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Original Paper

Direct Renin Inhibitor is Better than Angiotensin II Receptor Blocker for Intrarenal Arterioles

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Key Words
Renal pathology • Renin angiotensin system • Afferent arteriole • Renin inhibitor

Abstract
Background/Aims: We have reported that the long-term administration of angiotensin II receptor blockers (ARBs) induced unusual proliferative changes of renal afferent arteriolar smooth muscle cells (SMCs) in rats, associated with the overproduction of renin. In this study, we examined that a direct renin inhibitor (DRI: Aliskilen; Novartis Pharma Co, USA) might induce different changes on afferent arteriolar walls compared to ARBs. ***Method:*** Twenty one 6-weeks-old male spontaneous hypertensive rats (SHRs) were divided into the following three groups: high-dose DRI group (n=7), low-dose DRI group (n=5) and control group (n=9). The rats were fed a standard diet (0.4%NaCl) containing high-dose (150mg/kg/day), low-dose (30mg/kg/day) DRI and without DRI for 12 weeks. The kidneys were examined by histological and immunohistochemical studies. Systolic blood pressure, 24-h urine samples and blood samples were also examined. ***Results:*** The afferent arteriolar SMC walls in the two DRI groups showed no proliferative changes. The positive renin expression area was the largest in the high-dose DRI group among the three groups (14.3±4.0μm^2, 6.7±2.0μm^2, 2.6±0.9μm^2/glomerlus, p=0.020, p=0.008, p=0.017, respectively). ***Conclusion:*** The long-term DRI administration increases tissue and circulatory renin; however, afferent arteriolar proliferative changes as shown in ARBs were not induced.

Introduction

We have reported that the long-term administration of an angiotensin II receptor type1 blocker (ARB) on Zucker fatty rats induced unusual proliferative changes of smooth muscle cells (SMCs) in renal afferent arteriolar walls, and the changes were ascribed to the

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overproduction of renin [1]. We also found that the other ARB administration induces the same proliferative SMC changes and a marked renin-producing cell increase in afferent arterioles more frequently in juveniles than in adult spontaneous hypertensive rats (SHRs) in 2014 [2]. Racasan et al. described marked SMC hyperplasia and disruption of the wall structure of renal arteries and arterioles that were induced by ACEI or ARB administration in pregnant SHRs in 2004 [3].

In this study, we tried to examine a different type of Renin Angiotensin-Aldosterone system (RAAS) inhibitor as we mentioned above, a direct renin inhibitor (DRI: Aliskiren), which might induce the different changes on afferent arteriolar walls in spontaneous hypertensive rats (SHRs).

Materials and Methods

Animals

Twenty one 6-weeks-old male SHRs were purchased from Charles River (Yokohama, Japan). The rats were divided into the following three groups: the high-dose direct renin inhibitor (DRI) group (HD-DRI; n=7), the low-dose DRI group (LD-DRI; n=5) and the control group (n=9). The rats of the former two groups were fed a standard diet (0.4%NaCl) containing high-dose DRI (Aliskiren, Novartis Pharma Co. USA; 150mg/kg/day) and low-dose DRI (30mg/kg/day) for 12 weeks. The control group was fed the standard diet without DRI for 12 weeks. The body weight of the three groups was 150-179g in the HD-DRI, 163-176 g in the LD-DRI group and 165-180g in the control group, respectively. The rats were housed in a room at a temperature of 23±1°C with a 12-h light/dark cycle and were allowed free access to diet and water. All experiments were carried out in accordance with the Animal Experimentation Guidelines of Toho University.

