in apoE-KO mice. $P<0.05$, * vs. control; # vs. angiotensin II group.

AmpliTaq Gold DNA polymerase) in a total volume of 25 μl. The Thermal cycling conditions for the PCR reaction were 50 °C for 2 min; 95 °C for 10 min; and 40 cycles of melting at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. PCR reactions were monitored in real time using an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems Inc.). A standard curve for each target gene was generated with pooled reference RNA. Gene expression relative to GAPDH was determined using the standard curve method (ABI User bulletin #2, Applied Biosystems Inc.).

2.4. Noninvasive measurement of hemodynamics

A noninvasive Doppler method was used to quantify hemodynamic changes as described in detail previously (Hartley et al., 2000). In brief, mice were lightly anesthetized with 1.5% isoflurane (IMPAC 6, VetEquip, Pleasanton, CA). A lead II electrocardiogram and Doppler signals were obtained simultaneously using a Doppler data acquisition and processing system (Indus Instruments, Houston, TX). Blood flow velocity in the ascending aorta was measured by a 2-mm-diameter 20-MHz pulsed Doppler probe. Ascending flow velocity acceleration rate was calculated and used as an index of left ventricular contractility, average ascending flow velocity, as an index of cardiac output, and stroke distance, as an index of stroke volume.

2.5. Data analysis and statistics

Results are presented as mean ± standard error (S.E.M) for the number of animals (n) indicated. Comparison between 2 groups with different treatments was performed by Student *t*-test. Multiple comparison of mean values was performed by analysis of variance (ANOVA) followed by a subsequent Student–Newman–Keuls test for repeated measures. Differences were considered statistically significant when the P value was less than 0.05. The statistical analysis was performed using Statistica software (STASOFT, Tulsa, OK).

3. Results

3.1. Effects of fasudil on angiotensin II-induced cardiac hypertrophy

Infusion of angiotensin II in apoE-KO mice for 1 month significantly increased heart weight by 44% and heart/body weight ratio by 50% (Fig. 1). Treatment with fasudil attenuated angiotensin II-induced increases in heart weight and heart/body weight ratio. Angiotensin II resulted in an increase in cardiomyocyte size, which was significantly reduced by treatment with fasudil (Fig. 2). Histological examination revealed myocardial interstitial fibrosis in the mice receiving angiotensin II, but not in those with vehicle. Fasudil did not significantly affect angiotensin II-induced myocardial interstitial fibrosis (data not shown).

3.2. Effects of fasudil on angiotensin II-induced perivascular fibrosis

Angiotensin II resulted in a pronounced coronary perivascular fibrosis, which was dose-dependently attenuated by fasudil (Fig. 3). At the high dose of fasudil group, angiotensin II-induced coronary perivascular fibrosis was

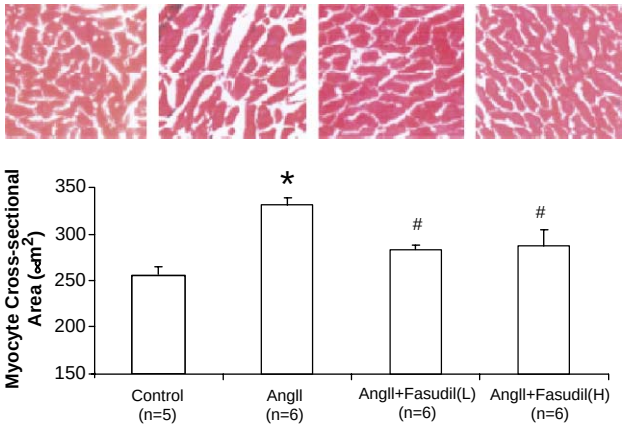

Fig. 2. Fasudil prevented angiotensin II-induced cardiac myocyte hypertrophy (top) measured by left ventricular myocyte size (bottom) in the heart of apoE-KO mice. $P<0.05$, * vs. control; # vs. angiotensin II group.

