

## Changes in renal vessels associated with long-term administration of angiotensin converting enzyme inhibitor in Zucker fatty rats

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### Abstract

**Background** Recently, we showed that long-term angiotensin receptor blocker (ARB) administration induced unusual proliferative changes in smooth muscle cells (SMCs) of afferent arterioles of the kidneys of Zucker fatty rats (ZFRs). In this study, we investigated renal afferent arteriolar changes induced by the long-term administration of an angiotensin converting enzyme inhibitor (ACEI) in ZFRs. **Materials and Methods** Fourteen 6-week-old male ZFRs were divided into two groups ( $n=14$ ): the ZFR+ACEI group ( $n=6$ ) was fed a standard diet containing ACEI (Enalapril, 2 mg/kg/day), and the ZFR control group ( $n=8$ ) for 12 weeks. Blood pressure and proteinuria were examined and morphological studies on kidneys were performed. **Results** Remarkable proliferative changes in the afferent arteriolar SMCs were frequently observed in the group given ACEI; ( $66.1 \pm 12.9\%$ ) compared with the control group ( $1.77 \pm 1.56\%$ ,  $P<0.001$ ). **Conclusions** It was indicated that long-term ACEI administration induced unusual proliferative changes in SMCs in afferent arterioles of ZFRs. These changes could reduce intraglomerular pressure by narrowing the lumens of afferent arterioles, but they could cause irreversible damage to the arterioles.

**Key words:** ACEI, Zucker fatty rat, afferent arteriole

### Introduction

Renin angiotensin aldosterone system (RAAS) inhibitors are widely used as anti-hypertensive agents. Moreover, they are known to have various organ-protecting effects without lowering blood pressure (1), and their nephro-protective effects are well known (2). However, most studies on the effects of angiotensin receptor blockers (ARBs) on the kidney were performed over a relatively short-term period (3, 4), and few studies

have focused on morphological changes in the afferent arterioles (5, 6), which are the most important resistance vessels for glomerular hemodynamics. Recently, we showed that long-term ARB administration induced unusual proliferative changes in smooth muscle cells (SMCs) of afferent arterioles in the kidneys of Zucker fatty rats (ZFRs) (7). These changes could narrow arteriolar lumens and reduce intraglomerular pressure, thereby potentially causing irreversible damage to the arterioles.

It well recognized that angiotensin converting enzyme inhibitors (ACEIs) provide renoprotection as well as ARBs (8–10). Therefore, in this study, we investigated afferent arteriolar changes induced by long-term administration of an ACEI in ZFRs.

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## Materials and Methods

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Fourteen 6-week-old male ZFRs were purchased from Charles River Japan. The rats were divided into two groups: the ZFR+ACEI group ( $n=6$ ) was fed a standard diet containing ACEI (Enalapril, 2 mg/kg/day, Sigma, Tokyo, Japan), and the ZFR control group ( $n=8$ ) for 12 weeks. Because this experiment was studied using the same methods as those of the experiment in reference #7 during the same period, it was valid to share the control data with the experiment of reference #7.

The body weight of each group was 210–230 g. The rats were housed in a temperature-controlled room at  $23 \pm 1$  °C and with a 12-h light/dark cycle, and they were allowed free access to diet and water. The experiments were carried out in accordance with the Animal Experimentation Guidelines of Toho University.

### *Blood pressure and biochemical measurement*

Beginning at 6 weeks of age, at intervals of 3 weeks, the body weight of each ZFR was recorded and its systolic blood pressure (SBP) and heart rate measured while in a conscious state using the indirect tail-cuff method (BP-98A; Softron, Tokyo, Japan) on a 37 °C preheated cloth jacket for about 10 min. The mean of three such recordings were taken as an individual rat's SBP and heart rate. Each ZFR was transferred to a metabolic cage for collection of a 24-h urine sample. All urine for each ZFR was collected for the measurement of urinary protein concentration. At 12 weeks into their specified diet, each ZFR was anesthetized with Inactin (100 mg/kg). Immediately after obtaining blood samples from the inferior vena cava of ZFRs, they were sacrificed and the kidneys isolated.