Histological analysis

Rats were anesthetized using thiobutabarbital (Inactin, Wako pure chemical industries Ltd, Osaka, Japan) (100 mg/kg). The kidneys were removed just after blood samples had been drawn. Each left kidney was cut along the long axis, including the hilum. One half of each removed kidney was used for light microscopic studies and immunohistochemistry, and the other half was used for electron microscopic studies. Specimens for light microscopic examination and immunohistochemistry were fixed in 10% neutral-buffered formalin solution and embedded in paraffin. Blocks for microscopic examination from each of the three groups were sectioned at 2 μm and stained with hematoxylin and eosin (HE), periodic acid-silver methenamine-HE (PASM-HE). The sections were examined by two pathologists independently.

Glomerular numbers were counted and the percentage of global sclerosis in all observed glomeruli in each specimen were calculated in the three groups

Twenty microphotographs were taken using a digital microscopic camera (Olympus BX61, Olympus Co., Tokyo, Japan) at 100-fold magnification in each rat kidney, in random areas that did not overlap. Two pathologists counted the total number of glomeruli and estimated the mesangial expansion of glomeruli, including the mesangial matrix increase and/or mesangial cell proliferation. The degree of lesions was scored as follows: 0, not remarkable; 1, mild; 2, moderate; 3, marked. The mesangial scores were calculated by multiplying each of the affected glomeruli by its degree of mesangial expansion, adding these numbers together and finally dividing the sum by the total number of glomeruli. Tubular atrophy and interstitial fibrosis were estimated as the percentage of the affected area occupying each microphotograph. These were scored as follows: 0, none; 1, 0–30%; 2, 31–50%; 3, ≥51%. These scores were summed up in each group and analysed statistically. The total numbers of the vertical and transverse cross-sections of arteries and arterioles were counted, and the numbers of arterioles with more than three layers of SMC walls in each microphotograph were enumerated. These were then summed and analysed statistically. All scoring was performed in a blind manner.

Immunohistochemistry

The paraffin blocks mentioned above were also used for immunohistochemistry. The sections underwent deparaffinization, rehydration, and treatment using antigen retrieval techniques for each

antigen. The primary antibody used was goat anti-rat renin antibody (gift from Prof. Tadashi Inagami, Vanderbilt University, Nashville, USA) 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. The area of the renin expression in each immunohitochemically stained section was measured using Adobe Photoshop CSS Extended (Adobe Ca, USA). The data was compared statistically among the three groups.

Electron microscopic studies

The specimens for electron microscopy were fixed in 5% glutaraldehyde solution, postfixed in osmium, embedded in Epon resin, sliced into ultrathin sections, and then examined under the electron microscope (JEM-1400; JOEL, Tokyo, Japan).

Blood pressure and Biochemical Measurements

Beginning at 6 weeks of age, and every 3 weeks thereafter, body weight was recorded and SBP was measured in all conscious rats using the indirect tail-cuff method (BP-98A; Softron, Tokyo, Japan) on a 37°C preheated cloth jacket for about 10 min. The means of three such recordings were taken from the individual rat's SBP and heart rate. Each rat was transferred to a metabolic cage for collection of a 24-h urine sample. All urine for each rat was collected for the measurement of protein and creatinine concentrations every 3 weeks. At 12 weeks, the rats were anesthetized with thiobutabarbital (100 mg/kg), and blood samples were obtained from the inferior vena cava, and the animals were sacrificed. The blood samples were used for blood chemistry (Mitsubishi Chemical, Tokyo, Japan) and prorenin/renin concentration measured by ELISA (RPRENKT-1, Innovative Research, Florida, USA).

Statistical analysis

Values are presented as means±Standard Deviation. One-way repeated-measures ANOVA was performed for each group followed by the Kruskal-Wallis test to assess the significance of results. Values of $p<0.05$ were considered to be significant. The Steal-Dwass test was used to assess differences between each group.