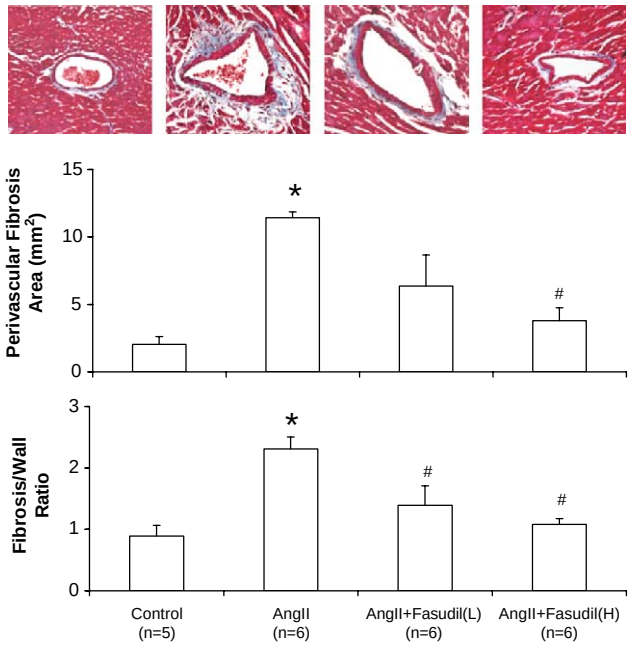

Fig. 3. Fasudil prevented angiotensin II-induced perivascular fibrosis (top) measured by perivascular fibrosis area (middle) and the ratio of perivascular fibrosis/vascular wall area (bottom) in the heart of apoE-KO mice. $P<0.05$, * vs. control; # vs. angiotensin II group.

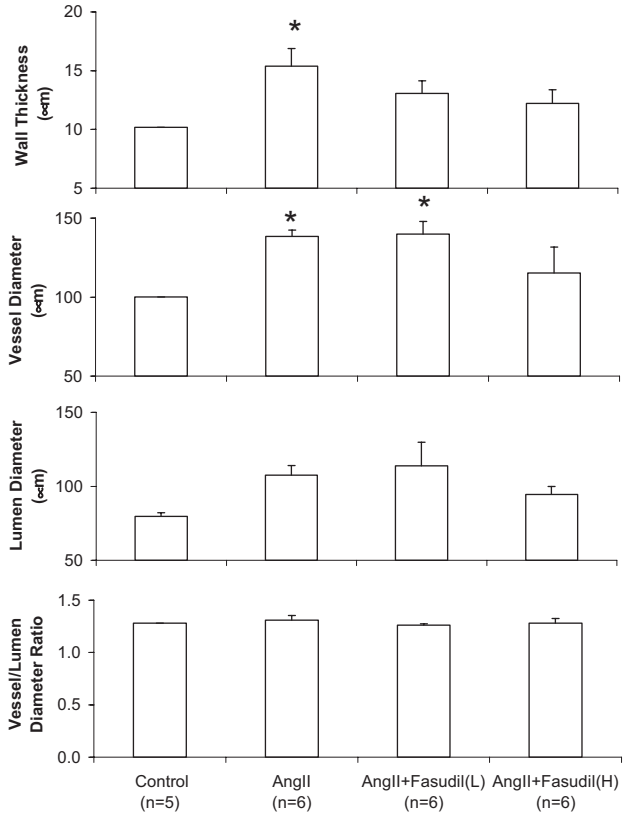

Fig. 4. Fasudil did not significantly affect angiotensin II-induced increase in vascular wall thickening and vessel and lumen diameters in the heart of apoE-KO mice. $P<0.05$, * vs. control group.