### *Histological analysis*

In each case the left kidney was cut along the long axis and one half was used for light microscopic studies, and the other used for immunohistochemistry. The specimens for light microscopic examination were fixed with 10% neutral-buffered formalin solution, and embedded in paraffin. Sections (2  $\mu\text{m}$ -thick) were stained with Periodic-acid silver methenamine and Hematoxylin-Eosin (PASM-HE). Dark-brown granules in smooth muscle cells after PASM-HE staining were identified as renin following immunohistochemistry (11).

In sections of the kidneys from each ZFR, morphological studies were performed by two experienced pathologists in a blinded trial. We took twenty photomicrographs using a digital microscopic camera (Olympus BX61) at 100 $\times$  magnification for each rat, in random areas (642  $\mu\text{m}$   $\times$  857  $\mu\text{m}$ ) that did not overlap. We tried to estimate the histopathological findings semi-quantitatively in the area (642  $\mu\text{m}$   $\times$  857  $\mu\text{m}$   $\times$  20) using PASM-HE sections. We counted the total number of glomeruli and also assessed the frequency of global glomerulosclerosis and focal segmental glomerulosclerosis (FSGS) in each photomicrograph. FSGS was diagnosed and classified according to the criteria accepted by the D'Agati et al. working group (12). We tried to estimate glomerular

hypertrophy by scoring the photomicrographs containing enlarged glomeruli with diameters exceeding 150  $\mu\text{m}$  (1: microphotographs filled with enlarged glomeruli, 0: filled with no enlarged glomeruli, 0.5: mixed with enlarged and not enlarged glomeruli). The mesangial expansion of glomeruli including the mesangial matrix increase and/or mesangial cell proliferation was scored as follows: 0: not remarkable, 1: mild, 2: moderate, or 3: marked. The mesangial scores were calculated by multiplying each of the affected glomeruli by the degree of mesangial expansion, and were then added in together. Tubular atrophy, interstitial fibrosis, and interstitial cell infiltration were estimated as the percentage of the affected area occupying each photomicrograph. These were scored as follows: 0: none, 1: 0–30%, 2: 31–50%, or 3: more than 51%. Interstitial cell infiltration scored as follows: 1: mild, 2: moderate, or 3: marked. The protein casts in the tubules were scored as 0: none, 1: sparse, 2: frequent, or 3: very frequent. These scores were summed up in each group and analyzed statistically. We counted the total number of the vertical and transverse cross-sections of these arterioles and also enumerated the number of arterioles with walls with more than three layers of SMC in each photomicrograph, added them up, and statistically analyzed them across the four groups. We excluded bifurcations, the start of preglomerular afferent or asymmetrically sectioned vessels, which were inadequate to estimate. We examined bigger arteries for changes in their walls.

### *Immunohistochemistry*

The additional sections from all the above paraffin blocks were used for immunohistochemistry. The primary antibodies used were goat anti-rat renin (a gift from Prof. Tadashi Inagami, Vanderbilt University, Nashville, USA), anti-rat  $\alpha$ -SMC actin (SMA, Sigma, MO, USA), monoclonal anti-smooth muscle myosin heavy chain isoform (SM-2, Yamasa, Code number 7601, Lot number 4808, Japan), and anti-rat endothelial aminopeptidase P monoclonal (JG12, Bender MedSystems, Vienna Austria) antibodies. The sections underwent deparaffinization, rehydration, and treatment using antigen retrieval techniques, which involved the use of pH 10 Target Retrieval Solution (TRS), treatment in an autoclave for 20 min at 105 °C, and reaction overnight for renin and SM2 and with pH 6 citric acid buffer for JG12.

Immunohistochemistry was performed using the ABC method (LSAB2 kit for use on rat specimens; Dako Japan, Tokyo, Japan). To verify antibody specificity, sections from each paraffin block were used as negative controls by omitting the primary antibody and replacing it with normal goat immunoglobulin.

### *Statistical analysis*

Mann-Whitney's *U* test was conducted for statistical analysis in accordance with each data characteristic and with a risk rate of 5% considered to be significant.