Results

Histological findings

The segmental, interlobar, and arcuate arteries demonstrated over three SMC layers were of the same size as well as regular arrangement of SMCs in the three groups. The intrarenal arteriolar changes, such as proliferation of SMC walls and SMC irregularities were also estimated. The numbers of observed arterioles were 176.3±4.5, 116.6±10.2, 101.4±5.0, respectively and there were no statistically significant differences among the three groups. The afferent arteriolar walls were mostly made of one to two SMC layers in the three groups, and rarely three SMC layers were observed in the deep cortex. The afferent arteriolar SMC walls in the two DRI groups and the control group showed no proliferative changes, and the SMCs showed no morphological abnormalities (such as irregularities in size or arrangement of SMC) in the three groups. The percentages of observed afferent arterioles with over three SMC layers were 3.0±0.2%, 5.1±4.0%, 5.0±2.7%, respectively, there were no statistically significant differences. Renin granules of the SMC in afferent arteriolar walls close to the glomerular hilus often increased extremely in the high-dose DRI group and they showed a slight increase in the low-dose DRI group (Fig.1).

Glomerular numbers counted in each specimen showed no statistical significant differences (386±19.4 in the high- dose DRI group, 380±37.5 in the low-dose DRI group and 410±56.3 in the control group). The percentage of global sclerosis in all observed glomeruli in each specimen also revealed no significant differences among the three groups (0.1±0.1%, 0.2±0.1%, 0.1±0.2%, respectively). Mesangial expansion, tubular atrophy, interstitial fibrosis

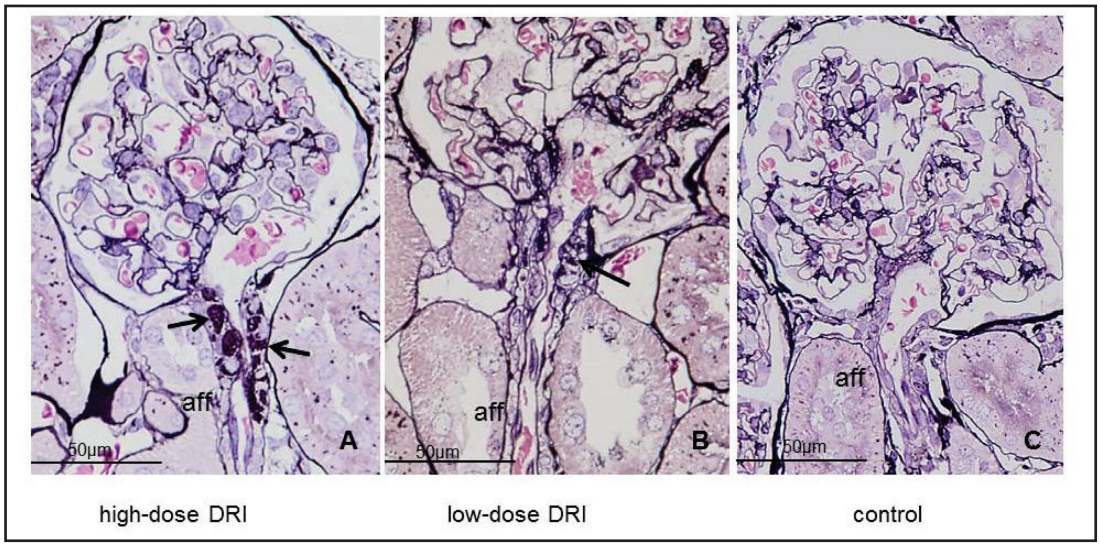

Fig. 1. Light microphotographs in the three groups. A: a glomerulus with afferent arteriole in the high-dose DRI rat. The amount of dark-brown renin granules in smooth muscle cells (SMCs) of afferent arteriolar walls only close to the glomerular hilus show an extreme increase (arrows). The SMCs in the arteriolar walls reveal no proliferative changes and they consist of regular arrangement. B: a glomerulus with afferent arteriole in the low-dose DRI rat. The accumulation of renin granules in the glomerular hilus is observed; however, the degree is not as increase as that in the high-dose DRI rats. The SMCs of the afferent arteriolar walls show one regularly arranged layer. C: a glomerulus with afferent arteriole in the control rat. No increase of renin granules are observed. aff: afferent arteriole; A~C: PASM-HE stain; original magnification x400.

