Efonidipine, a Calcium Channel Blocker, Alleviates Renal Injury Induced by Nitric Oxide Synthase Inhibition in Spontaneously Hypertensive Rats

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SUMMARY

This study was designed to evaluate the protective effects of efonidipine, an L- and T-type calcium channel blocker, against hypertensive renal injuries in spontaneously hypertensive rats (SHR) chronically treated with N-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibiter. Four groups of seventeen-week-old male SHR were treated for as follows：the control group, the L-NAME group given 80 mg/L L-NAME in drinking water, and the groups given 5 (low-dose) or 20 (high-dose) mg/kg/day efonidipine by gavage as well as 80 mg/L L-NAME in drinking. After three weeks, the rats were sacrificed and the degrees of renal injuries were compared. The L-NAME group developed renal dysfunction and renal tissue lesions such as glomerulosclerosis, arterial wall thickening and perivascular fibrosis and cell infiltration with the aggravation of hypertension. The high-dose efonidipine improved the renal function and alleviated the renal tissue lesions in SHR given L-NAME as well as lowering blood pressure. On the other hand, the low-dose efonidipine also exhibited such renoprotective effects without causing significant changes of blood pressure in L-NAME-treated SHR. Renal cortical mRNA expressions of transforming growth factor-β_1 (TGF-β_1) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were increased and decreased, respectively, in SHR given L-NAME. Such changes in the mRNA expressions were mitigated by either the low-dose or the high-dose efonidipine treatment. It is suggested that efonidipine is effective in inhibiting the progression of hypertensive renal injuries and the renoprotective effects are mediated by the mechanism independent of blood pressure reduction as well as by the antihypertensive action.

Key Words：hypertension, nephrosclerosis, calcium channel blockers, transforming growth factor-beta, peroxisome proliferator-activated receptors

INTRODUCTION

With the use of antihypertensive therapy, the morbidity and mortality from major cardiovascular complications of hypertension such as stroke have decreased. However, the occurrence of cardiovascular organ failures such as end stage renal disease and cardiac failure continues to increase. Regarding the animal model of hypertensive organ injuries, the development of nephrosclerosis is not obvious until much later stage of life in spontaneously hypertensive rats (SHR)[1]. In this respect, we have previously reported that SHR given N-nitro-L-arginine methyl ester (L-NAME), a nitric

oxide synthase (NOS) inhibitor, develop marked proteinuria and progressive hypertensive nephrosclerosis[2]. In addition, we observed that renin-angiotensin-aldosterone system (RAAS) is markedly enhanced in this L-NAME-treated SHR and reported that the inhibitors of RAAS such as angiotensin-converting enzyme (ACE) inhibitors and β-blockers are effective in alleviating the development of nephroscrelotic lesions[3,4].

In the process of hypertensive cardiovascular organ injuries, fibrotic and inflammatory tissue lesions develop along with proliferation and hypertrophy of the cells. In this respect, transforming growth factor-β_1 (TGF-β_1) is a profibrotic cytokine and peroxisome proliferator-activated receptor-γ (PPAR-γ) has been shown to exhibit anti-inflammatory, anti-fibrotic and anti-hypertrophic effects on the cardiovascular tissues. Therefore, the changes in these factors seem intriguing in the treatment of hypertension targeting the prevention of cardiovascular organ injuries.

Calcium channel blockers are widely used in the treatment of hypertension and other cardiovascular diseases. In this study, we investigated the therapeutic effects of a dihydropyridine calcium channel blocker, efonidipine, on the progression of hypertension and nephrosclerosis in relation to the expressions of TGF-β_1 and PPAR-γ in L-NAME-treated SHR.

METHODS

Male seventeen-week-old SHR (Charles River Laboratories, Tokyo) with body weight of 290 to 330 g were used in this study and they were maintained on a regular chow containing 0.3% NaCl. The experimental design was approved by the institutional animal care committee. The rats were divided into four experimental groups : group 1 rats (control, n=15) were given tap water ; group 2 rats (L-NAME, n=14) were given 80 mg/L L-NAME (Sigma Chemical Co., St. Louis, USA) in their drinking water ; group 3 rats (L-EFN, n=10) were given 80mg/L L-NAME as drinking and administered low-dose (5 mg/kg/day) efonidipine, a calcium channel blocker, by gavage ; group 4 rats (H-EFN, n=11) were given 80 mg/L L-NAME as drinking and administered high-dose (20 mg/kg/day) efonidipine. Efonidipine was provided by ZERIA Pharmaceutiocal Co., Ltd., Tokyo. Systolic

blood pressure was measured by the tail-cuff method, and the treatment was continued for three weeks. During the final week of treatment, all rats were placed in metabolic cages for 2 days to collect 24-hour urine samples.

