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Comparative Effects of Angiotensin II Type 1 Receptor Blockade with Angiotensin-Converting-Enzyme Inhibitor on Aortic Smooth Muscle Cell Phenotype in Spontaneously Hypertensive Rats

Akihisa Fujii

Second Department of Internal Medicine, Yamaguchi University School of Medicine, 1144 Kogushi, Ube, Yamaguchi, 755 Japan.
Phone: 0836-22-2248 Fax: 0836-22-2246
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Abstract To clarify the effects of renin-angiotensin system inhibition on aortic smooth muscle cell phenotype and fibrosis in spontaneously hypertensive rats (SHR), we treated 14-week-old SHR with a selective angiotensin II type 1 (AT1) receptor blockade, FK-739 (30mg/kg/day) or angiotensin-converting-enzyme (ACE) inhibitor, enalapril (10mg/kg/day) for 6 weeks. Both FK-739 and enalapril caused a significant decrease in blood pressure ($p < 0.001$) with no difference between the two treated groups. Immunoblot showed no significant difference in the expression of smooth muscle cell myosin heavy chain and nonmuscle myosin heavy chain (NMHC)-A in SHR aorta. In contrast, both FK- 739 and enalapril resulted in a significant decrease in the expression of NMHC-B compared with vehicle (vehicle vs FK-739 treated group ; $p < 0.0001$, vehicle vs enalapril treated group ; $p < 0.025$, respectively). Furthermore, FK-739 inhibited the expression of NMHC-B more significantly than enalapril ($p < 0.003$). Fractional fibrosis and a cross- sectional total cell number were not different statistically among three groups. These results suggest that, compared with ACE inhibition, AT1 receptor blockade may have a more potent effect on the phenotype change of smooth muscle cells in SHR aorta.

Key words: Angiotensin II type 1 receptor blockade, Smooth muscle cell, Myosin heavy chain isoform, Fibrosis, Hypertension

Introduction

Hypertension is a major risk factor of atherosclerosis and large artery walls become thicker and stiffer in hypertension 1). The proliferation of vascular smooth muscle cells is also an important component of many vascular diseases including atherosclerosis and restenosis after coronary angioplasty 2).

It is reported that smooth muscle cells have two phenotypes; contractile and synthetic types, and contain at least four types of myosin heavy chain isoforms: SM-1 (204 kDa), SM-2 (200 kDa), NMHC-A (196 kDa), and NMHC-B (198 kDa), which are useful molecular markers for phenotype change of smooth muscle cells 3)-5). NMHC-B is relatively increased as a result of phenotype change of smooth muscle cells from contractile type to synthetic type in injured arteries or arteriosclerotic lesions 6).

Changes in the extracellular matrix and fibrosis are part of the characteristic response of vascular tissue to hypertension, and they influence the biochemical characteristics of vascular smooth muscle cells 7)-8). Smooth

muscle cells obtain the ability to produce the extracellular matrix and growth factors by changing the phenotype to synthetic type 9). The extent of this structural transformation might be restored by using antihypertensive agents such as angiotensin-converting -enzyme (ACE) inhibitor 10).

Recent studies have shown that angiotensin II (Ang II) directly induced vascular smooth muscle cell hypertrophy and proliferation as well as the proliferation of extracellular matrix and fibrosis 11). ACE converts Ang I to Ang II and inactivates kinins and some peptides 12). It is also reported that some enzymes directly generate Ang II in such tissues like the vascular wall 13). Accordingly, selectively preventing the binding of Ang II to the receptor would provide a rational way to block the renin-angiotensin system. Recently, a nonpeptide antagonist to Ang II type 1 receptor (AT1) blockade (FK- 739) was developed 14). We have already demonstrated the effect of FK-739 with enalapril on left ventricular distensibility and collagen metabolism in SHR 15).

In this report, we compared the effect of an AT1 receptor blockade, FK-739 with that of an ACE inhibitor, enalapril on the isoform change of aortic smooth muscle cell phenotype and fibrosis in SHR aorta.

