

Renoprotective Effect of Azelnidipine in Rats

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To assess whether azelnidipine (AZN) exerts renoprotective effects, 20-week-old adult male stroke-prone spontaneously hypertensive rats (SHRsp) were treated with AZN 10 mg/kg/d ($n=6$), olmesartan (OLM) 3 mg/kg/d ($n=4$), hydralazine (HYD) 20 mg/kg/d ($n=3$), or water (control; $n=5$). Each test agent was administered by oral gavage for 12 weeks. Systolic blood pressure (SBP) was measured every 2 weeks and urinary protein excretion (UproV) every 3 weeks. At the age of 32 weeks, the rats were sacrificed and blood and kidneys collected for biochemical, histological, and immunohistochemical studies. All drug treatments significantly ($p<0.05$) reduced SBP, UproV, and blood biochemical parameters such as creatinine, total cholesterol, and blood urea nitrogen. Masson trichrome staining and immunohistochemical staining revealed significant ($p<0.05$) reductions of interstitial fibrosis, collagen type III, nicotinamide-adenine dinucleotide/nicotinamide-adenine dinucleotide phosphate oxidase, and p22^{phox} and p47^{phox} components expression in the AZN- and OLM-treated groups in comparison with rats treated with HYD and control animals. ED1, 4-hydroxy-2-nonenal (4-HNE), and heat shock protein (HSP)-47 expression was also reduced in the AZN- and OLM-treated groups versus in HYD and control animals. These results indicate that not only OLM but also AZN exerts renoprotective effects through inhibition of macrophage infiltration and antioxidant activity in SHRsp model of renal injury.

Key words azelnidipine; calcium channel blocker; antioxidant; hypertension; renoprotective effect

Hypertension plays one of the major roles in progression of renal damage, and agents that control hypertension may provide favorable protection for kidneys at risk of glomerular injury. Angiotensin II type 1 receptor blockers (ARB) are widely reported to possess renoprotective effects. Olmesartan (OLM), an ARB with high affinity to the type 1 receptor, alleviates tubulointerstitial injury by suppressing elevation of renal angiotensin II levels.¹⁾ It has also been shown that calcium channel blockers (CCBs), which are considered to act mainly as vasodilators, are effective against various renal injuries induced by angiotensin II; reports have demonstrated that these agents exert beneficial effects against angiotensin II-induced glomerular contraction, anti-natriuretic responses, and angiotensin II-mediated stimulation of mesangial cells.^{2–5)}

The calcium inflow mechanism dependent on L-type calcium channels is inhibited specifically by azelnidipine (AZN), a newly developed CCB, and this mechanism is an essential element of renovascular contraction induced by angiotensin II.^{6–8)} Therefore if calcium antagonists suppress calcium inflow, it may be possible to reduce renal events induced by elevated angiotensin II levels. Indeed, it has been shown that AZN significantly alleviates angiotensin II-induced peritubular ischemia.⁹⁾ Recent studies have demonstrated that AZN has blood pressure-independent vascular protective effects deriving presumably from its antioxidant activity.¹⁰⁾ Although the exact mechanism for renoprotective activity of CCBs remains unclear, it seems likely that AZN, an agent that suppresses inflow of calcium into cells, might exert antioxidative action and thereby elicit favorable effects on renal tissue.

Stroke-prone spontaneously hypertensive rats (SHRsp)

were selectively bred as a research tool in 1974.¹¹⁾ SHRsp undergo rapid increases in blood pressure at a young age, and develop severe and persistent hypertension, resulting in renal injury starting from about age 22 weeks.¹²⁾ The present study investigated whether AZN, a dihydropyridine-type CCB, elicits renoprotective effects when administered at the onset of renal injury in SHRsp.

MATERIALS AND METHODS

Animals and Treatment The experiments were performed in male SHRsp/Izm (Japan SLC, Shizuoka, Japan) aged 20 weeks. The rats were housed in standard rodent cages at constant ambient temperature (22 ± 1 °C) and humidity (85%), with 12-h cycles of light/d. They had free access to Funabashi SP diet (Funabashi Farm, Chiba, Japan) and tap water. The experimental protocol was inspected and approved by the Animal Care and Use Committee of Nagasaki University School of Medicine and the law and notification of the Japanese government prior to commencement of the study.

