Combination Therapy With Telmisartan and Spironolactone Alleviates L-NAME Exacerbated Nephrosclerosis With an Increase in PPAR- γ and Decrease in TGF- β_1

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SUMMARY

To investigate whether the receptor blockades of angiotensin II type 1 and aldosterone receptors can prevent renal tissue injury in relation to the renal tissue mRNA levels of peroxisome proliferation-activated receptors- γ (PPAR- γ) and transforming growth factor- β_1 (TGF- β_1) in spontaneously hypertensive rats (SHR) given N^G-nitro-L-arginine methyl ester (L-NAME), which is considered a model of malignant hypertension.

This study was performed in 5 groups of 17-week-old male SHR treated for 3 weeks as follows: group 1, control; group 2, L-NAME (50 mg/L in drinking); group 3, L-NAME plus aldosterone antagonist, spironolactone (SPRL, 100 mg/kg/day); group 4, L-NAME plus angiotensin II type 1 receptor blocker, telmisartan (TELM, 3 mg/kg/day); group 5, L-NAME plus combination therapy (COMB) with low-dose TELM (1 mg/kg/day) and SPRL (100 mg/kg/day).

Urinary protein excretion and the glomerular injury score were significantly reduced in the SPRL, TELM, and COMB groups as compared with the L-NAME group, while significant blood pressure reduction was observed only in the TELM group. In the TELM and COMB groups, the perivascular cell infiltration and fibrosis area were significantly reduced together with the PPAR- γ mRNA increase and TGF- β_1 mRNA decrease. The urinary excretion of nitric oxides was significantly recovered and the wall to lumen ratio of the interlobular artery was significantly reduced only in the COMB group compared with the L-NAME group.

Combined administration of 1 mg/kg/day telmisartan and 100 mg/kg/day spironolactone is thought to be effective in alleviating hypertensive renal injuries independently of blood pressure changes. The anti-inflammatory and antifibrotic effects due to PPAR- γ activation and TGF- β_1 inhibition may participate in the renoprotection of this combination therapy. (Int Heart J 2007; 48: 637-647)

Key words: Hypertension, Nephrosclerosis, Angiotensin II type 1 receptor blockers, Aldosterone antagonists, Peroxisome proliferator-activated receptors, Transforming growth factor beta

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THE renin-angiotensin-aldosterone system (RAAS) plays an important role in the development of hypertensive vascular injury. Numerous studies have demonstrated the significant contribution of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor blockers (ARB) in preventing renal and cardiovascular injuries.^{1,2)} In spontaneously hypertensive rats (SHR) given N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), we have observed severe hypertensive nephrosclerosis, proteinuria, and tubulo-interstitial fibrosis^{3,4)} with an increase in the mRNA of transforming growth factor- β_1 (TGF- β_1), a profibrotic cytokine.⁵⁾ We have reported that ACE inhibitors and ARB prevented and reversed severe hypertensive nephrosclerosis in this L-NAME/SHR model.^{6,7)} Furthermore, we have demonstrated that ACE inhibitors reduced the increased TGF- β_1 mRNA expression in the kidney of L-NAME/SHR using real time RT-PCR.⁵⁾

Recent studies have shown that the long-term inhibition of the RAAS by ACE inhibitors and ARB induces the rebound elevation of plasma aldosterone, which is known as the aldosterone breakthrough.^{8,9)} Some investigators observed that aldosterone caused renal and vascular injuries without altering blood pressure.^{10,11)} It has been reported that the administration of an ACE inhibitor combined with an aldosterone antagonist, spironolactone (SPRL), reduced urinary albumin excretion and the left ventricular mass index in type 2 diabetic patients with early nephropathy.⁸⁾

A family of ligand-activated nuclear transcriptional factors, peroxisome proliferator-activated receptors (PPARs), have been shown to affect vascular functions and structures.¹²⁾ Among the family of PPARs, PPAR- γ has been demonstrated to play an important role in the prevention of atherosclerosis, inflammation, fibrosis, and hypertrophy in the cardiovascular system.^{13,14)}

This study was designed to investigate the effects of the ARB telmisartan (TELM) with and without SPRL on the progression of renal injuries in malignant nephrosclerotic L-NAME/SHR. We also quantified the mRNA expressions of TGF- β_1 and PPAR- γ in the renal tissue and assessed their possible participations in the therapeutic effects of this combination therapy.