Methods

Chemicals and Reagents

All reagents were purchased from Sigma (St. Louis, MO, U.S.A.). FK-739 was a gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Enalapril was a gift from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan).

Antibodies

Antibodies against SM-1, SM-2, NMHC-A and NMHC-B were kind gifts from Drs. Adelstein and Kelly, Laboratory of Molecular Cardiology, NHLBI, U.S.A. Smooth muscle MHC antibodies specific for the 204 kDa (SM -1) and 200 kDa (SM-2) bovine aortic isoforms have been previously described 16). Antibodies against NMHC-A and NMHC-B have been described by Phillip et al 17). Mouse monoclonal antibody against α- smooth muscle actin (ASM-1) was obtained from American Research Products, Inc., (Belmont, MA, U.S.A.).

Animals and Experimental Model

Male SHRs were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Fourteen-week-old SHRs were treated for 6 weeks with FK-739 (30mg/kg/day of drinking water), enalapril (10mg/kg/day of drinking water) and vehicle. The doses used in this experiments were determined according to Yonezawa et al 15).

Hemodynamic Studies

Systolic blood pressure and heart rate (HR) were determined in a controlled-temperature room by tail-cuff plethysmography on unanesthetized rats at 26°C (Programmed Electro-Sphygmomanometer PE-300, NARCO BIO-SYSTEMS, Houston, TX, U.S. A.).

Rat Aorta

After anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), rats were killed with potassium chloride injected via the right jugular vein. After removal of the adventitia, part of the rat descending thoracic aorta was fixed in a mixed solution of 95 % ethanol and 1 % acetic acid according to the method of Aikawa et al. for immunohistological studies 18). The rest of the tissue was frozen in liquid nitrogen and stored at -80°C until use for immunoblot analysis.

Immunohistochemistry of Rat Aorta

Specimens fixed as described above were embedded in paraffin and sectioned (4μm). After deparaffinization and rehydration, immunoenzymatic staining was performed using a DAKO LSAB kit (Dako Co, CA, USA) according to the manufacturer's instructions. Briefly, sections were preincubated with 3 % hydrogen peroxide and normal rabbit serum to reduce nonspecific reaction. The primary

antibodies for the SM-1, SM-2, NMHC-A, and NMHC-B were used at dilutions of 1 to 200. Anti-α-smooth muscle actin antibody was used according to manufacturer's recommendation. Antibodies against SM-1, SM-2, NMHC-A, NMHC-B, and α-smooth muscle actin were applied and incubated for 24 hours at 4°C. Sections were incubated with biotinylated goat anti-rabbit immunoglobulin for 2 hours at room temperature and then with horse radish peroxidase-labeled streptavidin solution for 1-2 minutes. Then, sections were rinsed three times in 7.5 mM Tris-buffered saline, 0.05 % of Tween 20, pH 7.6, before each incubation step. Peroxidase activity was revealed by 3,3'-diaminobenzidine tetrahydrochloride with hydrogen peroxide. The slides were counterstained with hematoxylin solution, dehydrated, and mounted. Normal rabbit serum was used a place of the primary antibody as a negative control.

The cross-sectional total number of stained nuclei in aortic media of each sample was also microscopically counted by three independent investigators blinded to the specimen at a magnification of 200, and averaged for each animal. The coefficient of variance among investigators was 4.8 %.

SDS-PAGE and Immunoblotting of MHC Isoforms

Extracts from rat aortae were prepared according to Phillips et al. 17) with a Polytron homogenizer (Kinematica AG, Littau, Switzerland). Suspensions were centrifuged at 50,000 g for 20 minutes, and supernatants were used for electrophoresis. Protein concentrations in the tissue extracts were measured by Bradford's method 19). To normalize the loading condition, an equal amount (24 g) of total protein extracted from rat aorta was applied to each lane, and MHC isoforms were separated on sodium dodecyl sulfate (SDS) - 5 % polyacrylamide gels according to Kawamoto et al. 5). The proteins were electroblotted onto Immobilon-P (Millipore, Bedford, MA, U.S.A.). The membranes were immunologically stained by the method of Towbin et al. 20). All polyclonal antibodies were used at dilations of 1 to 1000. After immunoblotting, the film was scanned with a densitometric scanner (PAN-802, Johkoh Co, LTD, Tokyo, Japan) at 570 nm.