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## Results

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### *Physiologic and biochemical changes*

Table 1 summarizes the physiologic and biochemical data at 12 weeks after initiation of the two diet regimens. Body weight and the ratio of kidney weight to body weight were not significantly different between the two groups. Systolic blood pressure was lower in the ZFR+ACEI group than in the control group ( $93 \pm 15$ ,  $n=6$  vs.  $130 \pm 8.2$  mmHg,  $n=8$ ). Urinary protein excretion was significantly decreased in the ZFR+ACEI group compared with the control group ( $302 \pm 220$ ,  $n=6$  vs.  $12.4 \pm 3.9$  mg/day,  $n=8$ ). Serum glucose levels were not statistically different between the two groups.

**Table 1.** Physiologic and biochemical changes

	ZFR <sup>†</sup>	ZFR+ACEI
Body weight (g)	666 ± 72	522 ± 82*
Kidney weight (g/kg BW)	2.6 ± 1.4	2.7 ± 0.6
Systolic blood pressure (mmHg)	130 ± 8.2	93 ± 15*
Urine protein (mg/day)	302 ± 220	12.4 ± 3.9*
Serum glucose (mg/dl)	670 ± 562	198 ± 42

\*Significant difference from ZFR. <sup>†</sup>Quoted from reference#7.

**Table 2.** Histological findings

	ZFR <sup>†</sup>	ZFR+ACEI
<b>Glomerulus</b>		
Total numbers	79.14 ± 10.53	87.17 ± 9.02
Global sclerosis / Total glomeruli (%)	0.14 ± 0.38	0
FSGS / Total glomeruli (%)	0.41 ± 0.70	0.21 ± 0.50
Glomerular hypertrophy scores	0.43 ± 0.18	0.007 ± 0.02*
Mesangial expansion / Total glomeruli (%)	26.42 ± 12.05	14.11 ± 3.34*
Mesangial scores	0.15 ± 0.05	0.11 ± 0.02
<b>Tubules</b>		
Protein casts scores	0.37 ± 0.23	0*
Tubular atrophy scores	0.29 ± 0.19	0.47 ± 0.64
<b>Interstitial tissue</b>		
Fibrosis scores	0.29 ± 0.24	0.34 ± 0.53
Cell infiltration scores	0.22 ± 0.21	0.24 ± 0.42

\*Significant difference from ZFR group. <sup>†</sup>Quoted from reference#7. FSGS: focal segmental glomerulosclerosis.

### *Histological findings*

Tables 2 and 3 summarize the histological findings. The total numbers of glomeruli observed in photomicrographs of each rat showed no significant differences between the two groups. Global glomerulosclerosis was rarely observed, and its frequency between the two groups was not significantly different. FSGS, usually showing “NOS (not otherwise specified) variant” in morphological classification, without hyalinosis, was rarely observed in the two groups. The score of glomerular hypertrophy was significantly decreased in the ZFR+ACEI group compared with that of the ZFR control group ( $0.007 \pm 0.02$ ,  $n=6$  vs.  $0.43 \pm 0.18$ ,  $n=8$ ). The rate of mesangial expansion/total glomeruli was lower in the ZFR+ACEI group compared with the ZFR control group ( $14.11 \pm 3.34$ ,  $n=6$  vs.  $26.42 \pm 12.05$ ,  $n=8$ ; Table 2).