After three weeks, the rats were sacrificed under pentobarbital anesthesia (30 mg/kg, intraperitoneally). The kidneys were perfused and fixed with saline and neutral-buffered 8% paraformaldehyde solution. Then, the left cardiac ventricle, thoracic aorta and the kidneys were excised, weighed and served for the preparations of histological examination. Plasma and urinary concentrations of creatinine and protein were assayed by colorimetry and urinary nitric oxides (NOx) were determined by high performance liquid chromatography.

The kidneys were embedded in paraffin, and 2-μm frontal sections were cut for light microscopic histological examination. These sections were stained with hematoxylin-eosin, periodic acid-Schiff (PAS) and Masson trichrome 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 score (GIS) was assessed as described previously[4~7]. 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. Every glomeruli in the juxtamedullary layer of frontal section, generally 100 to 150 glomeruli, were evaluated in each rat and the scores were summed up. GIS was defined as the converted value of total score per 100 glomeruli. Namely, global sclerosis of every glomerulus corresponds to the maximum GIS of 300. 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 computer analyzer system (Image Quest, Hamamatsu Photonics, Hamamatsu and MacScope, Mitani Co., Fukui, Japan). 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

Table 1　Body weight, blood pressure and organ weights of rats at the end of 3-week treatment period.

Indices	Control (n = 15)	L-NAME (n = 14)	L-EFN + L-NAME (n = 10)	H-EFN + L-NAME (n = 11)
Body weight, g	351 ± 8	287 ± 10 **	303 ± 11	318 ± 9 †
Systolic blood pressure, mmHg	233 ± 4	271 ± 8 **	258 ± 10	175 ± 12 † †
Heart rate, bpm	459 ± 15	441 ± 32	470 ± 18	466 ± 13
Right kidney weight, g/kgBW	3.5 ± 0.1	3.6 ± 0.1	3.1 ± 0.3	3.4 ± 0.2
Left kidney weight, g/kgBW	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.2	3.9 ± 0.1
Left ventricular weight, g/kgBW	3.2 ± 0.1	3.7 ± 0.1 *	4.4 ± 0.2 †	3.6 ± 0.2

Data are mean ± SEM. BW：body weight. *$p < 0.05$, **$p < 0.01$ vs. control；† $p < 0.05$, † † $p < 0.01$ vs. L-NAME.

R is the radius of the outer circumference and r is the radius of the inner circumference, and R-r represents medial thickness[4,8]. The perivascular fibrosis area of interlobular artery was assessed by calculating the ratio of the collagen-stained area to the vascular wall and lumen area in the section stained with Masson trichrome. The infiltrating cell number was assessed by counting the number of filtrating cells such as lymphocytic cells, plasma cells, fibroblasts and macrophages in the perivascular space of interlobular artery using the Image Quest.

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, California, USA), is a method that provides reproducible quantitative PCR[9]. 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, California, USA). TaqMan probe which was modified with the fluorescent (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'-cgcctgcagagattcaagtcaa-3'
　　reverse promer：5'-gtcggttcatgtcatggatggt-3'
　　TaqMan probe：5'-agtggctgaaccaaggagacggaata-ca-3'
PPAR-γ,
　　forward primer：5'-cttggccatatttatagctgtcattatt-3'
　　reverse promer：5'-agcaggttgtcttggatgtcct-3'
　　TaqMan probe：5'-tggagaccgcccaggcttgct-3'
β-actin,
　　forward primer：5'-cgtgaaaagatgacccagatca-3'
　　reverse promer：5'-acacagcctggatggctacgta-3'
　　TaqMan probe：5'-tttgagaccttcaacaccccagcca-3'

One-way ANOVA, followed by Duncan's multiple range test, was performed to test for between-group significance. All data are expressed as the mean ± SEM. A probability level of ＜5％ was considered to indicate statistical significance.

RESULTS

Table 1 lists the data of physical measurements and organ weights in the four groups of rats in this study. The L-NAME group developed marked hypertension

Table 2 Chemical measurements of plasma and urine samples.