Fibrosis of Aorta Assessed by Azan Staining

Aortic fractional fibrosis was examined microscopically with Azan staining. To determine the degree of fibrosis, we randomly selected four fields from one cross-sectional cut of the aorta and calculated the ratio of Azan-stained fibrosis area to total aortic medial area using an NIH IMAGE (Research Service Branch, NIH, USA) analysis software according to the method of Kojima et al 21).

Statistical Analysis

All values were expressed as mean±SE. Comparisons among the three groups were made by one way ANOVA with Scheffe's post hoc analysis. A value of $p < 0.05$ was considered statistically significant.

Results

Hemodynamics and Total Cell Number in Aortic Media

There were no significant differences in body weight and HR among the three groups. In both FK-739 and enalapril treated groups, there was a significant decrease in systolic blood pressure ($p < 0.001$) in comparison with the vehicle, however, no significant difference was seen between the FK-739 and enalapril treated groups (Table 1). In addition, the cross-sectional total cell number (nuclei) in aortic media was not statistically different among the three groups.

Immunohistochemistry and Immunoblot Analysis of Smooth Muscle Cell Phenotypes in SHR Aorta

Immunohistochemical examinations have clearly disclosed that smooth muscle cells in SHR aortic media were specifically stained with both all of the antibodies against MHC isoforms and anti-α-smooth muscle actin antibody used in this experiment (Figure 1). Little intimal thickening was observed in

Table 1. Hemodynamics and cross-sectional total cell number per aortic media

	20-week-old SHR treated with		
	vehicle	Enalapril	FK-739
Number of rats	6	6	6
BW, g	353±4	344±6	360±4
HR, beats/min	334±9	341±9	349±5
SBP, mmHg	169±4	124±1*	135±3*
Number of cells	787±21	765±17	752±24

BW; body weight, HR; heart rate, SBP; systolic blood pressure. Data are given as mean ± SE. * $p < 0.001$ versus vehicle. Cross-sectional total cell number was determined from counted nuclei in rat aortic media.