The rats were treated with AZN (Sankyo, Tokyo, Japan) at a dosage of 10 mg/kg/d ($n=6$), olmesartan (OLM; Sankyo) 3 mg/kg/d ($n=4$), hydralazine (HYD; SIGMA, St. Louis, MO, U.S.A.) 20 mg/kg/d ($n=3$), or water (control group; $n=5$). AZN and OLM were suspended in 0.5% carboxymethylcellulose (039-01335; Wako Pure Chemical Industries, Osaka, Japan). Previous reports indicate that carboxymethylcellulose treatment has no effects on blood pressure, and has no apparent inhibitory effect on urinary protein excretion and renal injury in SHRsp.¹³⁾ Each agent was administered by gavage for 12 weeks. Systolic blood pressure (SBP) was

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measured every 2 weeks in conscious rats by the indirect tail-cuff method (BP-98A; Softron, Tokyo, Japan) without anesthesia. For metabolic study, each rat was placed in a metabolic cage designed to prevent feces-urine contact (Nalge Nunc International, Tokyo, Japan) after which 24-h urine samples were collected and urinary protein excretion (UproV) was measured every 3 weeks. At the age of 32 weeks, the rats were sacrificed and blood and kidneys collected. Blood samples were collected in blood collection tubes without heparin. Serum was obtained by blood centrifugation at 3000 rpm and 4 °C for 5 min. Serum creatinine (SCr) and total cholesterol (TC) levels were measured by automated enzymatic method (Alfresa Pharma, Osaka, Japan and Wako, respectively); blood urea nitrogen (BUN) by UV method (DENKA SEIKEN, Tokyo, Japan); albumin (Alb) by BCG method (Wako); and UproV by Pyrogallol Red method (Wako). All measurements were performed using a Hitachi 7180 Autoanalyzer.

Histological Examination Rat kidneys were removed and portions fixed in 4% paraformaldehyde (PFA). Other kidney portions were frozen in optimum cutting temperature (OCT) compound (Miles, Elkhart, IN, U.S.A.) and stored at -80 °C. Fixed renal tissues were embedded in paraffin and cut into 4- μ m-thick sections. The sections were stained with periodic acid-Schiff (PAS) stain and Masson trichrome stain to reveal histological changes and areas of interstitial fibrosis.

Immunohistochemical Study For immunohistochemistry analysis the following antibodies were used: mouse anti-ED1 antibody (MCA341R, Serotec, Oxford, U.K.) diluted 1/100 as marker of rat macrophages; mouse anti-heat shock protein (HSP)-47 antibody (SPA-470, Stressgen Biotechnologies, Victoria, BC, Canada) diluted 1/100 as indicator of collagen biosynthesis; rabbit anti-collagen type III antibody (AB757, CHEMICON International, Temecula, CA, U.S.A.) diluted 1/100; mouse anti-4-HNE antibody (MHN-100P, Japan Institute for the Control of Aging, Shizuoka, Japan) diluted 1/100 as marker of oxidative stress; and rabbit anti-p22^{phox} antibody (sc-20781; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1/50 and rabbit anti-p47^{phox} antibody (sc-14015, Santa Cruz Biotechnology) diluted 1/25 as marker of NADPH oxidase subunits.

For immunohistochemical examination, 4- μ m-thick paraffin-embedded tissues were deparaffinized and blocked for endogenous peroxidases and the slides immersed in 0.3% hydrogen peroxide in methanol for 20 min. PFA 4%- or acetone-fixed 4 μ m-thick frozen sections were also blocked for endogenous peroxidases by the same method.