METHODS

Male 17-week-old SHR (Charles River Laboratories, Tokyo) were housed and maintained in a temperature- and light-controlled room. All experiments were approved by our institutional animal care committee. The rats were divided into 5 experimental groups: group 1 rats (control, $n = 15$) were given tap water and regular chow containing 0.3% NaCl for 3 weeks; group 2 rats (L-NAME, $n = 14$) were given L-NAME (Sigma Chemical Co., St. Louis, USA) in their drinking

water (50 mg/L) for 3 weeks; group 3 rats (SPRL, $n = 10$) were administered the same dose of L-NAME and the aldosterone antagonist SPRL (100 mg/kg/day by gavage) for 3 weeks; group 4 rats (TELM, $n = 8$) were administered the same dose of L-NAME and the ARB TELM (3 mg/kg/day by gavage) for 3 weeks; and group 5 rats (COMB, $n = 8$) were administered the same dose of L-NAME plus combination therapy consisting of SPRL (100 mg/kg/day by gavage) and low dose TELM (1 mg/kg/day by gavage) for 3 weeks. The doses of spironolactone and telmisartan were selected based on earlier reports so that they would exhibit comparable antihypertensive effects.¹⁵⁻¹⁷⁾ During the final week of treatment, all rats were placed in metabolic cages for 2 days to measure 24-hour urinary excretions of creatinine, protein, and nitric oxides (NOx). Plasma and urinary creatinine levels were assayed by enzymatic colorimetry and urinary NOx levels were determined by high performance liquid chromatography.

Histological analysis of the kidney: The kidneys of each rat were fixed by perfusion with 10% paraformaldehyde, removed, and weighed. Sections were stained with hematoxylin-eosin, periodic-acid Schiff, and elastica-Masson for the assessment of glomerular and vascular injuries and perivascular lesions such as fibrosis and cell infiltration. Glomerular lesions were evaluated in the juxtamedullary cortex because juxtamedullary glomeruli are more vulnerable to sclerosis than glomeruli in the superficial layer of cortex. The glomerular injury rate (GIR) and the glomerular injury score (GIS) were assessed as described previously.¹⁸⁻²¹⁾ GIR is the percentage of glomeruli showing any degree of glomerular injury. In order to calculate GIS the glomerular injury was graded from 0 to 3+, in which 0 was no injury, 1+ was injury up to one third of the glomerulus, 2+ was one third to two thirds glomerular injury, and 3+ was injury of more than two thirds. GIS was defined as the sum of scores given to 100 glomeruli in each rat. Namely, global sclerosis of every glomerulus corresponds to the maximum GIS of 300.¹⁸⁻²¹⁾ The glomerular areas of the juxtamedullary layers of renal cortex and the glomerular cell number per glomerulus were measured, using a computer analyzer system (Image Quest, Hamamatsu Photonics, Hamamatsu and MacScope, Mitani Co., Fukui, Japan), by tracing the outlines of those glomeruli having a vascular pole and counting the numbers of nuclei in the glomeruli, as previously described.¹⁹⁻²¹⁾ The wall thickness was assessed as the ratio of media thickness to the outer radius of interlobular arteries. Inner and outer circumferences were measured by the same computer analyzer system (Image Quest). The values were corrected to the radius of each vessel, which were assumed to be circular, by the following calculation: wall thickness ratio = $(R-r)/R$, where R is the radius of the outer circumference and r is the radius of the inner circumference, and $R-r$ represents medial thickness.^{4,21)} The fibrosis area was assessed by calculating the ratio of the collagen-stained area to the vessel and perivascular area in the section stained with

elastica-Masson. The infiltrating cell number was assessed by counting the number of filtrating cells such as lymphocytic cells, plasma cells, fibroblasts, and macrophages in the unit area of perivascular space using the Image Quest.