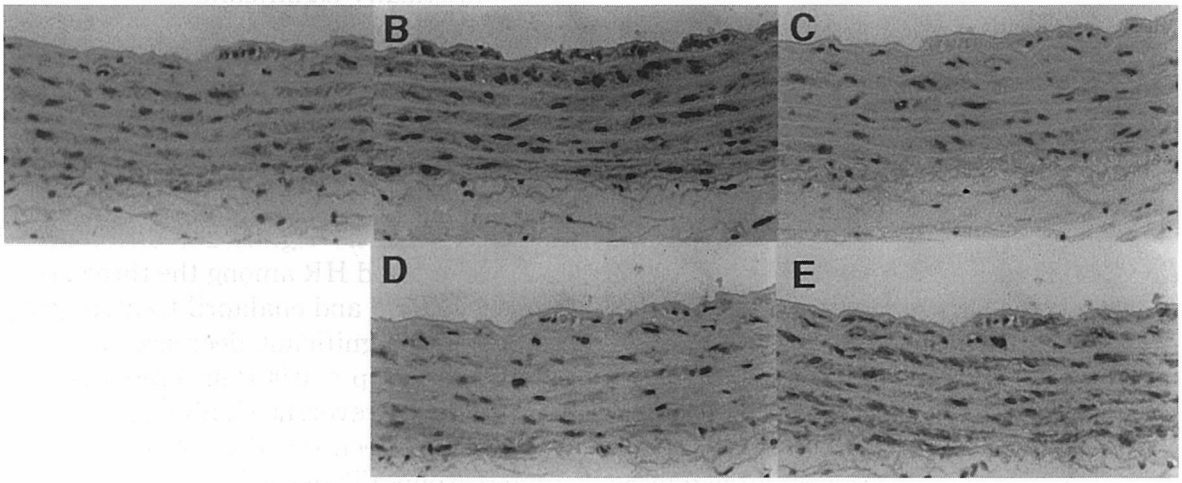

Fig. 1 Immunohistochemistry for smooth muscle specific myosin heavy chain (MHC) isoforms (SM-1 and SM-2), nonmuscle MHC isoforms (NMHC-A and NMHC-B) and α- smooth muscle actin in the aorta from vehicle SHR with serial sections. All of MHC isoforms and α-smooth muscle actin were expressed in smooth mucle cells in vehicle SHR aortic media. Little intimal thickening was observed in SHR aorta. A:α-smooth muscle actin, B:SM-1, C:SM-2, D:NMHC-A, E:NMHC-B. Magnification x 200.

SHR aorta and no stain with all of the anti-MHC antibodies and anti-α-smooth muscle actin antibody was noticed in the adventitia.

To examine further the effect of FK-739 and enalapril on the expression of smooth muscle MHC isoforms in SHR descending thoracic aorta, immunoblotting analysis was also performed. Figure 2 shows representative immunoblots of each MHC isoform in SHR aorta of vehicle, FK-739 and enalapril treated groups. All four MHC isoforms were specifically detected by the antibodies used.

Figure 3 shows the results of the densitometric analysis. No significant difference in the expressions of SM-1, SM-2 and NMHC -A were observed among the three groups, although there was a tendency of increase in the expressions of SM-1 and SM- 2, and a tendency of decrease in the expression of NMHC-A in both the FK-739 and enalapril treated groups as compared with the vehicle. In contrast, treatment with both FK-739 and enalapril resulted in a significant decrease in the expression of NMHC-B compared with vehicle in SHR aorta (FK-739 treated group vs vehicle; $p < 0.0001$, enalapril treated group vs vehicle; $p < 0.025$). Furthermore, the expression of NMHC-B was reduced more significantly in the FK-739 treated group in comparison with the enalapril treated group ($p < 0.003$, Figure 3).

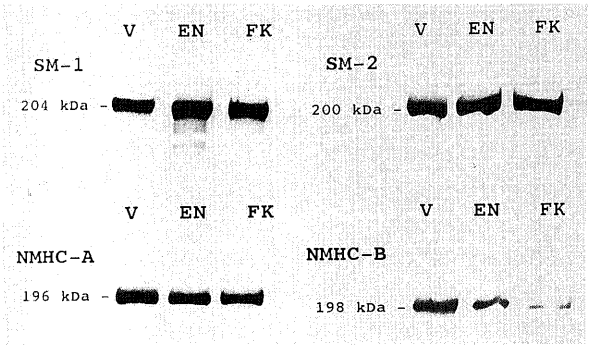

Fig. 2 Representative immunoblot of each different MHC isoforms in SHR aorta of vehicle, enalapril and FK-739 treated groups. All of four MHC isoforms were specifically detected with each antibody (SM-1 ; 204 kDa, SM-2 ; 200 kDa, NMHC-A ; 196 kDa, NMHC-B ; 198 kDa). Same amount (24μg) of total protein extracted from rat aorta was applied on each lane. V : vehicle, EN : enalapril treated group, FK : FK-739 treated group.

Effect of FK-739 and Enalapril on Fibrosis in SHR Aorta

We also made an assessment of the fibrosis of SHR aorta with Azan staining and compared it among the three groups.
Fractional fibrosis as determined from the ratio of Azan-stained fibrosis area to total aortic media showed that there was little difference in the fibrosis of aorta among the three groups (vehicle ; 34 ± 2 %, enalapril treated group ; 29 ± 1 % and FK-739 treated group ; 30 ± 2 %, n = 6, Figure 4).