Indirect immunohistochemical technique was used to assess ED1, 4-HNE, and HSP-47 expression in paraffin-embedded sections. To reduce nonspecific background staining, the sections were incubated with a blocking buffer containing 10% normal goat serum (X0907, DakoCytomation Denmark A/S, Glostrup, Denmark) 10% fetal calf serum (FCS; 011-16290M, Gibco Life Technology, Carlsbad, CA, U.S.A.), and 2% normal rat serum (X0912, DAKO) in phosphate-buffered saline (PBS) for 30 min. The sections were then reacted with the primary antibody, which was diluted in the same blocking buffer. After reacting with anti-ED1 antibody, anti-4-HNE antibody, or anti-HSP47 antibody at room temperature for 1 h, sections were reacted with horseradish peroxidase

(HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (P0161, DAKO) diluted 1/100 at room temperature for 30 min and with HRP-conjugated swine anti-rabbit immunoglobulin antibody (P0399, DAKO) at room temperature for 30 min.

The peroxidase anti-peroxidase (PAP) technique was used to assess type III collagen expression in fresh-frozen sections fixed in 4% PFA at room temperature for 15 min then incubated with blocking buffer similar to that described above for 30 min. After reacting with anti-type III collagen antibody at room temperature for 1 h, sections were further reacted with HRP-conjugated swine anti-rabbit immunoglobulin antibody (P0399, DAKO) diluted 1/50 at room temperature for 30 min, and rabbit PAP (Z0113, DAKO) diluted 1/100 at room temperature for 30 min. The avidin-biotin-peroxidase technique was used to detect p22^{phox} and p47^{phox} in fresh-frozen sections fixed in acetone at room temperature for 15 min. This method used a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) after reacting with each antibody at room temperature for 1 h. Reaction products were visualized by treating sections with H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (DAB; 347-00904, Dojindo Laboratories, Kumamoto, Japan). Finally, the sections were counterstained with methyl green and mounted. For all specimens, negative controls were prepared with each blocking buffer in place of the primary antibody.

Data Processing and Statistical Analysis For semi-quantitative estimation of the blue area of Masson trichrome staining and positive area of expression of each protein, images were digitized using image analysis software (Win Roof Mitanicorp, Chiba, Japan). Images were transformed into a matrix of 2250 \times 1800 pixels and viewed at \times 100 magnification; the area of each section was 0.89 mm². The area of cortical interstitium was measured by light microscopic examination. For each group, 20 such areas were selected. In a total of 80 fields, we calculated the percentage of areas staining positive for HSP-47, type III collagen, p22^{phox}, p47^{phox}, and interstitial fibrosis by Masson trichrome stain. We also counted the number of ED1-positive macrophages at 20 fields under \times 200 magnification in each group.

All data are expressed as mean \pm S.E.M. Differences among groups were examined for statistical significance by repeated measures analysis of variance (ANOVA; Fisher's PLSD). *p*-values <0.05 were considered to denote the presence of statistically significant difference.

RESULTS

Blood Pressure SBP values in the rats during the 12-week experimental period are shown in Fig. 1. SBP in the control group increased progressively throughout this period, whereas in the HYD-, AZN-, and OLM-treated groups similar and significant (*p*<0.05) decreases of this parameter were noted compared with control. Eleven weeks after initiation of treatment, SBP in the HYD, OLM, AZN and control groups was 218.4 \pm 25.0, 222.8 \pm 11.8, 229.0 \pm 12.9, and 309 \pm 7.3 mmHg, respectively.

UproV Measurements Persistent hypertension induces an elevation of intraglomerular pressure. Glomerular hyperfiltration yielded by this condition eventually results in proteinuria. Thus 24-h urine protein excretion can be used as an

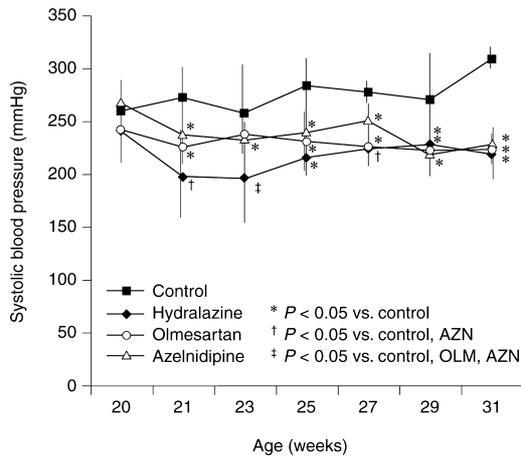


Fig. 1. Systolic Blood Pressure (SBP) Values

In comparison with control animals given vehicle (water), rats treated with AZN, OLM, or HYD exhibited significantly decreased SBP throughout the 12-week experimental period. No significant difference was observed in the level of SBP achieved with each drug.