were estimated by the microphotographs as described in the method. Mesangial scores in the three groups were 0.17±0.020, 0.13±0.02, 0.21±0.04, respectively. The scores showed no statistically significant differences. The scores of tubular atrophy (6.66±0.47, 8.00±2.36, 6.10±5.06, respectively) and the scores of interstitial fibrosis (6.00±2.44, 5.60±2.15, 3.20±3.05, respectively) were also not significantly different.

Immunohistochemical studies

Renin expression in the high-dose DRI group was observed frequently in the SMCs of the afferent arteriolar walls close to the glomerular hilus. The amount of renin expression sometimes increased in the high-dose DRI group, and in the low-dose DRI group (Fig. 2). The amount of renin expression in the hilus of glomeruli increased; however, the outer layer SMCs of the afferent arterioles apart from the hilus rarely showed an increase. The average measured area of positive renin expression per glomerulus in the sections of each group seen by immunohistochemical study was the largest in the high-dose DRI group among the three groups (14.3±4.0μm^2, 6.7±2.0μm^2, 2.6±0.9μm^2, respectively; the high-dose DRI group versus the low-dose DRI group: p=0.020, the high-dose DRI group versus the control group: p=0.008, the low-dose DRI group versus the control group: p=0.017; Fig. 3).

Electron microscopic studies

In the high-dose DRI group, the SMCs of the afferent arterioles close to the glomerular hilus sometimes contained extremely abundant renin granules (Fig, 4). The hyperplasia of JGA was not observed and lacis cells of JGA contained no renin granules. The SMCs of afferent arteriolar walls showed no proliferative and activated changes

Endothelial cells along the inner lumens of afferent arterioles morphologically showed no abnormal changes. Glomerular endothelial cells, mesangial matrix, mesangial cells and epithelial cells also showed no remarkable changes.

Laboratory findings

The laboratory data in the three groups are shown in Table 1. Systolic blood pressure (SBP) in the high-dose DRI group was significantly lower compared to those of the low-dose DRI group and the control group (P=0.0123, p=0.0024). The averages of blood urea nitrogen in the two DRI groups were higher compared to that in the control group; however, serum creatinine levels and creatinine clearances showed no significant differences among the three groups. The daily amount of proteinuria in the two DRI groups were significantly lower than that of the control group.

Discussion

We have already reported that the proliferative changes in afferent arteriolar walls in rats were induced by the long-term administration of ARBs in 2011 [1] and 2014 [2]. We considered that the increased renin secretion by the feedback mechanism had a key role