Discussion

In this study, we treated 14-week-old SHR with either FK-739 or enalapril for 6 weeks, and demonstrated that : (1) both FK- 739 and enalapril had similar effects on hemodynamics, (2) with both immunohistochemistry and immunoblot, two smooth muscle MHC isoforms (SM-1 and SM-2) and two nonmuscle MHC isoforms (NMHC-A and NMHC-B) were expressed in SHR aortic

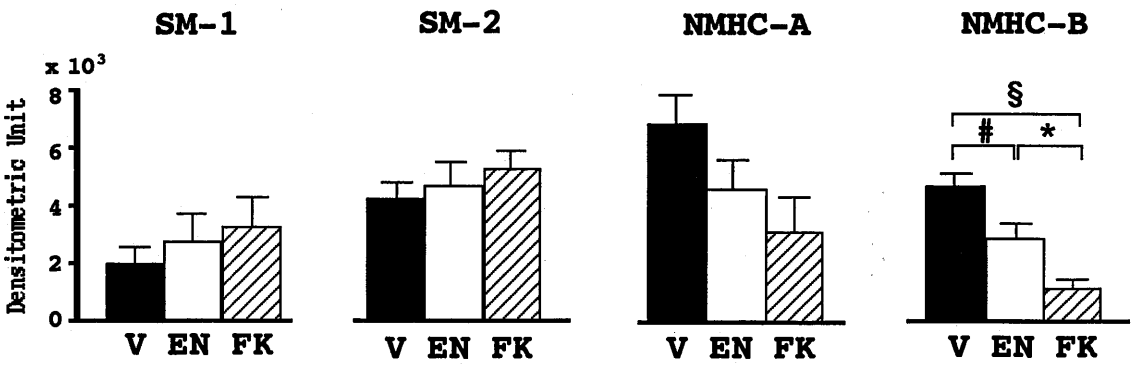

Fig. 3 The results of the densitometric analysis of immunoblotting. V : vehicle, EN : enalapril treated group, FK : FK-739 treated group. Data are given as mean ± SE. (n = 5) #p < 0.001, * p < 0.0035, $p < 0.0004.

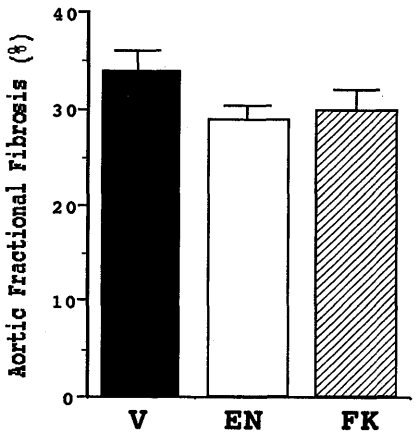

Fig. 4 Effect of enalapril and FK-739 on cross-sectional fractional Azan-stained fibrosis. Fractional fibrosis was determined with NIH image. There was no statistical difference in the fibrosis of aortic media among the three groups. V : vehicle, EN : enalapril treated group, FK : FK-739 treated group. Data are given as mean ± SE. (n = 9)

media, (3) there was a significant decrease in the expression of NMHC-B in both FK-739 and enalapril treated groups as compared with the vehicle. In addition, FK-739 induced a significant decrease in the expression of NMHC-B as compared with enalapril. (4) There were no significant differences in total cell number per cross-sectional aortic media, aortic fibrosis and the expressions of SM-1, SM-2 and NMHC-A in SHR aorta among the vehicle, FK-739 and enalapril treated groups.

There are several reports on the distribution of the MHC isoforms in vascular smooth muscle cells in different species.
Kuro-o et al. showed that SM-1 and SM-2 were important in the identification of differentiated smooth muscle cells, and SMemb/NMHC-B were most abundantly expressed in rabbit embryonic smooth muscle, in proliferating smooth muscle cells of experimental atherosclerotic or arteriosclerotic lesions, and in cultured smooth muscle cells 22). Unlike the findings for rabbit, Aikawa and colleagues have recently showed the presence of three isoforms, smooth muscle 204 and 200 kDa MHCs and SMemb/NMHC-B in both human foetal and adult aortas 18). Frid et al., despite detecting the 204 and 200 kDa smooth muscle MHC, and a nonmuscle MHC -A-like type in adult human aorta, detected a nonmuscle-B-like isoform which showed considerable expression in developing human aortic smooth muscle, but appeared to be down-regulated with development and almost absent in the adult aortic media 24). On the other hand, with the smooth muscle and nonmuscle MHC specific antibodies used in this study, Phillips et al. demonstrated the presence of two smooth muscle (204 kDa and 200 kDa) and significant amounts of two nonmuscle (A and B) MHC isoforms in normal human adult and foetal aortic media by immunoblot and immunohistochemistry 17). Our results in 20-week-old SHR's aortic media were in the agreement with those of Phillips et al. 17).