indicator for glomerular permeability. UproV values in rats during the 12-week experimental period are shown in Fig. 2. In the control group, UproV progressively increased throughout the experimental period, whereas in the HYD, AZN, and OLM groups this parameter was significantly ($p < 0.05$) decreased compared with control starting from 2 weeks after initiation of treatment and remained as such thereafter, demonstrating that impaired glomerular permeability was improved by HYD, AZN, and OLM administration. No significant difference in this effect was found among the HYD, AZN, and OLM groups.

Biochemical Measurements Biochemical measurements in the rats during the 12-week treatment period are shown in Fig. 3. Blood urea nitrogen (BUN) is a waste product from proteins consumed as an energy source *in vivo*, and serum creatinine (SCr) is one of the end products of metabolism mainly produced from creatine through non-enzymatic dehydration reaction in muscle. Values of BUN and SCr are used as indices of renal filtration rate. BUN and SCr in the

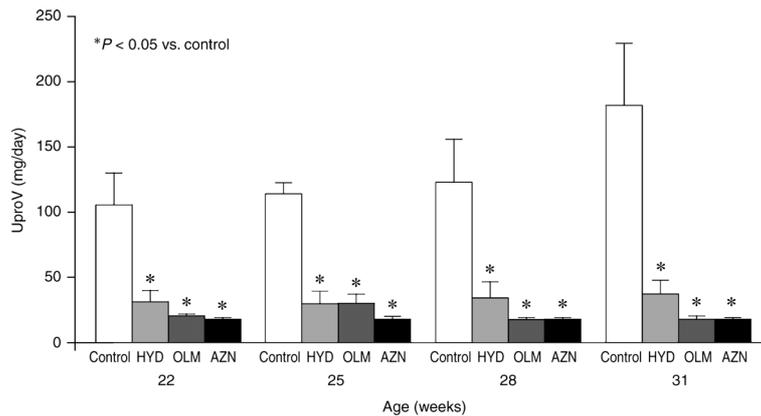


Fig. 2. Urinary Protein Excretion

SHRsp rats in the control group had markedly higher proteinuria than those treated with antihypertensive agents (AZN, OLM, or HYD). Furthermore, urinary protein level in AZN- and OLM-treated rats was lower than that in HYD-treated animals, although the difference was not significant.

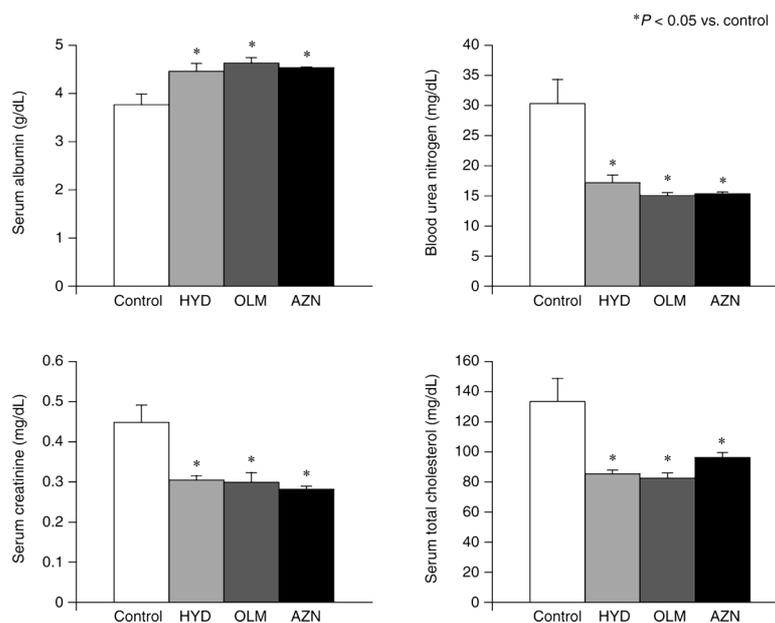


Fig. 3. Biochemical Analysis

Compared with control SHRsp rats, animals treated with AZN, OLM, or HYD exhibited marked reductions of blood urea nitrogen (BUN), serum creatinine (SCr), and total cholesterol (TC) and elevation of serum albumin (Alb).