Indices	Control (n = 15)	L-NAME (n = 14)	L-EFN + L-NAME (n = 10)	H-EFN + L-NAME (n = 11)
Serum creatinine, mg/dL	0.20 ± 0.02	0.51 ± 0.06**	0.33 ± 0.01 ††	0.30 ± 0.02 ††
C-Cr, mL/min/kg	9.5 ± 1.0	3.3 ± 0.1**	5.4 ± 0.6 †	7.4 ± 0.4 ††
Urinary protein, mg/day	19 ± 1	120 ± 13**	69 ± 19 †	24 ± 3 ††
Urinary NOx, μmol/day	5.0 ± 0.5	0.6 ± 0.2**	1.1 ± 0.2	1.3 ± 0.3

Data are mean ± SEM. C-Cr, creatinine clearance. *p < 0.05, **p < 0.01 vs. control ; † p < 0.05, †† p < 0.01 vs. L-NAME.

Table 3 Histological findings of the kidneys of rats at the end of 3-week treatment period.

| Indices	Control (n = 15)	L-NAME (n = 14)	L-EFN + L-NAME (n = 10)	H-EFN + L-NAME (n = 11)
Glomerular injury score	9 ± 2	197 ± 15**	30 ± 5 ††	18 ± 4 ††
Wall thickness ratio	0.48 ± 0.03	0.58 ± 0.03*	0.47 ± 0.02 †	0.40 ± 0.03 ††
Perivascular fibrosis area	0.35 ± 0.04	0.91 ± 0.11**	0.36 ± 0.02 ††	0.37 ± 0.08 ††
Perivascular cell number number/$10^4 \mu m^2$	47 ± 7	110 ± 10**	47 ± 3 ††	50 ± 7 ††

Data are mean ± SEM. *p < 0.05, **p < 0.01 vs. control ; † p < 0.05, †† p < 0.01 vs. L-NAME.

and reduced body weight as compared with the control SHR group. The high-dose efonidipine alleviated this aggravation of blood pressure elevation and body weight reduction, while the low-dose efonidipine did not significantly affect the blood pressure and body weight of L-NAME-treated SHR. The kidney weight was not significantly affected by administration of either L-NAME or efonidipine. The L-NAME treatment increased the cardiac left ventricular weight of SHR. The low-dose efonidipine increased but the high-dose efonidipine did not significantly changed the left ventricular weight of L-NAME-treated SHR.

Table 2 presents the results of chemical measurements of plasma and urine samples. As compared with the control SHR, the plasma creatinine concentration was increased in the L-NAME group, however, the increases were mitigated in the L-EFN and the H-EFN groups. The creatinine clearance was also markedly lower in the L-NAME group than in the control group, and either the low-dose or the high-dose efonidipine increased the creatinine clearance of L-NAME-treated SHR. The L-NAME group showed marked proteinuria as compared with the control group, however, this was significantly reduced in the L-EFN and the H-EFN groups. Regarding the urinary metabolites of nitric oxides, the L-NAME treatment markedly reduced the

urinary NOx excretion in SHR and either the low-dose or the high-dose efonidipine did not significantly affect the urinary NOx excretion in L-NAME-treated SHR.