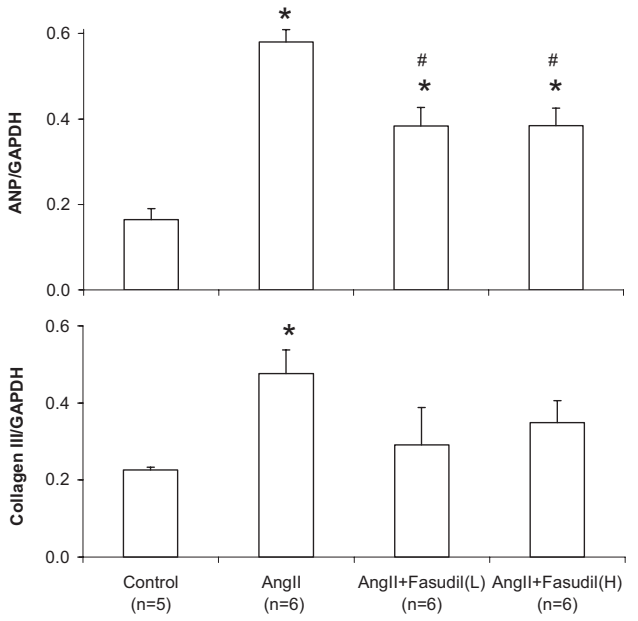

Fig. 5. Fasudil alleviated angiotensin II-induced up-regulation of the gene expressions of ANP (top) and collagen III (bottom) in the heart of apoE-KO mice. $P<0.05$, * vs. control; # vs. angiotensin II group.

dramatically reduced to a level that was not significantly different from that in control apoE-KO mice without treatment of angiotensin II. Intra-myocardial coronary arteries in angiotensin II-treated apoE-KO mice showed medial thickening with increased outer diameter of the vessel (Figs. 3 and 4). Luminal diameters tended to increase slightly, but the effect was not statistically significant.

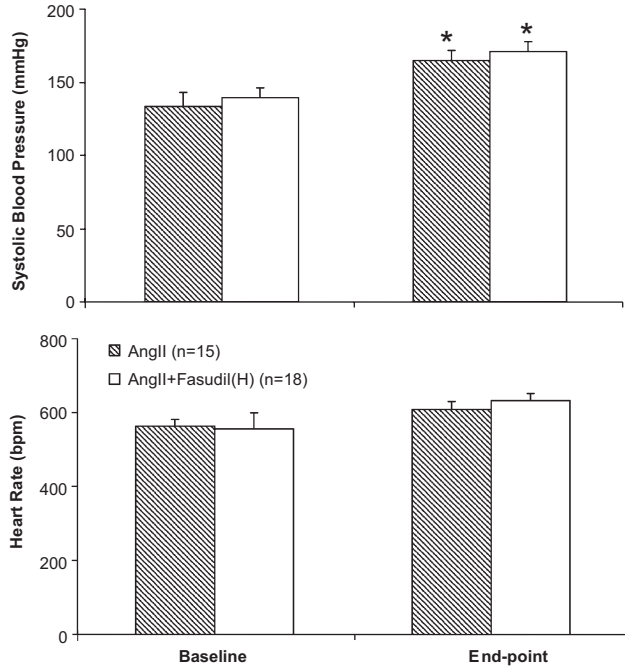

Fig. 6. Fasudil did not significantly affect blood pressure (top) and heart rate (bottom) in apoE-KO mice either before (baseline) or after infusion of angiotensin II. $P<0.05$, * vs. corresponding baseline.

Treatment with fasudil did not significantly alter these angiotensin II-induced vascular changes.

3.3. Effects of fasudil on angiotensin II-induced myocardial gene expression

Cardiac expressions of ANP and collagen type III mRNA were significantly up-regulated in mice treated with angiotensin II compared to those with vehicle, which were attenuated by fasudil (Fig. 5).

3.4. Effects of fasudil on angiotensin II-induced hemodynamic changes

Infusion of angiotensin II in apoE-KO mice increased systolic blood pressure with no significant effect on heart rate (Fig. 6). Treatment with fasudil had no effect on blood pressure or heart rate at baseline or following 28 days of angiotensin II treatment. Infusion of angiotensin II in apoE-KO mice for 1 month significantly decreased aortic flow acceleration rate as an index for left ventricular contractility, aortic flow velocity as an index for cardiac output, and stroke distance of the aortic flow as an index for stoke volume measured (Fig. 7). Treatment with fasudil prevented above angiotensin II-induced cardiac dysfunction.