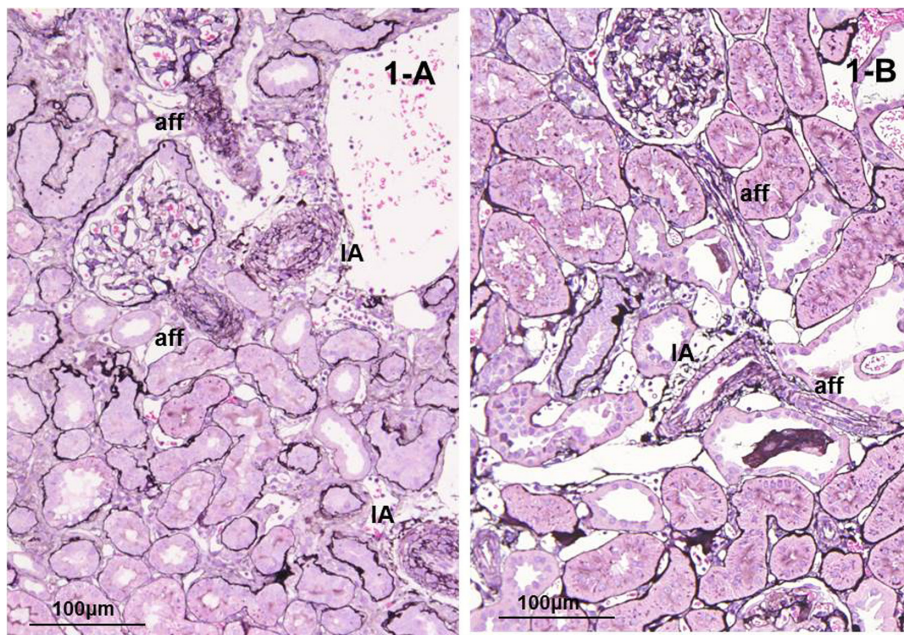
The scores for protein casts were significantly decreased in the ZFR+ACEI group compared with the control group ( $0$ ,  $n=6$  vs.  $0.37 \pm 0.23$ ,  $n=8$ ). The scores for tubular atrophy and interstitial changes had no statistically significant differences between the ZFR+ACEI and the ZFR control groups (Table 2).

Table 3 summarizes the vascular changes. The total numbers of interlobular arteries (IAs) observed in the photomicrographs per kidney in each group showed no significant difference between the two groups. In afferent arterioles, extreme proliferative changes in the arteriolar walls were frequently observed in the ACEI+ZFR group. The SMCs of arteriolar walls in the ACEI+ZFR group showed marked irregularities in size, shape, and arrangement. The layers of SMCs in the walls showed an extreme increase and concentric multiplication in vertical cross-sections, which resulted in a marked narrowing of the arteriolar lumens (Fig. 1-A, Fig. 2-A, B).

**Table 3.** Vascular changes

	ZFR <sup>†</sup>	ZFR+ACEI
Artery		
Total numbers of IAs	10 ± 4.54	15.7 ± 5.0
IAs with increased smooth muscle cell layers (%)	4.58 ± 7.83	37.2 ± 14.0*
Arteriole		
Vertical sections	40.57 ± 15.96	59.33 ± 12.8
Transverse sections	35 ± 6.11	46 ± 14.6
Total numbers of arterioles	75.57 ± 17.80	105.7 ± 25.8
Numbers of the arterioles with more than three layers of smooth muscle cell walls (%)	1.77 ± 1.56	84.2 ± 42.1*

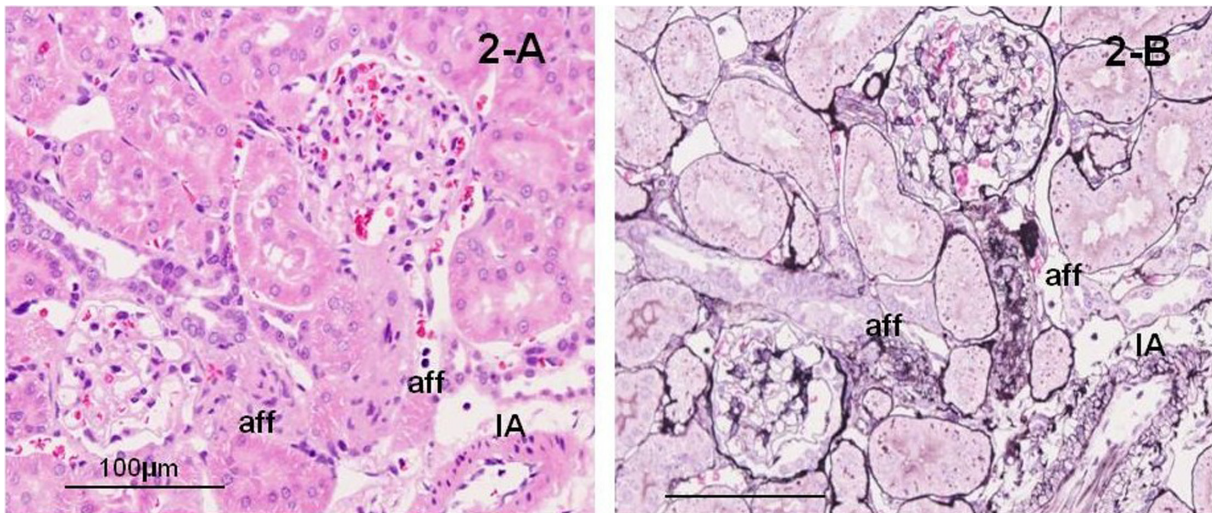
\*Significant difference from ZFR group. <sup>†</sup>Quoted from reference#7. IA: interlobular artery.