Figure 1 presents the representative micrographs of glomeruli in each group. The L-NAME-treated SHR showed marked sclerosis and hyalinosis of glomeruli, while the glomerular lesions were minimal in the control SHR (Figure 1, panels A and B). Such glomerular sclerosis was much improved in the L-EFN or the H-EFN groups (Figure 1, panels C and D). The results of histological analyses were presented in Table 3. The L-NAME treatment markedly increased the GIS in SHR, however, this was prominently improved in the L-EFN or the H-EFN group. Figure 2 presents the micrographs of interlobular arteries. The L-NAME group showed the increased wall thickness ratio of interlobular artery as compared with the control group (Figure 2, panels A and B). and the increase was inhibited by either the low-dose or the high-dose efonidipine treatment (Figure 2, panels C and D). Both the fibrosis area and the number of infiltrating cells in the perivascular area of interlobular artery were increased in the L-NAME group as compared with the control group (Figure 2, panels A and B). and these increases were suppressed in the L-EFN or the H-EFN group (Figure 2, panels C and D).
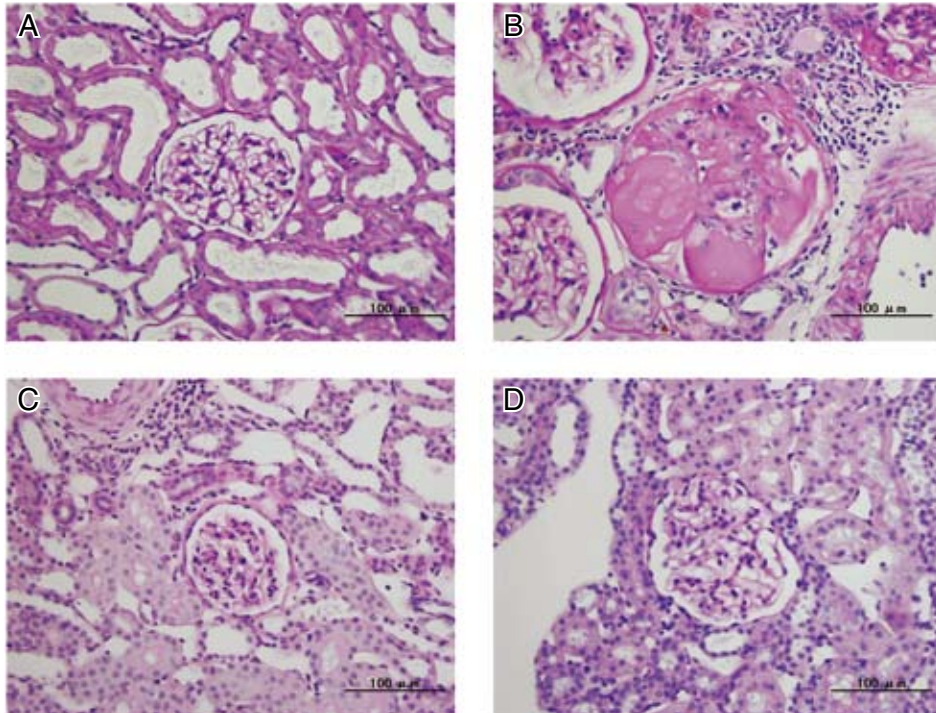
Figure 1 Representative PAS-staining micrographs of glomeruli in each group. A：SHR without L-NAME or efonidipine, B：L-NAME-treated SHR, C：SHR given L-NAME and low-dose（5 mg/kg）efonidipine, D：SHR given L-NAME and high-dose（20 mg/kg）efonidipine.
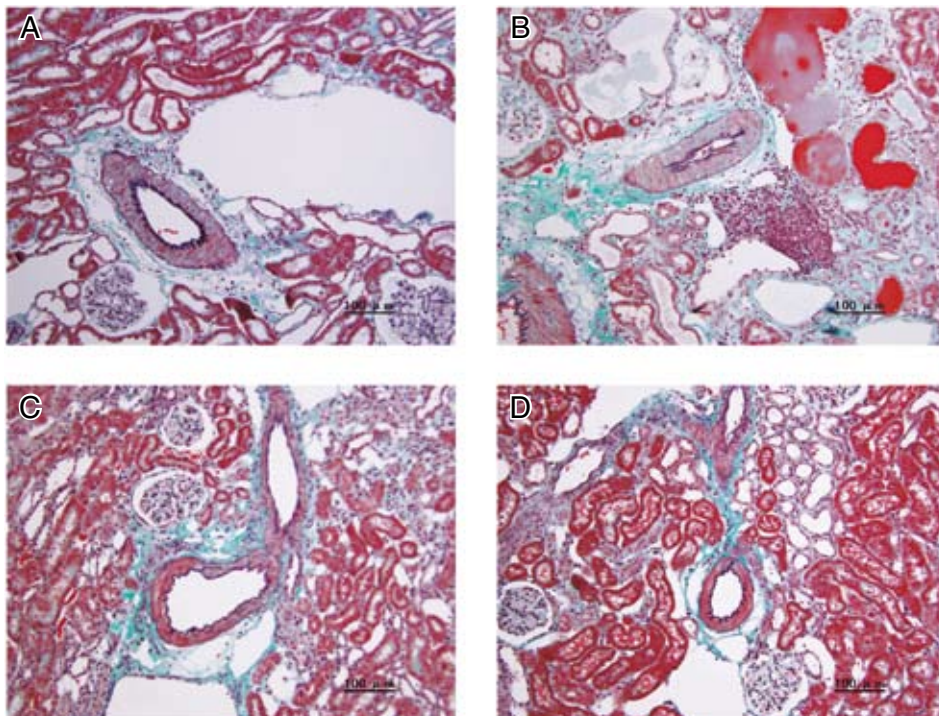
Figure 2 Representative Masson trichrome-staining micrographs of interlobular arteries and perivascular areas in each group. A：SHR without L-NAME or efonidipine, B：L-NAME-treated SHR, C：SHR given L-NAME and low-dose（5 mg/kg）efonidipine, D：SHR given L-NAME and high-dose（20 mg/kg）efonidipine.
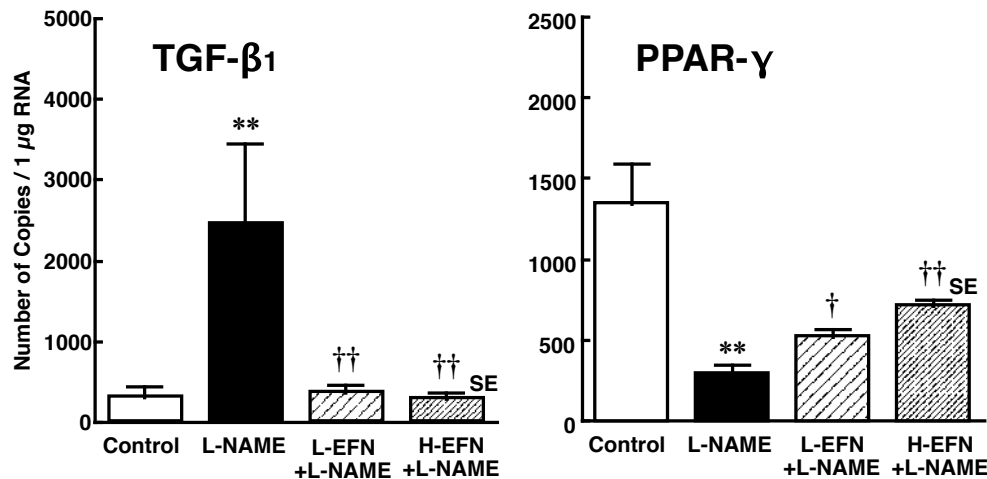
Figure 3 Levels of mRNA for transforming growth factor-β_1 (TGF-β_1) and, peroxisome proliferation-activated receptor-γ (PPAR-γ) in the renal cortical tissues. *p＜0.05, **p＜0.01 vs. control ; †p＜0.05 vs. L-NAME. L-NAME, N-nitro-L-arginine methyl ester ; L-EFN, low-dose efonidipine (5 mg/kg/day) ; H-EFN, high-dose efonidipine (20 mg/kg/day).