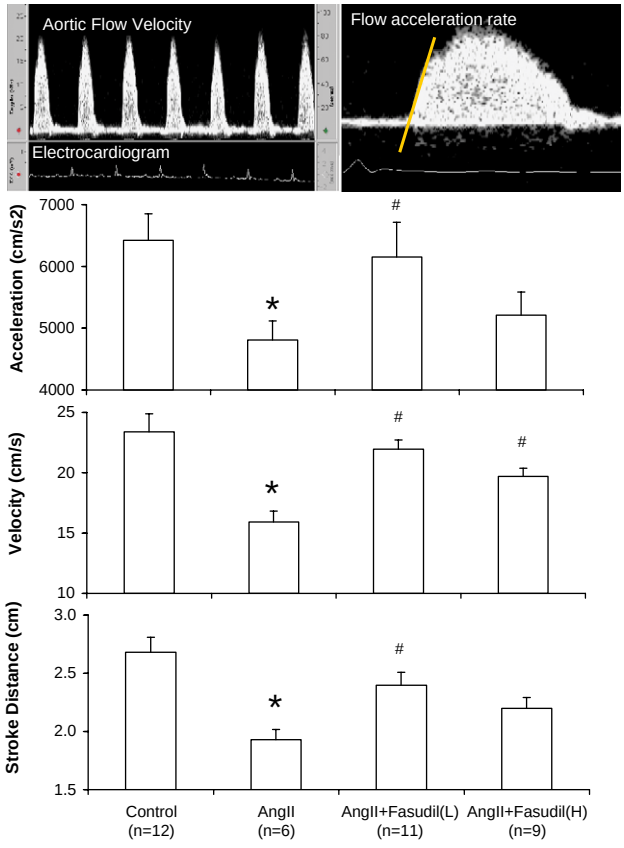

Fig. 7. Fasudil improved Angiotensin II-induced cardiac dysfunction in apoE-KO mice: representative original Doppler flow velocity signals and electrocardiogram (top left) and illustration of the calculation of aortic flow acceleration rate (top right), left ventricular contractility measured by aortic flow acceleration rate (second panel), cardiac output measured by mean aortic flow velocity (the third panel), and cardiac stoke volume measured by aortic flow stroke distance (bottom). $P<0.05$, * vs. control; # vs. angiotensin II group.

4. Discussion

In the present study, a 30-day infusion of angiotensin II in apoE-KO mice resulted in cardiac hypertrophy, and myocardial interstitial and coronary artery perivascular fibrosis. These morphological changes were associated with increased expression of ANP and collagen type III as well as impaired left ventricular ejection phase indices. Without significant effect on systemic blood pressure, treatment with fasudil attenuated cardiac hypertrophy, prevented perivascular fibrosis, and restored left ventricular systolic function.

Infusion of angiotensin II modestly increased blood pressure, which can elevate cardiac afterload, thus, contributing to cardiac hypertrophy. However, the cardioprotective effects of fasudil were clearly independent of blood pressure, which was not altered by fasudil. In addition to its vasoconstrictor effects, angiotensin II activates Rho by signaling through AT1 receptors, resulting in organization of actin into striated myofibrils (Aoki et al., 1998), induction of myofibrillogenesis, and stimulation of cardiomyocyte growth (Aceto and Baker, 1990; Baker and Aceto, 1990), DNA synthesis and cell migration (Seasholtz et al., 1999a,b). A role for Rho-kinase was also demonstrated by using a dominant negative Rho-kinase that can block angiotensin II-induced myocardial hypertrophy and myofibrillar assembly (Hoshijima et al., 1998). Further support for the role of Rho-kinase in cardiac hypertrophy comes from data indicating that Rho activates fetal genes, such as ANP, and is associated with cardiomyocyte growth (Sah et al., 1996; Thorburn et al., 1997). Indeed in the present study, angiotensin II treatment in apoE-KO mice up-regulated ANP mRNA in the hypertrophied heart, which were attenuated by fasudil. These results, which are consistent with several other reports (Aikawa et al., 2000; Aoki et al., 1998; Higashi et al., 2003), provide further evidence for the role of Rho-kinase in mediating angiotensin II-induced cardiac hypertrophy.