**Fig. 1.** Microphotographs with low magnification in the two groups. Calibration Bar 100 µm. 1-A: a microphotograph of the kidney in the ZFR+ACEI rat. Arteriolar walls show extreme mural thickening and multiplication of smooth muscle cells (SMC) layers. Interlobular arteries also show an increase of the layers in the vessel walls. 1-B: ZFR control rat shows arterioles with one layer of SMCs, and interlobular artery shows just two layers. aff: afferent arteriole, IA: interlobular artery, PASM-HE stain.

The frequency of arterioles with more than three SMC layers out of all observed arterioles in each rat were  $84.2 \pm 42.1\%$  ( $n=6$ ) in the ZFR+ACEI group, which was significantly higher compared with that of the ZFR control group ( $1.77\% \pm 1.56\%$ ,  $n=8$ ; Table 3). Hyaline deposition was not detected in the arteriolar walls. Walls of segmental, interlobar, and arcuate arteries showed no remarkable changes in the two groups; however, the wall of IAs sometimes showed the same proliferative changes as found in afferent arterioles in the ZFR+ACEI group from photomicrographs in each rat.

The efferent arterioles sometimes showed irregularities in the SMCs, but they showed no proliferative changes or luminal narrowing in either group. Endothelial cells in arteries and arterioles in both groups showed no evident morphological changes except for infrequent swelling of the cytoplasm.



**Fig. 2.** Microphotographs with higher magnification in the ZFR+ACEI rats. Calibration Bar 100  $\mu\text{m}$ . 2-A: the markedly thickened arteriolar walls are shown with HE stain, but the difference of the lesion in the arteriolar walls with arteriosclerosis is not clear. 2-B: PASM-HE stained serial section of the 2-A. The afferent arterioles show an extreme increase of SMC layers and luminal narrowing. SMCs show irregularities in size, shape and arrangement, and the many brown granules which are considered as renin are shown in the SMC walls. aff: afferent arteriole, IA: interlobular artery.