Figure 3 depicts the mRNA expressions of TGF-β_1 and PPAR-γ in the renal cortical tissues of rats quantified by the real-time PCR analyses. As we have previously reported, the TGF-β_1 mRNA expression in the kidney was markedly increased in L-NAME-treated SHR[3,10], however, the expression was suppressed in the L-EFN or the H-EFN group of this study. On the other hand, the mRNA expression of PPAR-γ in the kidney was reduced in the L-NAME group as compared with the control group, and this reduction was significantly alleviated by either the low-dose or the high-dose efonidipine treatment.

DISCUSSION

Hypertension is a major risk factor for cardiovascular organ injuries. In addition, cardiovascular risk factors such as aging, dyslipidemia and oxidative stress cause endothelial dysfunction and decreased NO production which further promote cardiovascular tissue injuries. SHR treated with L-NAME, a NOS inhibitor, have been shown to develop hypertensive target organ injuries[2,11]. With regard to the hypertensive renal injury, we have previously reported the progression of glomerulosclerosis and vascular lesions in the kidneys of SHR given L-NAME with aggravation of hypertension[2], while such renal lesions cannot be seen until much later stage of life in SHR without L-NAME treatment. Thus, it is assumed that L-NAME-treated SHR can serve as an experimental animal model of hypertensive nephrosclerosis. In the present study, we examined the protective effects of a calcium channel blocker, efonidipine, against the progression of hypertensive renal injuries in L-NAME-treated SHR.