Fibrosis is a common pathologic feature of myocardial remodeling (Weber et al., 1991). In the present study, we observed that angiotensin II induced myocardial interstitial and perivascular fibrosis accompanied by up-regulation of collagen type III gene expression in the hypertrophied heart in apoE-KO mice. Interstitial and perivascular fibrosis in the hypertrophied myocardium is likely a result of cardiac fibroblast growth and collagen accumulation. Rho-kinase has been shown to mediate the induction of connective tissue growth factor by angiotensin II (Iwanciw et al., 2003). Inhibition of Rho-kinase has been shown to attenuate cardiac (Satoh et al., 2002a), hepatic (Iwamoto et al.,

2000; Tada et al., 2001) and renal (Ikegaki et al., 2001; Miyata et al., 2000; Nagatoya et al., 2002; Satoh et al., 2002b) fibrosis. These reports together with our data that fasudil prevented perivascular fibrosis provide strong evidence that Rho-kinase mediates the fibrogenic effects of angiotensin II. Inhibition of cardiac expression of collagen type III in fasudil-treated mice in the present study may contribute to the antifibrotic mechanism of fasudil. Angiotensin II is also a potent stimulator of plasminogen activator inhibitor-1 (PAI-1) expression, a major inhibitor of tissue and urokinase plasminogen activators that plays an important role in fibrogenesis (Feener et al., 1995). It has been known that angiotensin II-induced induction of PAI-1 expression is dependent on the AT1-receptor and the Rho/Rho-kinase pathway plays a critical role (Kobayashi et al., 2002a,b). Inhibition of Rho-kinase by Y-27632 prevented angiotensin II-induce PAI-1 expression and attenuated cardiac remodeling (Kobayashi et al., 2002a,b). Thus, inhibition of PAI-1 mediated fibrogenesis could be another mechanism for the antifibrotic effect of fasudil. In addition, inflammatory cell infiltration, in specific, macrophages also thought to play an important role in the fibroproliferative response by releasing fibrogenic growth factors. Rho-kinase inhibitor has been reported to suppress serum levels of proinflammatory cytokines, interleukin-6 (IL-6), keratinocyte chemoattractant (KC) and granulocyte colony-stimulating factor (G-CSF) (Bao et al., 2004), thus, inhibiting macrophage infiltration (Ikegaki et al., 2001; Miyata et al., 2000). Therefore, the antifibrotic effects of fasudil could also be partially attributed to its antiinflammatory action.

Our data show that angiotensin II-induced fibrosis is much more pronounced in the perivascular region than in the myocardial interstitial space, findings consistent with other reports (Kurisu et al., 2003; Weber et al., 1991). Treatment with fasudil completely prevented perivascular fibrosis and significantly attenuated cardiac hypertrophy, but had limited effect on myocardial interstitial fibrosis. One explanation for this finding is that the mechanism for myocardial interstitial fibrosis is different from that of perivascular fibrosis and cardiac hypertrophy, in which Rho-kinase may play a more important role. It has been reported that angiotensin II-induced TGF-β1 expression may act as an autocrine/paracrine stimulus for collagen formation in cardiac fibroblasts (Lee et al., 1995), but did not appear to be involved in hypertrophy of cultured neonatal cardiomyocytes (Kim et al., 1995). Thus, our finding that fasudil had minimal effects on myocardial interstitial fibrosis suggests that Rho-kinase may not be involved in angiotensin II-induced cardiac fibroblast stimulation. It has also been reported that activation of AT2 receptors attenuates perivascular fibrosis by a kinin/nitric oxide-dependent mechanism, but had no effect on cardiomyocyte hypertrophy (Kurisu et al., 2003). In contrast, it is well known that angiotensin II induces cardiac hypertrophy requires activation of AT_1 receptors (Aoki et al., 1998). Thus, an alternative explanation is that activation of Rho/Rho-kinase by angiotensin II attenuates AT_2 receptor-mediated inhibition of perivascular fibrosis. Fasudil, by blocking Rho-kinase, restores the negative feedback loop.