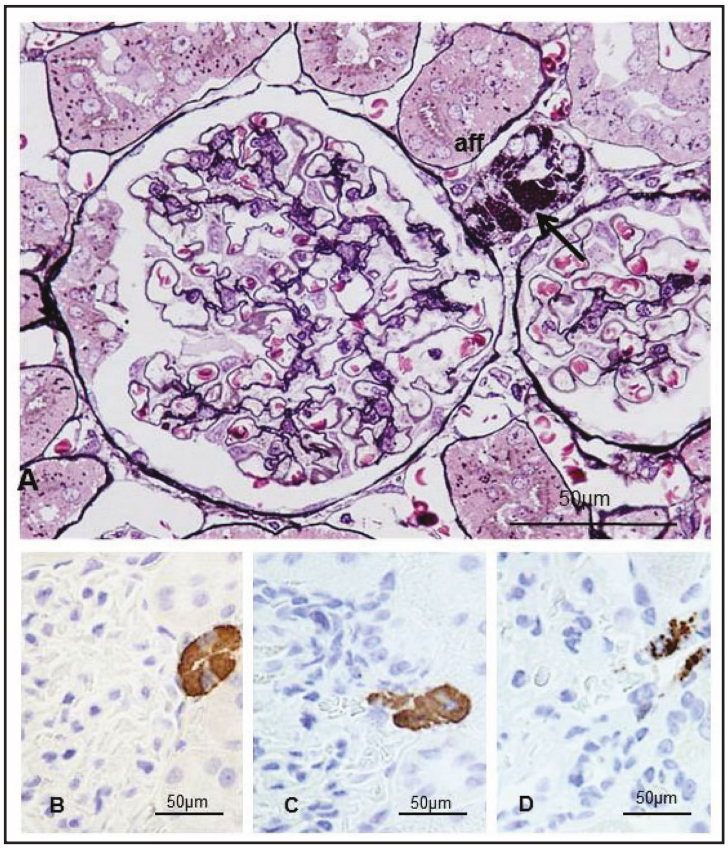

Fig. 2. Renin expression in the three groups. A: A light microphotograph shows a glomerulus associating alongside afferent arteriolar SMCs filled with dark-brown renin granules (arrow) in the high-dose DRI rat. B: an afferent arteriole shows the extreme increase of renin expression in the high-dose DRI rat. C: An afferent arteriole shows an increased renin expression in the low-dose DRI rat. D: An afferent arteriole shows renin expression in the control rat. G=glomerulus; aff=afferent arteriole; A: PASM-HE stain; B~D: immunohistochemistry; original magnification x400.

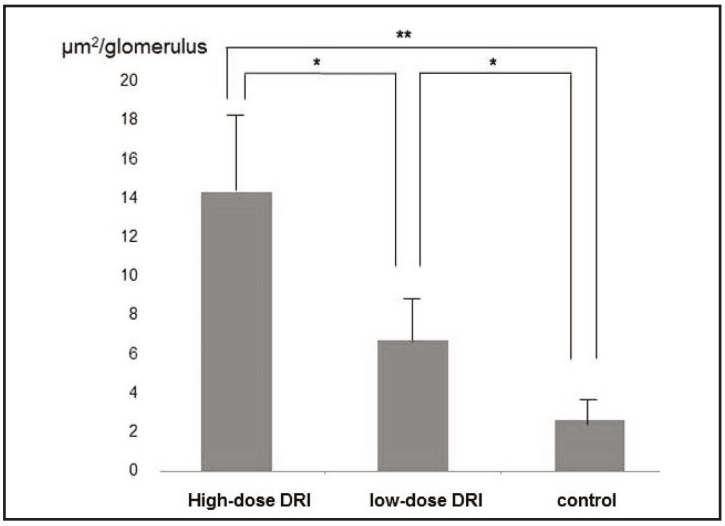

Fig. 3. Renin positive expression (positive area/ glomerulus) in the three groups. Bars show the averaged positively stained area/ glomerular numbers in each specimen within each group by immunohistochemistry. The bar in the high-dose DRI group shows significantly the highest amount among the three groups.