Quantification of mRNA in the kidney by real-time PCR: The total RNA of specimens was extracted from the renal cortical tissue using TRIzol reagent (Life Technologies, Rockville, Maryland, USA) according to the manufacturer's procedure. The first-strand cDNA was made from the total RNA using the SuperScript preamplification system (Life Technologies) with random hexamers. Excess oligomers were removed using a centrifugal filter, Microcon YM-10 (Millipore Co., Bedford, Massachusetts, USA). Real-time PCR analysis, using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), is a method that provides reproducible quantitative PCR.²²⁾ Cleavage of the sequence-specific probe (TaqMan Probe) by 5' nuclease activity of the TaqDNA polymerase releases the reporter dye, resulting in an increase in emission at the corresponding wavelengths. With each cycle, the fluorescence intensity of additional reporter dye molecules is monitored by the system. Threshold cycles were selected in the line in which all samples were in logarithmic phase. The quantity of PCR products was calculated from the threshold cycle value. This real-time detection generates quantitative data based on PCR at early cycles when PCR fidelity is highest. TGF- β_1 and PPAR- γ RNA levels were quantified as the ratios to β -actin using this real-time PCR system. All oligonucleotides were obtained by chemical synthesis using the PerSeptive 8900 (PE Biosystem, Fremont, CA, USA). TaqMan probes modified with the fluorescent dye were purchased from GRENER JAPAN, Inc., Tokyo, Japan. The nucleotide sequences of the PCR primers and TaqMan probes were as follows:

TGF- β_1 ,

forward primer: 5'-cccagcatctgcaaagctc-3'

reverse promoter: 5'-gtcaatgtacagctgccgca-3'

TaqMan probe: 5'-gtggagctgaagcaatagttggtgtccag-3'

PPAR- γ ,

forward primer: 5'-cttgccatatttatagctgtcattatt-3'

reverse promoter: 5'-agcaggtgtcttggtgtcct-3'

TaqMan probe: 5'-tggagaccgcccaggcttgct-3'

β -actin,

forward primer: 5'-cgtgaaaagatgacccagatca-3'

reverse promoter: 5'-acacagcctggatggctacgta-3'

TaqMan probe: 5'-tttgagacctcaacacccagcca-3'

Statistics: One-way ANOVA followed by Duncan's multiple range test²³⁾ were performed to test for between-group significance. All data are expressed as the

mean $\pm$ SEM. A probability level of $< 5\%$ was considered to indicate statistical significance.

RESULTS

Table I lists the physical and biochemical measurements in the 5 groups of rats. We have previously observed that L-NAME significantly increased blood pressure, urinary protein excretion, and TGF- β_1 mRNA, and reduced body weight, creatinine clearance, and urinary NOx excretion.³⁻⁵⁾ In this study, SPRL, high-dose TELM, and COMB significantly alleviated the body weight decrease by L-NAME. High-dose TELM, but not SPRL or COMB, significantly reduced blood pressure as compared with the L-NAME group. Urinary protein excretion was significantly reduced in the SPRL, high-dose TELM, and COMB groups as compared with the L-NAME group. This reduction in proteinuria was more prominent in the TELM and COMB groups than in the SPRL group. The reduction of creatinine clearance was significantly alleviated by high-dose TELM and COMB. However, SPRL failed to produce a significant increase in creatinine clearance of L-NAME/SHR. A significant increase in urinary NOx excretion was observed in the COMB group.

Histological findings in the kidney: In histological analysis, we have previously reported that L-NAME/SHR showed glomerular sclerosis, thickening of the interlobular arteries, and perivascular fibrosis and cell infiltration.³⁻⁵⁾ Table II lists the results of morphological evaluation of the glomeruli in the present study. Both GIR and GIS were significantly improved in the SPRL, high-dose TELM, and COMB groups. Accompanying this, the glomerular areas and the cell numbers of juxtamedullary glomeruli were significantly increased by SPRL, high-dose

Table I. Physical Findings and Laboratory Data at the End of 3-Week Treatment Period