Long-term cardiac hypertrophy can lead to ventricular dysfunction. Indeed, in the present study, we observed that treatment with angiotensin II reduced left ventricular contractility, indexed by the acceleration rate of the ascending aortic flow, and decreased cardiac output, indexed by the ascending aortic flow velocity (Hartley et al., 2000). The ascending aorta flow velocity and acceleration rate can be influenced by aortic stiffness that was significantly increased by angiotensin II (Tham et al., 2002a). A stiff aorta with reduced compliance is expected to increase aortic flow velocity and acceleration. Thus, the observed reduction of the aortic flow velocity and acceleration rate may underestimate the effect of angiotensin II-induced cardiac dysfunction.

Angiotensin II-induced coronary vasoconstriction and perivascular fibrosis can result in myocardial ischemia, which could contribute to impaired cardiac function. In the present study, regions of micro-infarction were observed in the heart from one apoE-KO mouse treated with angiotensin II (data not show). Furthermore, collagen accumulation in the heart increases myocardial stiffness, which can also lead to diastolic, and ultimately, systolic dysfunction. Long-term inhibition of the renin–angiotensin system has been reported to result in both regression of myocardial fibrosis and improvement in diastolic distensibility in hypertensive rats (Brilla et al., 1996) and humans (Diez et al., 2002). The improvement of ejection phase indices by fasudil in the present study may be related to the observed attenuation of cardiac hypertrophy and prevention of perivascular fibrosis. Although fasudil had little effect on myocardial interstitial fibrosis, the improvement of diastolic dysfunction and the regression of cardiomyocyte hypertrophy could be independent of the reduction of collagen content as it is shown in hypertensive patients (Brilla et al., 2000). Collagen cross-linking has been reported to affect myocardial stiffness and function (Norton et al., 1997), which was not measured in the present study.

In summary, our results provide further evidence that Rho-kinase contributes to angiotensin II-induced cardiac hypertrophy, coronary perivascular fibrosis, and impaired left ventricular ejection in apoE-KO mice. Fasudil, a Rho-kinase inhibitor, attenuated angiotensin II-induced cardiac hypertrophy, prevented perivascular fibrosis, and improved ventricular ejection. Thus, inhibition of Rho-kinase may be a useful therapeutic strategy to attenuate progression of heart failure resulting from cardiac hypertrophy.

References

Aceto, J.F., Baker, K.M., 1990. [Sar1]angiotensin II receptor-mediated stimulation of protein synthesis in chick heart cells. Am. J. Physiol. 258, H806–H813.

Aikawa, R., Komuro, I., Nagai, R., Yazaki, Y., 2000. Rho plays an important role in angiotensin II-induced hypertrophic responses in cardiac myocytes. Mol. Cell. Biochem. 212, 177–182.

Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., Kaibuchi, K., 1997. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275, 1308–1311.

Aoki, H., Izumo, S., Sadoshima, J., 1998. Angiotensin II activates RhoA in cardiac myocytes: a critical role of RhoA in angiotensin II-induced premyofibril formation. Circ. Res. 82, 666–676.

Baker, K.M., Aceto, J.F., 1990. Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. Am. J. Physiol. 259, H610–H618.

Bao, W., Hu, E., Tao, L., Boyce, R., Mirabile, R., Thudium, D.T., Ma, X.L., Willette, R.N., Yue, T.L., 2004. Inhibition of Rho-kinase protects the heart against ischemia/reperfusion injury. Cardiovasc. Res. 61, 548–558.

Brilla, C.G., Matsubara, L., Weber, K.T., 1996. Advanced hypertensive heart disease in spontaneously hypertensive rats. Lisinopril-mediated regression of myocardial fibrosis. Hypertension 28, 269–275.

Brilla, C.G., Funck, R.C., Rupp, H., 2000. Lisinopril-mediated regression of myocardial fibrosis in patients with hypertensive heart disease. Circulation 102, 1388–1393.

