Angiotensin II Type 1 Receptor Antagonist Downregulates Nonmuscle Myosin Heavy Chains in Spontaneously Hypertensive Rat Aorta

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Abstract—The aim of this study was to clarify the differences between the angiotensin II type 1 (AT1) receptor antagonist and the angiotensin-converting enzyme (ACE) inhibitor on smooth muscle and nonmuscle myosin heavy chain isoforms in aortic smooth muscle cells of Wistar-Kyoto rats and spontaneously hypertensive rats. All 4 myosin heavy chain isoforms are heterogeneously expressed in the smooth muscle cells of the aortic tunica media in 20-week-old rats, and the contractile-type myosin heavy chains are highly expressed in smooth muscle cells of the aortic tunica media compared with the synthetic-type myosin heavy chains. Both the AT1 receptor antagonist and the ACE inhibitor had the same effects on hemodynamics, smooth muscle cell hypertrophy and proliferation, fibrosis, and vascular remodeling in spontaneously hypertensive rats. However, the AT1 receptor antagonist had a more potent effect on the downregulation of the synthetic-type myosin heavy chains than the ACE inhibitor in spontaneously hypertensive rat aortic tunica media. In contrast, these effects of the AT1 receptor antagonist and the ACE inhibitor on hemodynamics, morphology, fibrosis, and expression of myosin heavy chain isoforms in smooth muscle cells of the aortic tunica media were not observed in Wistar-Kyoto rats. Thus, within 6 weeks, the AT1 receptor antagonist might modulate the cellular composition of myosin heavy chain isoforms in smooth muscle cells more efficiently than the ACE inhibitor, without morphological changes in the spontaneously hypertensive rat aorta. (Hypertension. 1999;33:975-980.)

Key Words: angiotensin ■ aorta ■ hypertension, arterial ■ muscle, smooth ■ myosin

Arterial hypertension is known to result in vascular remodeling.1 The proliferation of smooth muscle cells (SMC) is also an important component of many vascular diseases.2,3 Rat vascular SMC contain high levels of both smooth muscle (SM) myosin heavy chain (MHC) and α-SM actin and very low levels of nonmuscle myosin heavy chain (NMHC).4 In addition, they contain at least 4 MHC isoforms: SM-1 (204 kDa), SM-2 (200 kDa), NMHC-A (196 kDa), and NMHC-B (198 kDa).5 The relative ratios between SM-MHCs and NMHCs are not only determinants of the contractile properties of SM but are also a useful molecular marker for phenotypic changes in SMC. The dedifferentiation process of SMC, known as phenotypic modulation, contributes to the development and/or progression of atherosclerotic diseases.2,3 SM-MHCs have been shown to be important in the identification of differentiated SMC. On the other hand, it has been demonstrated that NMHCs are most abundantly expressed in embryonic SM and proliferating SMC of atherosclerotic lesions.7,8 Medial hypertrophy is associated with changes in the gene expression of vascular SMC, leading to a synthetic phenotype characterized by the accumulation of NMHC.3 Angiotensin II (Ang II) plays a key role in regulating both the tone and growth of vascular SMC and is directly involved in vascular remodeling.9 Although Ang II interacts with 2 major receptor subtypes, AT1 and AT2, the AT1 receptor has been shown to primarily mediate Ang II–induced vascular events such as hypertrophy, the proliferation of SMC, and extracellular matrix formation.10 Angiotensin-converting enzyme (ACE) converts Ang I to Ang II and inactivates kinins, and some enzymes directly generate Ang II in tissues such as the vascular wall.11 Thus, selectively preventing the binding of Ang II to the AT1 receptor would provide a rational way of blocking the renin-angiotensin system. The extent of the phenotypic modulation of SMC might be restored by using antihypertensive agents such as ACE inhibitor.12 However, the mechanism by which Ang II stimulates the phenotypic modulation of SMC and how this may contribute to the development of vascular hypertrophy in hypertension are still unclear.