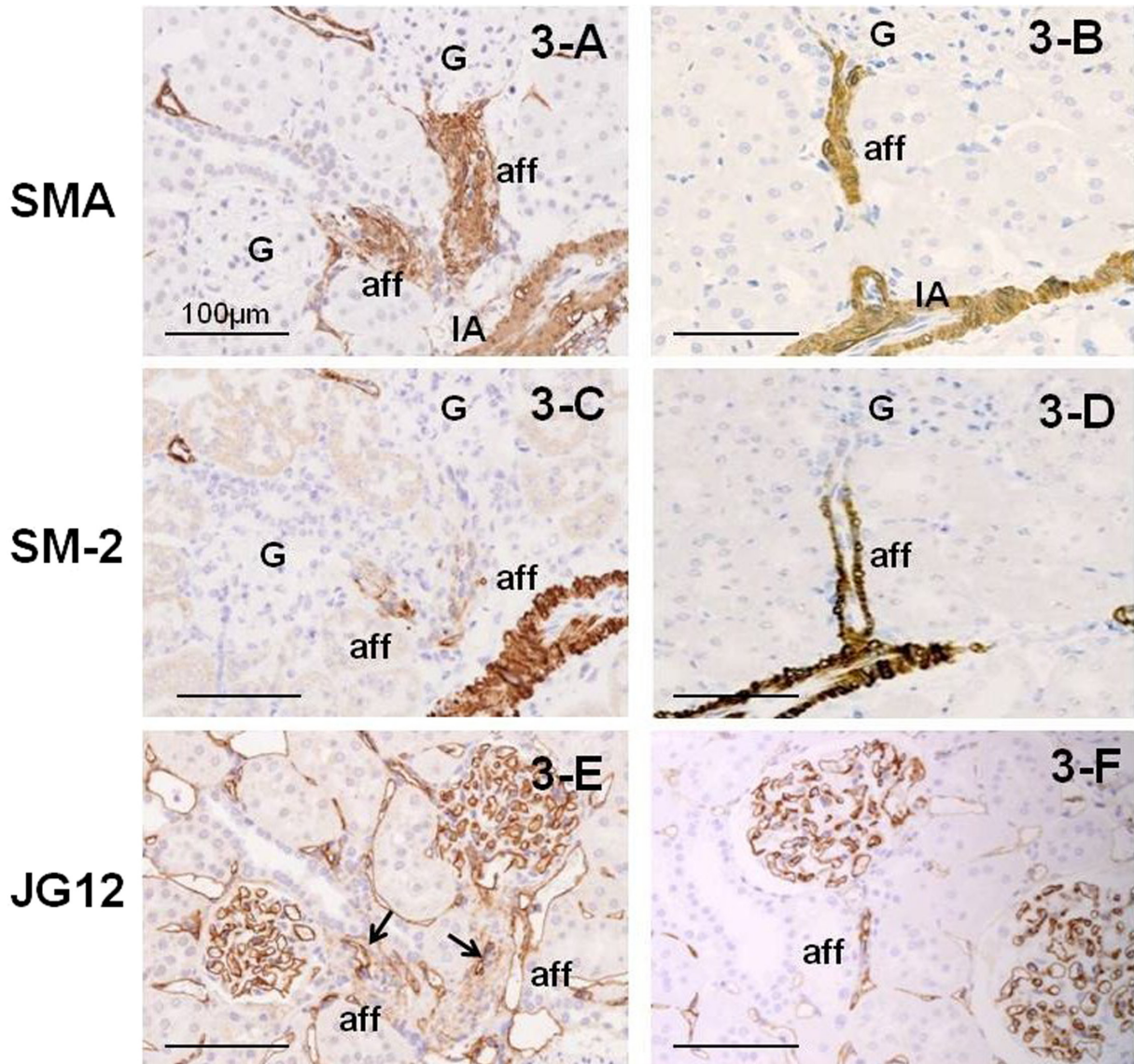
### *Immunohistochemistry findings*

The expression of  $\alpha$ -SMC actin was clearly observed in arterial and arteriolar walls in both groups (Fig. 3-A, B); however, expression of SM-2, the myosin heavy chain isoform, was extremely decreased in afferent arterioles in the ZFR+ACEI group (Fig. 3-C) compared with the clear positive expression in afferent arterioles of the ZFR control group (Fig. 3-D). The expression of SM-2 in the IAs was clearly positive in both groups (Fig. 3-C, D). The expression of endothelial marker JG12 was shown clearly along the glomerular capillaries, afferent arteriolar capillaries (arrows), and peritubular capillaries in the ZFR+ACEI group (Fig. 3-E) and in ZFR controls (Fig. 3-F). The expression of renin was markedly increased in afferent arterioles of the ZFR+ACEI group (Fig. 4-A). In the ZFR control group, renin expression (arrow) was infrequently observed only locally in the preglomerular areas (Fig. 4-B).