Diez, J., Querejeta, R., Lopez, B., Gonzalez, A., Larman, M., Martinez Ubago, J.L., 2002. Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. Circulation 105, 2512–2517.

Feener, E.P., Northrup, J.M., Aiello, L.P., King, G.L., 1995. Angiotensin II induces plasminogen activator inhibitor-1 and-2 expression in vascular endothelial and smooth muscle cells. J. Clin. Invest. 95, 1353–1362.

Funakoshi, Y., Ichiki, T., Shimokawa, H., Egashira, K., Takeda, K., Kaibuchi, K., Takeya, M., Yoshimura, T., Takeshita, A., 2001. Rho-kinase mediates angiotensin II-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. Hypertension 38, 100–104.

Hartley, C.J., Reddy, A.K., Madala, S., Martin-McNulty, B., Vergona, R., Sullivan, M.E., Halks-Miller, M., Taffet, G.E., Michael, L.H., Entman, M.L., Wang, Y.X., 2000. Hemodynamic changes in apolipoprotein E-knockout mice. Am. J. Physiol., Heart Circ. Physiol. 279, H2326–H2334.

Hattori, T., Shimokawa, H., Higashi, M., Hiroki, J., Mukai, Y., Kaibuchi, K., Takeshita, A., Morikawa, K., Ichiki, T., Takahashi, S., 2004a. Long-term treatment with a specific Rho-kinase inhibitor suppresses cardiac allograft vasculopathy in mice. Circ. Res. 94, 46–52.

Hattori, T., Shimokawa, H., Higashi, M., Hiroki, J., Mukai, Y., Tsutsui, H., Kaibuchi, K., Takeshita, A., 2004b. Long-term inhibition of Rho-Kinase suppresses left ventricular remodeling after myocardial infarction in mice. Circulation 109, 2234–2239.

Higashi, M., Shimokawa, H., Hattori, T., Hiroki, J., Mukai, Y., Morikawa, K., Ichiki, T., Takahashi, S., Takeshita, A., 2003. Long-term inhibition of Rho-Kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. Circ. Res. 93, 767–775.

Hoshijima, M., Sah, V.P., Wang, Y., Chien, K.R., Brown, J.H., 1998. The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes. Involvement of Rho kinase. J. Biol. Chem. 273, 7725–7730.

Ikegaki, I., Hattori, T., Yamaguchi, T., Sasaki, Y., Satoh, S.I., Asano, T., Shimokawa, H., 2001. Involvement of Rho-kinase in vascular remodeling caused by long-term inhibition of nitric oxide synthesis in rats. Eur. J. Pharmacol. 427, 69–75.

Iwamoto, H., Nakamuta, M., Tada, S., Sugimoto, R., Enjoji, M., Nawata, H., 2000. A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. J. Hepatol. 32, 762–770.

Iwanciw, D., Rehm, M., Porst, M., Goppelt-Struebe, M., 2003. Induction of connective tissue growth factor by angiotensin II: integration of signaling pathways. Arterioscler. Thromb. Vasc. Biol. 23, 1782–1787.

Kim, N.N., Villarreal, F.J., Printz, M.P., Lee, A.A., Dillmann, W.H., 1995. Trophic effects of angiotensin II on neonatal rat cardiac myocytes are mediated by cardiac fibroblasts. Am. J. Physiol. 269, E426–E437.

Kobayashi, N., Horinaka, S., Mita, S., Nakano, S., Honda, T., Yoshida, K., Kobayashi, T., Matsuoka, H., 2002a. Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. Cardiovasc. Res. 55, 757–767.

Kobayashi, N., Nakano, S., Mita, S., Kobayashi, T., Honda, T., Tsubokou, Y., Matsuoka, H., 2002b. Involvement of Rho-kinase pathway for angiotensin II-induced plasminogen activator inhibitor-1 gene expression and cardiovascular remodeling in hypertensive rats. J. Pharmacol. Exp. Ther. 301, 459–466.