In this report, to investigate the role of Ang II in vascular remodeling and the phenotypic modulation of SMC in the aorta, we compared the effects of the AT1 receptor antagonist with that of the ACE inhibitor on the MHC isoform expression of aortic SMC as well as the morphology of and fibrosis in rat aortas.

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Methods

The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine and were performed according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine and the law (No. 105) and notification (No. 6) of the Japanese government.

Chemicals, Reagents, Antibodies, and Purified Myosin

All reagents were purchased from Sigma. The nonpeptide AT1 receptor antagonist FK-739 (FK) and enalapril (EN) were gifts of Fujisawa Pharmaceutical Co, Ltd (Osaka, Japan) and Banyu Pharmaceutical Co, Ltd (Tokyo, Japan), respectively. Specific antibodies against SM-1, SM-2, NMHC-A, and NMHC-B were the kind gift of Drs Robert S. Adelstein, Hiroshi Ito, and Christine A. Kelly, and purified turkey gizzard SM myosin for SM-1 and SM-2 and purified human platelet myosin for NMHC-A were the kind gift of James R. Sellers, National Institutes of Health (Bethesda, Md). Mouse monoclonal antibody against α-SM actin was obtained from American Research Products, Inc. Mouse monoclonal antibody to actin (anti-actin), which reacts with all 6 isoforms of vertebrate actin, was obtained from F. Hoffman-La Roche Ltd. Biotinylated-goat anti-rabbit immunoglobulin and biotinylated-goat anti-mouse immunoglobulin were obtained from Zymed Laboratories, Inc.

Animals and Experimental Model

Fourteen-week-old male Wistar-Kyoto rats (WKY; n=56) and spontaneously hypertensive rats (SHR; n=75) were used in the experiments (Charles River Japan, Inc, Kanagawa, Japan). WKY and SHR were randomized into 3 groups each and treated for 6 weeks with vehicle (tap water; WKY and SHR groups), EN (10 mg/kg per day in drinking water; WKY+EN and SHR+EN groups), and FK (30 mg/kg per day in drinking water; WKY+FK and SHR+FK groups). The doses used in the experiments were determined according to Yonezawa et al.

Hemodynamic Studies, Morphometry, and Fibrosis in the Tunica Media of Aortas

On unanesthetized rats, the systolic blood pressure and heart rate were determined in a controlled-temperature room by the tail-cuff method. After a 6-week treatment period, the rats were weighed and killed with a sodium pentobarbital overdose and perfusion fixed for 5 minutes at a pressure of 90 mm Hg with 4% paraformaldehyde buffered with 0.1 mol/L NaHPO4. The thoracic aortas were excised, fixed, and dried in the same buffer for 24 hours, then dehydrated and embedded in paraffin. The paraffin slices (4 μm thick) were stained with hematoxylin-cosin and Azan staining solutions. The cross-sectional area, the total cell number in the aortic tunica media, and the wall thickness/lumen ratio (the medial thickness to the internal diameter) were determined (magnification ×10 for cross-sectional area and lumen diameter; magnification ×100 for cell counting). The cross-sectional fractional Azan-stained fibrosis was measured from 5 randomly selected fields in 1 cross section of aorta (magnification ×100) with NIH IMAGE 1.60, according to Kojima et al.

Tissue Preparation and Immunohistochemistry

The other rats were also killed by an intraperitoneal sodium pentobarbital overdose. For the immunohistological studies, a certain amount of rat descending thoracic aorta was fixed in a mixed solution of 95% ethanol and 1% acetic acid. Care was taken not to damage either the endothelium or the medial layer. The rest of the rat thoracic aortas were carefully rinsed with phosphate-buffered saline to remove adventitial fat and connective tissue by blunt dissection, frozen with liquid nitrogen, and stored at −80°C until use for the immunoblot analysis. Fixed specimens were paraffin-embedded and sectioned in 4-μm slices. After deparaffinization and rehydration, immunoenzymatic staining was performed with the use of the DAKO LSAB kit (Dako Co) according to the manufacturer’s instructions. The primary antibodies for 4 MHC isoforms were used at a dilution of 1:200. Anti-α-SM actin antibody was used according to the manufacturer’s recommendation. As a second antibody, a 1:200 dilution of biotinylated-goat anti-rabbit immunoglobulin was used for 4 MHC isoforms, and a 1:200 dilution of biotinylated-goat anti-mouse immunoglobulin was used for α-SM actin. Normal rabbit serum was used for 4 MHC isoforms as a negative control, and mouse IgG was used for α-SM actin. The slides were counterstained with hematoxylin solution.