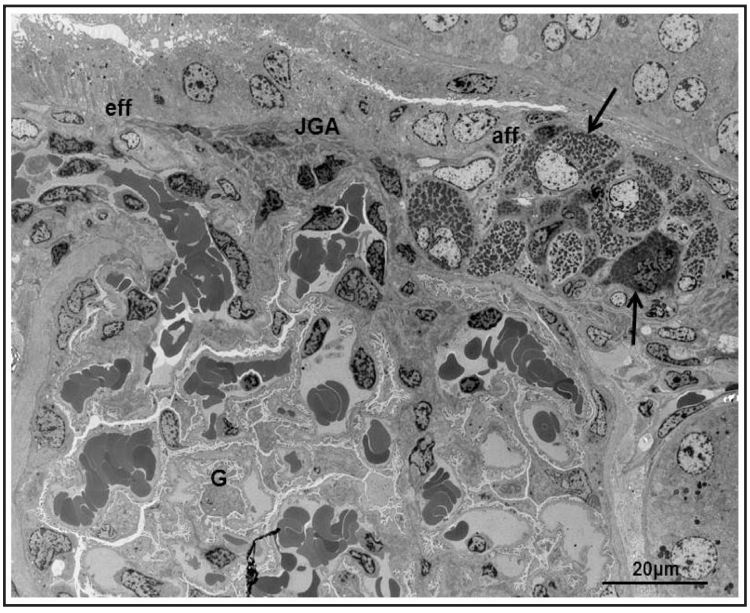

Fig. 4. An electron microphotograph in the high-dose DRI rat. The afferent arteriolar SMCs contain abundant renin granules(arrows). The JGA does not show hypertrophy and lacis cells do not contain renin granules. The efferent arteriolar SMCs also do not contain them. G: glomerulus; aff: afferent arteriole; eff:efferent arteriole; JGA: juxtaglomerular apparatus.

Table 1.

	High-dose DRI group	Low-dose DRI group	Control group	P-value
Systolic BP (mmHg)	146.1±5.9	188.7± 3.6	187.6± 14.2	a:0.0123; b:0.0024
Body weight (g)	298.0± 11.7	3798± 12.3	393.5± 17.0	a:0.0167; b:0.0041
BUN (mg/dl)	21.7±1.9	21.3±10.6	18.6±2.7	b:0.0215; c:0.0155
Serum creatine (mg/dl)	0.29±0.03	0.45±0.35	0.21±0.05	n.s.
Ccr/BW (ml/min/kg)	13.2±3.2	11.7±0.7	16.6±6.5	n.s.
Prorenin/renin (ng/ml)	72.0±3.1	15.4±2.0	6.2±1.7	a:0.0244; b:0.0244; c:0.0379
Proteinuria (mg/day)	6.1±2.0	9.9±2.0	26.5±12.5	b:0.0024; c:0.0075

a: High-dose DRI group vs. Low-dose DRI group; b: High-dose DRI group versus Control group; c: Low-dose DRI group versus Control group; BP=blood pressure; Ccr=creatinine clearance: BW=body weight. Data shown are mean±SD

for the proliferative changes of the renal afferent arteriolar SMCs in two studies mentioned above. We suspected that the increased intrarenal prorenin/renin induced the proliferation of SMCs in afferent arteriolar walls through prorenin receptors (PRR) by intracellular signal transductions of mitogen-activated protein kinases (MAPKs) pathways [4, 5].

We never found the multicentric proliferation in this study, the same as the lesions induced by ARBs of afferent arteriolar walls in the high-dose and low-dose DRI groups by light microscopy. SMCs in the afferent arteriolar walls in the high and low-dose DRI groups showed no evident cellular irregularities compared with the SMCs in which changes were often observed in the long-term administrations of ARBs in the previous studies [1, 2]. The renin granules contained in the SMCs of afferent arteriolar walls close to the glomerular hilus extremely increased in the two DRI groups dose-dependently. This renin increase in the renal tissue is induced by the feedback of RAA system, which is the same as shown in the administration of the other RAA system blockers. Plasma concentrations of prorenin/renin also increased in the two DRI groups. The over-produced prorenin/renin from the DRI administration is generally released into the renal tissue fluid; the flow of lymph to the renal venous stream [6, 7]. Then, the concentration of prorenin/renin in interstitial tissue fluid around the hilus is considered to be extremely high. As previously mentioned, the extreme increase of prorenin/renin in renal tissue and plasma was similarly induced by ARBs, ACEIs and DRI; however, our study showed the proliferative changes of afferent arteriolar SMCs

were not observed in the rats with DRI administration. We have to point out that aliskiren has higher specificity for humans than rats [8]. We have to use a higher dosage of aliskiren for lowering blood pressure of SHRs, about 50-fold of human dosage was needed. However, the blood pressure was significantly lower in the high-dose DRI group and their plasma prorenin/renin levels showed an increase in this study. We consider insufficient dosage of aliskiren does not attribute to our results.