	Control (n = 15)	L-NAME (n = 14)	SPRL (n = 10)	TELM (n = 8)	COMB (n = 8)
Body weight, g	351 $\pm$ 4	287 $\pm$ 10**	312 $\pm$ 9†	350 $\pm$ 5‡¶	346 $\pm$ 3‡¶
Systolic blood pressure, mmHg	233 $\pm$ 4	271 $\pm$ 8**	248 $\pm$ 13	181 $\pm$ 10‡¶	249 $\pm$ 6
Kidney weight, g/kg body weight	7.2 $\pm$ 0.2	7.3 $\pm$ 0.2	6.5 $\pm$ 0.2	6.8 $\pm$ 0.2	7.1 $\pm$ 0.2
Creatinine clearance, mL/min/kg body weight	9.5 $\pm$ 1.0	1.3 $\pm$ 0.1**	3.3 $\pm$ 0.5	4.8 $\pm$ 0.4†	6.4 $\pm$ 1.0‡¶
Urinary protein excretion, mg/day	19.1 $\pm$ 0.9	119.8 $\pm$ 13.3**	69.9 $\pm$ 13.2†	12.5 $\pm$ 1.7‡¶	20.8 $\pm$ 2.8‡¶
Urinary NOx excretion, μ mol/day	5.0 $\pm$ 0.5	0.6 $\pm$ 0.2**	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	2.7 $\pm$ 0.7†¶

Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ versus Control; † $P < 0.05$, ‡ $P < 0.01$ versus L-NAME; ¶ $P < 0.05$ versus SPRL. L-NAME indicates N^G-nitro-L-arginine methyl ester; SPRL, spironolactone (100 mg/kg/day); TELM, telmisartan (3 mg/kg/day); COMB, combination of telmisartan (1 mg/kg/day) and spironolactone (100 mg/kg/day); and NOx, nitrites.

Table II. Histological Evaluation of the Juxtamedullary Glomeruli

	Control (n = 15)	L-NAME (n = 14)	SPRL (n = 10)	TELM (n = 8)	COMB (n = 8)
Glomerular injury rate, %	8.1 ± 1.4	39.3 ± 5.1**	11.3 ± 2.4‡	4.9 ± 1.1‡	5.8 ± 1.5‡
Glomerular injury score	8.8 ± 1.7	54.8 ± 7.0**	14.2 ± 3.1‡	5.3 ± 1.1‡	6.3 ± 1.6‡
Glomerular area, ×10 ³ mm ²	14.5 ± 0.6	10.3 ± 0.5*	13.8 ± 0.6†	16.0 ± 0.7†¶	16.2 ± 0.4†¶
Glomerular cell number	93 ± 3	77 ± 4**	92 ± 3‡	91 ± 4‡	88 ± 3†

Data are means ± SEM. * $P < 0.05$, ** $P < 0.01$ versus Control; † $P < 0.05$, ‡ $P < 0.01$ versus L-NAME; ¶ $P < 0.05$ versus SPRL. L-NAME indicates N^G-nitro-L-arginine methyl ester; SPRL, spironolactone (100 mg/kg/day); TELM, telmisartan (3 mg/kg/day); and COMB, combination of telmisartan (1 mg/kg/day) and spironolactone (100 mg/kg/day).

Table III. Histological Evaluation of the Interlobular Arteries and Perivascular Lesions in the Kidney

	Control (n = 15)	L-NAME (n = 14)	SPRL (n = 10)	TELM (n = 8)	COMB (n = 8)
Wall thickness ratio	0.48 ± 0.03	0.58 ± 0.03*	0.53 ± 0.02	0.54 ± 0.02	0.47 ± 0.02†
Perivascular fibrosis area, × 10 ³ μm ²	3.5 ± 0.4	9.1 ± 1.1*	3.0 ± 0.4†	1.9 ± 0.3†	2.3 ± 0.2†
Perivascular cell number	47 ± 7	110 ± 10*	51 ± 7†	24 ± 3†¶	26 ± 3†¶

Data are means ± SEM. * $P < 0.05$, ** $P < 0.01$ versus Control; † $P < 0.05$, ‡ $P < 0.01$ versus L-NAME; ¶ $P < 0.05$ versus SPRL. L-NAME indicates N^G-nitro-L-arginine methyl ester; SPRL, spironolactone (100 mg/kg/day); TELM, telmisartan (3 mg/kg/day); and COMB, combination of telmisartan (1 mg/kg/day) and spironolactone (100 mg/kg/day).

TELM, and COMB. The increase in glomerular area was less prominent in the SPRL group than in the TELM and COMB groups.