SDS-PAGE and Immunoblots for MHC Isoforms and Actin

The rat thoracic aorta tissue extracts used for electrophoresis were prepared according to Takahashi et al., and the protein concentrations were measured. The same amount of protein extracted from rat aortas was loaded on each lane, and actin was used for the internal standard to standardize the loading condition. Actin was separated on SDS–10% PAGE, and MHC isoforms were separated on SDS–5% PAGE according to Kawamoto and Adelstein. The proteins were then electroblotted and stained with Konica Immunostaining HRP-1000 (Konica Co, Ltd). Anti-actin and all of the sera containing polyclonal antibodies were used at a dilution of 1:1000. After immunoblotting, the film was scanned with a densitometric scanner at 570 nm.

Statistical Analysis

All values are expressed as mean±SEM. The experimental groups were compared with ANOVA followed by Scheffé’s multiple comparisons. A value of P<0.05 was considered statistically significant. The ratios of MHC isoforms to actin obtained by measuring dimensionless quantities (band densities) from multiple similar experiments were combined by a calculation of the fold increase versus either the vehicle WKY or SHR group under each experimental condition.

Results

Body Weight, Hemodynamics, Morphology, and Fibrosis

Body weight and heart rate were unaltered among the 6 groups (Table). In the 3 WKY groups as well as the SHR+FK and SHR+EN groups, the systolic blood pressure was significantly lower than that of the vehicle SHR group. However, both the SHR+FK and SHR+EN groups still showed significantly higher blood pressures than the 3 WKY groups. In addition, there was no significant difference in blood pressure between the SHR+FK and SHR+EN groups or among the 3 WKY groups.

Figure 1 shows that the cross-sectional area in the vehicle SHR group was significantly higher than that in the 3 WKY groups (P<0.05; Figure 1A). However, there was no significant difference in the cross-sectional area not only among the 3 WKY groups but also among the 3 SHR groups. In addition, no difference in cross-sectional area was observed among the 3 WKY, SHR+EN, and SHR+FK groups. The cross-sectional total cell number (nuclei) in the vehicle SHR group was slightly higher than that in the other 5 groups, but the difference was not statistically significant among all 6 groups (Figure 1B). The wall thickness/lumen ratio in the 3 SHR groups was significantly higher than that in the 3 WKY groups (P<0.05; Figure 1C); however, no difference was observed in the wall thickness/lumen ratio not only among the 3 WKY groups but also among the 3 SHR groups. Cross-sectional fractional Azan-stained fibrosis showed few differences among the 6 groups (Figure 1D).
Immunohistochemical Localization of MHC Isoforms in Rat Aortas

The photographs in Figure 2 show that all 4 of the MHC isoforms in the SMC cytoplasm of the tunica media from the WKY and SHR were specifically and heterogeneously stained with specific antibodies against MHC isoforms and α-SM actin (brown against a pale blue background). The staining of 20-week-old WKY and SHR aortas with peptide-specific antibodies for 4 different MHC isoforms showed prominent cellular staining of SMC. In addition, no staining of NMHCs was observed in rat endothelial cells or adventitia. Little intimal thickening of the descending thoracic aortas was observed in either WKY or SHR.

Effect of Ang II Inhibition on MHC Isoforms in Rat Aortas

Myosin purified from turkey gizzard exhibited 2 major bands, 204 kDa and 200 kDa, which correspond to SM-1 and SM-2, respectively (Figure 3A, lane 1). In contrast, myosin purified from human platelet exhibited only a single band, 196 kDa for NMHC-A (Figure 3A, lane 2). Both WKY and SHR aortas also showed 2 major bands, which migrated at the same molecular weight as purified turkey gizzard myosin: 204 kDa for SM-1 and 200 kDa for SM-2, respectively (Figure 3A, lanes 3 and 4). We could not determine either NMHC-A or NMHC-B in WKY and SHR aorta with Coomassie blue staining. In contrast, immunoblotting using specific antibodies for actin and all 4 MHC isoforms clearly demonstrated that actin and all 4 MHC isoforms could be specifically detected in rat descending thoracic aortas as a single band with each antibody (Figure 3B).