Kurisu, S., Ozono, R., Oshima, T., Kambe, M., Ishida, T., Sugino, H., Matsuura, H., Chayama, K., Teranishi, Y., Iba, O., Amano, K., Matsubara, H., 2003. Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. Hypertension 41, 99–107.

Lee, A.A., Dillmann, W.H., McCulloch, A.D., Villarreal, F.J., 1995. Angiotensin II stimulates the autocrine production of transforming growth factor-beta 1 in adult rat cardiac fibroblasts. J. Mol. Cell. Cardiol. 27, 2347–2357.

Miyata, K., Shimokawa, H., Kandabashi, T., Higo, T., Morishige, K., Eto, Y., Egashira, K., Kaibuchi, K., Takeshita, A., 2000. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler. Thromb. Vasc. Biol. 20, 2351–2358.

Nagatoya, K., Moriyama, T., Kawada, N., Takeji, M., Oseto, S., Murozono, T., Ando, A., Imai, E., Hori, M., 2002. Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. Kidney Int. 61, 1684–1695.

Norton, G.R., Tsotetsi, J., Trifunovic, B., Hartford, C., Candy, G.P., Woodiwiss, A.J., 1997. Myocardial stiffness is attributed to alterations in cross-linked collagen rather than total collagen or phenotypes in spontaneously hypertensive rats. Circulation 96, 1991–1998.

Sah, V.P., Hoshijima, M., Chien, K.R., Brown, J.H., 1996. Rho is required for Galphaq and alpha1-adrenergic receptor signaling in cardiomyocytes. Dissociation of Ras and Rho pathways. J. Biol. Chem. 271, 31185–31190.

Satoh, S., Ikegaki, I., Toshima, Y., Watanabe, A., Asano, T., Shimokawa, H., 2002a. Effects of Rho-kinase inhibitor on vasopressin-induced chronic myocardial damage in rats. Life Sci. 72, 103–112.

Satoh, S., Yamaguchi, T., Hitomi, A., Sato, N., Shiraiwa, K., Ikegaki, I., Asano, T., Shimokawa, H., 2002b. Fasudil attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction. Eur. J. Pharmacol. 455, 169–174.

Seasholtz, T.M., Majumdar, M., Brown, J.H., 1999a. Rho as a mediator of G protein-coupled receptor signaling. Mol. Pharmacol. 55, 949–956.

Seasholtz, T.M., Majumdar, M., Kaplan, D.D., Brown, J.H., 1999b. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ. Res. 84, 1186–1193.

Shimokawa, H., 2002. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J. Cardiovasc. Pharmacol. 39, 319–327.

Tada, S., Iwamoto, H., Nakamuta, M., Sugimoto, R., Enjoji, M., Nakashima, Y., Nawata, H., 2001. A selective ROCK inhibitor, Y27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. J. Hepatol. 34, 529–536.

Tham, D.M., Martin-McNulty, B., Wang, Y.X., Da Cunha, V., Wilson, D.W., Athanassious, C.N., Powers, A.F., Sullivan, M.E., Rutledge, J.C., 2002a. Angiotensin II injures the arterial wall causing increased aortic stiffening in apolipoprotein E-deficient mice. Am. J. Physiol., Regul. Integr. Comp. Physiol. 283, R1442–R1449.

Tham, D.M., Martin-McNulty, B., Wang, Y.X., Wilson, D.W., Vergona, R., Sullivan, M.E., Dole, W., Rutledge, J.C., 2002b. Angiotensin II is

associated with activation of NF-kappaB-mediated genes and down-regulation of PPARs. Physiol. Genomics 11, 21–30.

Thorburn, J., Xu, S., Thorburn, A., 1997. MAP kinase- and Rho-dependent signals interact to regulate gene expression but not actin morphology in cardiac muscle cells. EMBO J. 16, 1888–1900.

Weber, K.T., Brilla, C.G., Janicki, J.S., Reddy, H.K., Campbell, S.E., 1991. Myocardial fibrosis: role of ventricular systolic pressure, arterial hypertension, and circulating hormones. Basic Res. Cardiol. 86, 25–31.