Either the low-dose or the high-dose efonidipine was effective in alleviating the renal dysfunction and renal tissue injuries such as glomerulosclerosis and vascular lesions. L-NAME-treated SHR develop marked hypertension and the high-dose efonidipine significantly lowered the blood pressure. Although the low-dose efonidipine failed to alter the blood pressure in L-NAME-treated SHR, the insignificant blood pressure reduction may have related to the renoprotective effects. In addition, the effects such as suppression of TGF-β_1 and induction of PPAR-γ may have participated to the alleviation of hypertensive renal injuries.

We have reported that L-NAME-treated SHR develops hypertensive nephrosclerosis with marked enhancement of RAAS[8]. According to the hyperfiltration theory, increases in glomerular capillary pressure, referred to as glomerular hypertension, play an important role in the progression of renal dysfunction[12,13]. Intraglomerular capillary pressure is affected by the tone of the glomerular arterioles as well as by the level of systemic arterial pressure. Because angiotensin II is a potent constrictor of the efferent arterioles, inhibitors of RAAS, which block the generation and/or action of angiotensin II, are thought to be effective at alleviating glomerular hypertension in L-NAME-treated SHR[14].

Indeed, we have reported that RAAS inhibitors such as ACE inhibitors and angiotensin II receptor blockers improve nephrosclerosis exacerbated by L-NAME in SHR[3,10,15,16]. By contrast, calcium channel blockers tend to dilate the afferent arterioles and therefore are less effective at reducing intraglomerular pressure, when compared with specific inhibitors of the RAAS[17]. In this respect, it has been demonstrated that the glomerular efferent arterioles express the T-type calcium channels. Efonidipine, used in this study, blocks the T-type calcium channels in addition to the L-type channels[18], and efonidipine has been shown to dilate the efferent arterioles as well as the afferent arterioles in the experiment using isolated perfused kidney[19]. Also in the clinical study, we have reported that efonidipine reduced proteinuria in patients with nondiabetic renal diseases[20] and this may be related to a reduction in glomerular capillary pressure caused by the efferent arteriole dilation. Therefore, such effects of efonidipine on the glomerular hemodynamics may have contributed to the renoprotective effects observed in this study.

The mRNA expression of TGF-β_1 was increased by L-NAME treatment in SHR, and the increase was inhibited by the low-dose and the high-dose efonidipine. Because both angiotensin II and aldosterone have been shown to increase TGF-β_1 generation, the increased renal cortical TGF-β_1 expression in L-NAME-treated SHR is supposed to have been caused by the enhanced RAAS[21,22]. TGF-β_1 is a cytokine which inhibits the immigration and proliferation of vascular cells on one side. On the other side, TGF-β_1 increases the production of extracellular matrices and promotes the fibrosis of cardiovascular and renal tissues[23,24]. On the other hand, the mRNA expression of PPAR-γ was decreased in L-NAME-treated SHR as compared with SHR, and the decrease was alleviated by the low-dose and the high-dose efonidipine. PPAR-γ has been shown to exhibit anti-inflammatory, anti-fibrotic actions and anti-hypertrophic effects on the cardiovascular tissues[25,26]. The ligands of PPAR-γ, such as pioglitazone, have been shown to exhibit blood pressure-lowering action by blockade of calcium uptake by vascular smooth muscle and antiproteinuric effect by increasing the gene transcription of nephrin[27,28]. Thus, it is suggested that the inhibition of TGF-β_1 expression and the resto-

ration of PPAR-γ expression have participated in the renoprotective actions of efonidipine in L-NAME-treated SHR.

The left ventricular weight was increased by the L-NAME treatment in SHR, however, the weight was unchanged by the high-dose efonidipine and was rather increased by the low-dose efonidioine. We have previously shown that the necrotic and fibrotic lesions develop in the cardiac tissue of L-NAME-treated SHR[11,29]. Although the histological evaluation of cardiac tissue was not performed in this study, a reduction of such changes may have resulted in the increase of left ventricular weight in the L-NAME-treated SHR given low-dose efonidipine.

In conclusion, efonidipine may effectively inhibit the progression of hypertensive renal injuries. This renoprotective effects are thought to be brought about by its antihypertensive effect. In addition, the mechanism such as TGF-β_1 suppression and PPAR-γ restoration may partly participate to the protective effects of efonidipine against hypertensive renal injuries.

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