The densitometric analysis of immunostaining for 4 different MHC isoforms showed that in the 3 WKY groups, there were no effects of the 2 drugs on the expression of any MHC isoform in aortic SMC (Figure 4A to 4D). In addition, a 6-week treatment with the 2 drugs did not affect the expression of SM-MHCs in either the SHR+EN or the SHR+FK group (Figure 4E and 4F). In contrast, a 6-week treatment with FK resulted in a significant downregulation of the expression of NMHC-A by 50% (P<0.005) and of NMHC-B by 24% (P<0.05) in the SHR+FK group, but not in the SHR+EN group, compared with the vehicle SHR group (Figure 4G and 4H).

Discussion

It has been reported that medial SM hypertrophy can be explained by the presence of cellular hypertrophy without hyperplasia in 5-month-old WKY and SHR. Reductions in the vascular SM content with drug treatment have been
shown to be due to reductions in SMC size, not to reductions in cell number. In the present study, we also showed that medial SM hypertrophy is caused by cell hypertrophy rather than cell proliferation in SHR aortas (Figure 1).

Several studies indicate that the coexistence of 2 distinct phenotypes in the normal vascular wall and the compositions of the 4 MHC isoforms are regulated in the developmental stage of the tissues and species examined. Although the expression of NMHCs has been associated with a dedifferentiated SMC phenotype, the contractile and synthetic phenotypes described for vascular SMC might represent only 2 points on a continuous spectrum of SM phenotypic expression. In agreement with Soltis, our results indicate the presence and heterogeneous expression of 4 distinct MHC isoforms in the SMC of rat aortic tunica media, and SM-MHCs are highly expressed in SMC compared with NMHCs (Figures 2 and 3).

The important findings of the present study are the discrimination of the AT1 receptor antagonist and the ACE inhibitor in relation to qualitative changes in the MHC isoform composition of vascular SMC in the SHR aortic tunica media. In this study, there was little intimal thickening in the vehicle SHR group (Figure 2). Furthermore, a 6-week treatment with either EN or FK induced similar significant decreases in systolic blood pressure, but only FK induced a significant downregulation of NMHC expression in the aortic tunica media in SHR (Figure 4). In contrast, the cellular SM-MHC content in SMC of the aortic tunica media did not change in response to the drug treatment in SHR. These results indicate that Ang II might play an important role in generating high blood pressure without SMC proliferation in SHR aortas. In addition, the inhibition of the renin-angiotensin system by

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**Figure 2.** Immunohistochemical staining of α-SM actin and 4 different MHC isoform expressions in the tunica media of the descending thoracic aortas of 20-week-old WKY and SHR with serial sections. Shown are WKY (A through E); SHR (F through J); α-SM actin (A and F); SM-1 (B and G); SM-2 (C and H); NMHC-A (D and I); and NMHC-B (E and J). Magnification ×400.

**Figure 3.** Coomassie blue and immunoblot staining of each MHC isoform in the descending thoracic aorta from 20-week-old WKY and SHR. A, Representative Coomassie blue staining of SDS–5% PAGE (7.5 μg of each protein was loaded on lanes 1 to 4). B, Representative immunoblots of 4 MHC isoforms and actin. A total of 2.5 μg of protein extracted from rat descending thoracic aorta was loaded on the gels, followed by immunoblotting.
the AT1 receptor antagonist for 6 weeks could result in a relative increase in the cellular contents of the contractile type of MHC isoforms by preventing the expression of the synthetic type of MHC isoforms of vascular SMC in SHR aortic tunica media. The effects of FK seen in SHR were not observed in WKY. These results suggest that the AT1 receptor antagonist might induce a greater phenotypic modulation of aortic SMC than the ACE inhibitor. This modulation by the AT1 receptor antagonist causes the aortic SMC to be more like the contractile type than the synthetic type of SMC, and no morphological changes occur in the SHR during the 6 weeks of treatment.