Table III shows the results of histological evaluation of the vascular area in the kidney. The perivascular fibrosis area and the number of cells infiltrating into the perivascular space were significantly decreased in the SPRL, high-dose TELM, and COMB groups as compared with the L-NAME group. These perivascular lesions were improved more markedly by high-dose TELM and COMB than by SPRL. On the other hand, a significant reduction in the wall thickness ratio was seen only in the COMB group.

Messenger RNA quantification of TGF-β₁ and PPAR-γ: The Figure shows the mRNA levels of TGF-β₁ and PPAR-γ in renal cortical tissue. In the L-NAME group, TGF-β₁ mRNA was markedly increased and PPAR-γ mRNA was markedly decreased as compared with the control group. This increase in TGF-β₁ mRNA and decrease in PPAR-γ mRNA were significantly alleviated in the high-dose TELM and COMB groups. However, the changes were not statistically significant in the SPRL group.

Figure. Levels of mRNA for transforming growth factor- β_1 (TGF- β_1) and peroxisome proliferation-activated receptors- γ (PPAR- γ) in renal cortical tissue. * $P < 0.05$, ** $P < 0.01$ versus Control; † $P < 0.05$ versus L-NAME. L-NAME indicates N^G-nitro-L-arginine methyl ester; SPRL, spironolactone (100 mg/kg/day); TELM, telmisartan (3 mg/kg/day); and COMB, combination of telmisartan (1 mg/kg/day) and spironolactone (100 mg/kg/day).

DISCUSSION

Prevention of glomerular capillary pressure elevation is important for the inhibition of glomerular sclerosis in hypertension, and ACE inhibitors and ARBs alleviate glomerular hypertension by reducing the constrictive effect of angiotensin II on the efferent arterioles. Angiotensin II also enhances the production of free radicals, promotes cell growth, and increases the synthesis of inflammatory and profibrotic cytokines.^{24,25)} Furthermore, recent studies have revealed that aldosterone also has mitogenic and profibrotic properties.^{26,27)} These nonhemodynamic effects of angiotensin II and aldosterone are also deleterious to the kidney and cause inflammation, fibrosis, and scarring of the renal tissue, thereby resulting in the deterioration of renal function.²⁸⁾ Therefore, inhibition of RAAS with ACE inhibitors, ARBs, and aldosterone receptor antagonists may protect against the nonhemodynamic effects of angiotensin II and aldosterone that might otherwise promote the progression of renal tissue injuries.

In the present study, SPRL, an aldosterone antagonist, alleviated the glomerular injuries and perivascular fibrotic and inflammatory lesions in L-NAME/SHR without significant blood pressure reduction. We have reported that L-NAME/SHR develops hypertensive nephrosclerosis with marked enhancement of RAAS.⁴⁾ Therefore, the blockade of increased aldosterone is thought to be effective in mitigating the renal tissue injuries in L-NAME/SHR by a mechanism independent of blood pressure changes. Blasi, *et al*²⁹⁾ have also reported that

epplerenone, an aldosterone antagonist, reduced renal inflammation and fibrosis in rats given aldosterone and salt. Thus, it is suggested that aldosterone blockade offers protective effects against hypertensive renal disease.

TELM is an ARB which blocks the angiotensin II type 1 receptor and subsequently reduces stimuli for aldosterone production. In the present study, the high-dose of TELM reduced proteinuria and perivascular inflammation in L-NAME/SHR more prominently than SPRL. This may be a result of significant blood pressure reduction. However, the blockade of angiotensin II actions and subsequent aldosterone reduction may have jointly inhibited renal injuries in L-NAME/SHR. As well as the animal experiments, an ARB has been shown to reduce proteinuria and slow the progression of renal dysfunction in patients with nondiabetic renal disease.³⁰⁾