## Discussion

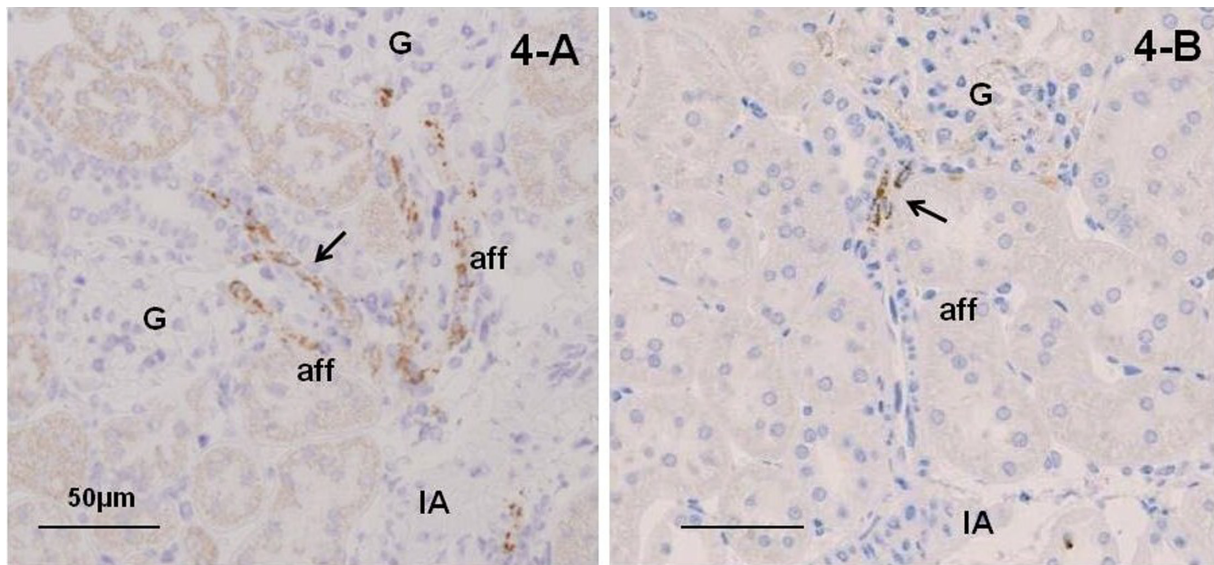
We reported previously that the administration of ARB resulted in pronounced increases in renin expression from the glomerulus hilus toward IAs as observed using immunohistochemical methods (7, 13). This study also showed that administration of an ACEI resulted in unexpected changes in afferent arteriolar SMCs, including unprecedented proliferative changes, disparities in cell size, and pronounced sequence irregularities. In 2004, Raccasan et al. described marked SMC hyperplasia in renal arterioles after ACEI or ARB administration (14). Additionally, in the 1980s and 90s, several pathotoxicological studies using rats and monkeys reported that an ACEI, captopril, commonly induced JGA hyperplasia and afferent arteriolar thickening; however, these studies did not describe the arteriolar changes in microscopic detail (15, 16). The proliferative changes in the afferent arteriolar walls in this study were quite different from the changes that have been observed in other hypertensive, inflammatory, or drug-induced vascular lesions.

In this study, the expression of SMA in the affected arteriolar walls was preserved; however, the expression



**Fig. 3.** Immunohistochemical examinations in the two groups. Calibration Bar 100  $\mu$ m. 3-A: expression of  $\alpha$ -smooth muscle actin (SMA) shows marked thickening of afferent arteriolar walls and shows an extreme luminal narrowing in the ZFR+ACEI rat. 3-B: ZFR control rat with the expression of SMA. 3-C: expression of SM-2 is markedly decreased in the afferent arterioles in the ZFR+ACEI rat; however, the expression of SM-2 is preserved in the interlobular artery. 3-D: expression of SM-2 in afferent arterioles and the interlobular artery are clearly shown in the ZFR control rat. 3-E, F: expression of endothelial marker, JG12, show clearly along the glomerular capillaries, afferent arterioles (arrows) and peritubular capillaries in the ZFR+ACEI rat (3-E) and the ZFR control rat (3-F). 3-A~F: G: glomerulus, aff: afferent arteriole, IA: interlobular artery.