Several mechanisms might be responsible for the differences in the effects of FK and EN on the NMHC isoform expression of SMC in SHR aortic tunica media. The effects of FK seen in SHR were not observed in WKY. These results suggest that the AT1 receptor antagonist might induce a greater phenotypic modulation of aortic SMC than the ACE inhibitor. This modulation by the AT1 receptor antagonist causes the aortic SMC to be more like the contractile type than the synthetic type of SMC, and no morphological changes occur in the SHR during the 6 weeks of treatment.

Several mechanisms might be responsible for the differences in the effects of FK and EN on the NMHC isoform expression of SMC in SHR aortic tunica media. There could be a difference in the inhibitory effects of both FK and EN on the local renin-angiotensin system in the vascular wall.11 In addition, there may be a difference between the blocking actions of FK and EN on the AT1 receptor in aortic SMC. The mitogenic effects of Ang II on vascular SMC depend on the ratio of AT1 to AT2 receptors. In addition, most of the known functional effects of Ang II (vascular SMC proliferation and migration) are mediated by the AT1 receptors, whereas the stimulation of the AT2 receptor inhibits cell growth.10,20 It has been suggested that the lack of AT1 receptors in vascular SMC of SHR might be partly responsible for the increased growth of vascular SMC and vascular remodeling through the AT1 receptors in SHR.21 Furthermore, Sabri et al22 have also suggested that in Ang II–induced hypertension, changes in vascular SMC phenotypes are triggered primarily through the AT1 receptors. In addition, kinin metabolism, including nitric oxide and prostaglandins and other systems,11 might be involved in the difference in action between FK and EN on the NMHC isoform expression of SMC in SHR aortic tunica media.

Moreover, it has been reported that SMC that express NMHC isoforms can form connective tissue molecules in the aorta,3 and the downregulation of NMHC-B inhibits vascular SMC proliferation.23 In addition, in a study by Albaladejo et al,24 aortic collagen content was observed to be significantly lower in SHR treated with the ACE inhibitor quinapril for 12 weeks. In our study, fractional cross-sectional aortic fibrosis as assessed by Azan staining in SHR treated for 6 weeks with either FK or EN remained basically unchanged compared with the vehicle SHR. The 6-week treatment of SHR with either FK or EN was shorter than that described previously,24 and it is possible that the treatment was not long enough to prevent aortic fibrosis in SHR.

In summary, this study has indicated that 6-week treatments with either the AT1 receptor antagonist or the ACE inhibitor are similarly effective with regard to SHR hemodynamics, SMC hypertrophy and proliferation, extracellular matrix formation, and vascular remodeling. The AT1 receptor antagonist, however, inhibits the expression of NMHCs, but

Figure 4. Results of densitometric analysis of immunoblotting for 4 MHC isoforms in 20-week-old WKY and SHR after a 6-week treatment with vehicle, EN, or FK. The ratios of each MHC isoform to actin were obtained by combining dimensionless quantities (band densities) from 6 independent experiments for WKY and 8 for SHR by calculating the fold increase vs the vehicle group. Bar graphs show mean±SE fold increase (decrease) over the vehicle group. *P<0.005 and #P<0.05 vs the vehicle SHR group. Shown are WKY (A through D); SHR (E through H); SM-1 (A and E); SM-2 (B and F); NMHC-A (C and G); and NMHC-B (D and H).
not SM-MHCs, more significantly than the ACE inhibitor, which might modulate the relative composition of contractile-type and synthetic-type MHC isoforms and might affect the contractile properties of aortic SMC even before morphological changes occur in the SHR aorta. Further investigations are necessary to clarify the physiological significance of the AT₁ and AT₂ receptor subtypes on aortic SMC, as well as the long-term effect of the AT₁ receptor antagonist therapy on vascular remodeling in hypertension.

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References

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