Sartore et al. reported that, in both Ang II infusion and Ang II-dependent hypertension rabbit model (one-kidney, one-clip Goldblatt procedure), there was a remarkable change in smooth muscle cell phenotype 23). These results, including ours, indicated that Ang II might play an important role in generating high blood pressure, smooth muscle cell phenotype change in SHR, and the inhibition of renin-angiotensin system with either AT1 receptor blockade or ACE inhibitor may increase the contractile type of smooth muscle cell by preventing the phenotype change of smooth muscle cells.

Furthermore, we showed that FK-739 induced a significant decrease in the expression of NMHC-B in SHR aorta as compared with enalapril. Several mechanisms may result in the difference of the effect of FK-739 and enalapril on phenotype change of aortic smooth muscle cells in SHR. First, there may be a difference in the inhibitory effect of both FK-739 and enalapril on the local renin-angiotensin system in vascular wall. Second, it is also possible that there may be a difference in the blocking action of FK-739 and enalapril on AT2 receptor in aortic smooth muscle cells, since recent studies suggest that AT2 receptor is mainly expressed in proliferative smooth muscle cells, such as after balloon injury 25). In addition, kinin metabolism including nitric oxide and prostaglandins may be involved in the difference seen in the action

of both FK-739 and enalapril on MHC isoforms and the phenotype change of smooth muscle cells in SHR aorta 26).

With a nonpeptide antagonist to Ang II type 1 receptor (AT1) blockade: FK-739 14), we have already demonstrated that, compared with ACE inhibition, FK-739 may have additional effects on left ventricular distensibility and collagen metabolism in the regression of left ventricular hypertrophy in SHR 15). Pierre et al. showed that aortic collagen content was significantly lower in SHR treated with quinapril for 12 weeks, suggesting that Ang II receptor stimulation increases collagen synthesis by acting directly on smooth muscle cells 27). It is also reported that smooth muscle cells which express NMHC isoforms, such as the synthetic type, can form connective tissue molecule in aorta 9). These results suggest that phenotype change of smooth muscle cells regulates extracellular matrix formation and fibrosis. In our study, fractional cross-sectional aortic fibrosis assessed by Azan staining in SHR treated with FK-739 or enalapril was decreased only by about 10 %, and was not statistically significant compared with the vehicle. It is possible that the 6-week-treatment of either FK-739 or enalapril was shorter than that of a previous report 27), and not long enough to prevent aortic fibrosis in SHR.

By using in vitro motility assays, which are thought to correlate with the unloaded shortening velocity (Vmax) of muscle fibers, Umemoto et al. demonstrated that the relative velocity of smooth muscle myosin filaments on the actin filaments was about 5 times faster than that of cytoplasmic (nonmuscle) myosin filaments 28). It is likely that the change of the composition of MHC isoforms by reducing NMHC-B with FK-739 or enalapril in aortic media may change the contractile property of smooth muscle by affecting the actin-myosin interaction. It would be important to examine the effect of inhibition of NMHC-B by FK-739 or enalapril on aortic smooth muscle mechanics.

In summary, the results of this study indicated that 6-week-treatment of chronic ACE inhibition and AT1 receptor blockade are similarly effective on the hemodynamics in SHR. AT1 receptor blockade, however, selectively and significantly inhibited the expression of NMHC-B more than ACE inhibitor with little effect on aortic fibrosis and cell proliferation of smooth muscle cells in SHR aortic media. These results suggest that the renin-angiotensin system is important in the development of hypertension in SHR. In addition, AT1 receptor blockade may have an additional effect on the phenotype change of aortic smooth muscle cells. Further investigation will be necessary to clarify the physiological significance of the phenotype change of aortic smooth muscle cells and the effect of long term therapy of AT1 receptor blockade on vascular smooth muscle cells and fibrosis in hypertension.

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