of SM-2, a marker of mature myosin heavy chain, disappeared in immunohistochemical assays (17). The findings suggested that the SMCs in the walls of afferent glomerular arterioles characterized by abnormal growth may have undergone transformation and de-differentiation. The cause of SMC activation or de-differentiation in hypertensive situations has been reported as endothelial damage from high blood pressure, because the endothelial cells are a defensive wall against many trigger factors for de-differentiation, such as PDGF, including blood flow (18, 19). However, in this study there were no abnormal morphological findings in endothelial cells, nor were there any endothelial abnormalities on immunostaining of endothelial markers as JG12. The protective effect of RAAS inhibitors on endothelial cells was maintained. Therefore, in this study, the transformation



**Fig. 4.** Renin expression (arrows) in the two groups. Calibration Bar 100  $\mu\text{m}$ . 4-A: expression of renin extremely increases in the outer layer of the afferent arterioles and extends from the glomerular hilus to the interlobular artery in the ZFR+ACEI rat. 4-B: expression of renin is shown just locally at the preglomerular area of the afferent arteriole in the ZFR control group. G: glomerulus, aff: afferent arteriole. IA: interlobular artery.

and de-differentiation in SMCs was not involved in the mechanisms underlying atherosclerosis.

Gomez et al. showed that the need for renin continues, as in mice treated with hypotensive agents, additional smooth muscle-like cells undergo transformation and seems to de-differentiate (20). However, in the present study we found a remarkable increase in the numbers and layers of afferent arteriolar SMCs and marked irregularities in the arrangement and morphology of SMCs. This phenomenon could not be explained from the transformation of preexisting cells, and instead needed cell proliferation. We reported previously that extremely increased expression of renin by immunohistochemistry and elevated plasma prorenin/renin concentrations significantly were seen in ARB-treated rats (13), although plasma prorenin/renin concentrations were not evaluated in the present study. Therefore, there is the possibility that increased renin induced by inhibitors of the RAAS could have directly stimulated the proliferation of SMCs in afferent arterioles, causing the pronounced medial thickening observed in the present study. Previous reports have also shown that increases in prorenin during the culture of human vascular SMCs stimulated growth factors such as extracellular signal-regulated kinase, resulting in the growth of SMCs (21). In addition, the *in vitro* stimulation of proliferation of SMCs by prorenin has been reported (22). That is, it could appear that the de-differentiation of SMCs in afferent arteriolar walls resulted directly from pronounced increases in renin and prorenin due to negative feedback, not arteriosclerosis induced by endothelial damage, nor transformation of preexisting SMCs.

ACEIs and ARBs are the most used drugs for RAAS inhibition. Clinical and experimental evidence indicates that ACEIs and ARBs could have renoprotective effects independent of their antihypertensive effects (23). ACEIs reduce angiotensin II (Ang II) and elevate bradykinin (BK) and Ang 1–7 resulting in the opposition of Ang II actions (24). On the other hand, ARBs selectively and completely block Ang II actions via AT1 receptors, allowing Ang II binding to AT2 receptors with beneficial AT2-mediated vasodilator, antiproliferative, and antifibrogenic effects (25, 26). Therefore, from the result of our studies, the proliferative changes in SMCs of afferent arterioles could not be prevented by BK, Ang 1–7, or AT2 agonists.

We did not observe change in arterioles after administration of an ACEI for 12 weeks; therefore, there is the possibility that the changes in afferent arterioles induced by RAAS inhibitors were reversible. However,



these changes may also result in luminal narrowing of afferent arterioles, and in the more extreme cases may lead to glomerular destruction and potential loss of renal function. Given the current recommendations for the long-term clinical use of large doses of RAAS inhibitors, greater numbers of patients with renal impairment may become a problem in the future. The mechanism involved in RAAS inhibitor-induced changes in afferent glomerular arteriolar SMCs must be studied in greater detail.

In conclusion, we observed that the long-term ACEI administration induced unusual proliferative changes of SMCs in afferent arterioles of ZFRs. These changes could reduce intraglomerular pressure by narrowing the lumens of afferent arterioles, but they could cause irreversible damage to the arterioles. The present study seeks to contribute to that end.

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## Conflict of Interest

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The authors declare that they have no conflict of interest.

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