The decline of blood pressure was larger in the ARB treated group in our report in 2014, that also used the same age SHRs, than aliskiren group in this study [2]. However, the increase of the plasma levels of prorenin/renin after the ARB treatment was smaller than the aliskiren treatment in this study. We consider that plasma prorenin/renin level and the amount of renal tissue prorenin/renin granules do not show a pararell change as known in diabetic nephropathy [9, 10]. Moreover, a degree of lowering blood pressure does not directly reflect the change of plasma prorenin/renin levels induced by RAS blockers.

We suspect that the reason for the different morphological responses of the afferent arteriolar SMCs to DRI administration is that the DRI has different pharmacological actions from ARBs and ACEIs.

Prorenin and renin binding to PRR induces an activation of the two major pathways [11]. The first is the classical pathway; activation of tissue RAA system depends on angiotensin II [5, 11]. RAA system inhibitors such as ARBs and ACEIs inhibit activation of this angiotensin II-dependent RAA system, and DRI also inhibits this angiotensin II-dependent pathway. The second, PRR induces intracellular signal transduction of MAPKs, extracellular signal regulated kinase (ERK) 1/2 activation independent from angiotensin II by the binding of prorenin/renin [5]. Prorenin is reported to induce human aortic SMC migration through PRR expression [12]. Lie G also reported that the recombinant rat prorenin induces vascular SMC proliferation by binding PRR and activating intracellular ERK1/2 and Act pathways [13]. Moreover, PRR is reported to be needed for cell survival through maintaining normal functions of vacuolar proton ATPase in mouse podocytes [5] and murine cardiomyocytes [14], as well as needed for inhibitory effects of vascular inflammation in murine vascular SMCs [15].

However, Huang Y, et. al. reported that recombinant human and rat renin induce phosphorylation of ERK1/2 in mesangial cells and subsequent cell proliferation, these effects were not altered by the addition of ARB, ACEI [16, 17], and Saris et.al described prorenin induced intracellular signaling in cardiomyocytes angiotensin-independently, and this regulation was not altered in the presence of direct renin inhibitor aliskiren [18]. On the other hand, Feldman et al. reported the direct renin inhibitor aliskiren extensively distributed to the kidney, localizing in the glomeruli and vascular walls, and they reported aliskiren significantly suppressed renal PRR gene expression in diabetic rats [19]. Ferri et al. also showed aliskiren to reduce prorenin receptor expression and activity in cultured human aortic smooth muscle cells [20]. Therefore, it is still unknown if aliskiren suppresses intra-renal vascular PRR expression or not.

Urinary protein excretion decreased by the long-term administration of DRI with or without lowering systemic blood pressure in this study. Aliskiren has reported to reduce proteinuria with monotherapy [21]. There are results of clinical trials showing the additional reno-protective effects of aliskiren treatment combined with ARB or cardio-protective effects of aliskiren combined with ACEIs more than monotherapy [22, 23]. We consider the one of reno-protective effects of aliskiren is induced by plasma renin activity reduction which differs from the other blockers of RAA system. Aliskiren works in the upper stream of the angiotensin cascade differently than ARB or ACEI and strongly works in the intra-renal local blockade of the RAA system related pathways.

Data of blood urea nitrogen increased in the two DRI groups significantly; however, creatinine levels and creatinine clearances showed no significant differences among the three groups. Moreover, histological differences regarding global glomerulosclerosis, mesangial expansion or tubular atrophy were not observed among the three groups.

Conclusion

A long-term administration of DRI induced an increase of tissue and circulatory prorenin/renin the same as ARB treatment; however, the remarkable proliferation and irregularity of afferent arteriolar SMCs as ARB treatment were not induced.

Disclosure Statement

This research received no specific grants from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare that they have no conflicts of interest.

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