The COMB group in this study was given SPRL and a low-dose of TELM which did not significantly lower the blood pressure. However, the renal dysfunction, proteinuria, glomerular injuries, and perivascular lesions were improved comparably to the high-dose TELM group in which the blood pressure was significantly lowered. In addition, the wall thickness of interlobular arteries was significantly reduced in the COMB group but not in the high-dose TELM group, and the increase in NOx was observed only in the COMB group. Considering that L-NAME is a competitive inhibitor of NOS, the protective effect against the endothelial injury may have been greater in the CMB group than in the other groups. Thus, the simultaneous blockades of angiotensin II and aldosterone at the level of their respective receptors may be an effective strategy for the prevention of hypertensive renal injuries. Moreover, the effects seem to be brought about, in part, by a mechanism independent of blood pressure reduction. Also in the heart, the combination of ARB and spironolactone has been shown to suppress cardiac remodeling such as myocyte hypertrophy and perivascular fibrosis in hypertensive rats.³¹⁾

PPAR- γ has been shown to exhibit anti-inflammatory, antifibrotic, and anti-hypertrophic effects on the cardiovascular tissues.¹⁴⁾ It has been also reported that PPAR- γ activators such as pioglitazone and rosiglitazone reduce blood pressure, inflammation, and endothelial dysfunction in angiotensin II infused rats.³²⁾ The hypotensive effects of thiazolidinedione derivatives have also been observed in hypertensive and diabetic patients.³³⁻³⁵⁾ TELM is a distinctive ARB in terms of its activation of PPAR- γ . Benson, *et al* have demonstrated that a conventional dose of TELM and a high dose of irbesartan, but not other ARBs, could activate PPAR- γ and induce adipocyte differentiation.³⁶⁾ They speculate that the similarities in chemical structures between TELM and pioglitazone may participate in the activation of PPAR- γ by TELM. In the present study, the high-dose TELM and COMB administrations significantly increased PPAR- γ mRNA levels in the renal

cortical tissues and reduced the perivascular fibrosis and cell infiltration. The improvement of renal tissue injuries in the high-dose TELM group may be brought about by blood pressure reduction. However, in the COMB group, the increase in PPAR- γ mRNA may have contributed to the alleviation of vascular wall thickening, perivascular fibrosis, and inflammation because blood pressure was not significantly altered. The PPAR- γ mRNA increases were comparable between the high-dose TELM and COMB groups, and SPRL increased PPAR- γ mRNA insignificantly. Therefore, it is also speculated that the increase in PPAR- γ mRNA was induced as the result of the improvement of renal tissue injuries. On the other hand, it has recently been pointed out that rosiglitazone possibly increases the risk of myocardial infarction.³⁷⁾ Therefore, the therapeutic benefits of PPAR- γ activation should be evaluated considering the comprehensive effects on the cardiovascular system including the heart, kidneys, and vessels.

TGF- β_1 is a cytokine which inhibits the migration and proliferation of vascular cells on the one hand. On the other hand, TGF- β_1 increases the production of extracellular matrices and promotes the fibrosis of cardiovascular and renal tissues.^{38,39)} Angiotensin II is known as a stimulator of TGF- β_1 production. We have previously reported that TGF- β_1 mRNA expression was increased in the renal tissue of L-NAME/SHR and that this was inhibited by the administration of an ACE inhibitor.⁵⁾ In the present study, the renal TGF- β_1 mRNA level was markedly decreased in the TELM and COMB groups given an ARB, while the decrease was not significant in the SPRL group. The decrease in TGF- β_1 mRNA may be also mediated by the increase in PPAR- γ by TELM, because pioglitazone, a ligand for PPAR- γ , was shown to reduce TGF- β_1 expression and ameliorate renal injury in diabetic rats.⁴⁰⁾ In the SPRL group in this study, the increases in PPAR- γ mRNA and TGF- β_1 mRNA were not significant and the alleviation of renal tissue injuries was less marked than in the TELM and COMB groups. We believe that, in addition to the blood pressure changes and angiotensin II receptor blockade, modification of factors such as PPAR- γ and TGF- β_1 is involved in the mechanism of the renoprotective effects of TELM.

In summary, the combination of low dose ARB with an aldosterone antagonist alleviated the renal injuries in L-NAME/SHR without reducing blood pressure. The renoprotective effects of this combination therapy were comparable to the hypotensive dose of ARB. The anti-inflammatory and antifibrotic effects of PPAR- γ activation and TGF- β_1 suppression may be involved in the mechanism of this renoprotection. It is suggested that the dual blockade of angiotensin II and aldosterone in RAAS is effective in preventing hypertensive renal injuries.

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