HYD-, AZN-, and OLM-treated groups were significantly ($p < 0.05$) lower than those in the control group, indicating that impaired glomerular filtration was improved by administration of these agents.

Albumin (Alb) is a high-molecular weight protein synthesized in the liver. Following renal damage, Alb leakage from glomeruli results in low levels in serum. We observed that blood levels of Alb in the HYD-, AZN-, and OLM-treated groups were significantly higher than those in the control group ($p < 0.05$), indicating that impaired glomerular filtration was improved by HYD, AZN, and OLM administration.

TC is often increased when renal function is impaired. The underlying mechanism is considered due to excess of LDL and VLDL synthesis compensatory to reduction of serum Alb. The levels of TC in the HYD-, AZN-, and OLM-treated groups were significantly higher than those in the control group ($p < 0.05$), indicating that impaired glomerular filtra-

tion was improved by HYD, AZN, and OLM administration.

No significant difference in the above biochemical measurements was found among the HYD, AZN, and OLM groups.

Morphologic Examination PAS staining of kidney sections did not reveal any histological changes at 16 weeks, whereas interstitial fibrosis was observed at 24 weeks of age in SHRsp. In contrast, Wistar-Kyoto rats (WKY) showed no histological change (data not shown). Sclerotic change was observed in almost all glomeruli in the control group: in interstitium, evidence of severe atherosclerotic lesions, atrophic tubules, tubular dilatation, and cellular infiltration was observed (Fig. 4a). Whereas, in the HYD group (Fig. 4b) slightly better morphology was observed and in the OLM (Fig. 4c) and AZN (Fig. 4d) groups markedly better morphology was observed *versus* control.

Masson trichrome staining revealed that the area of inter-

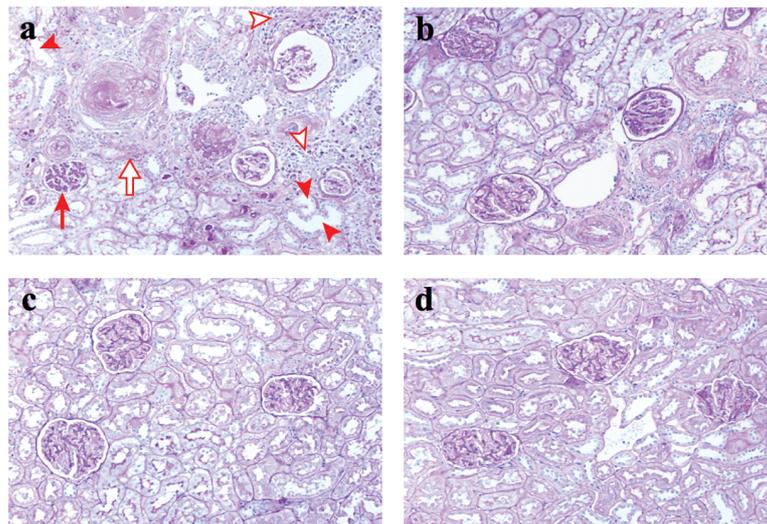


Fig. 4. PAS Staining

PAS staining revealed segmental and global glomerular sclerosis (arrow) and tubulointerstitial injury (tubular dilatation, atrophy of tubular epithelial cells: arrowheads; fibrosis: open arrow; and infiltration of inflammatory cells: open arrow heads) in SHRsp treated with vehicle (water) (a). Segmental glomerular sclerosis and tubulointerstitial injury were less observed in HYD-treated rats (b). Glomerular and tubulointerstitial injury was markedly ameliorated in SHRsp treated with OLM (c) and AZN (d).

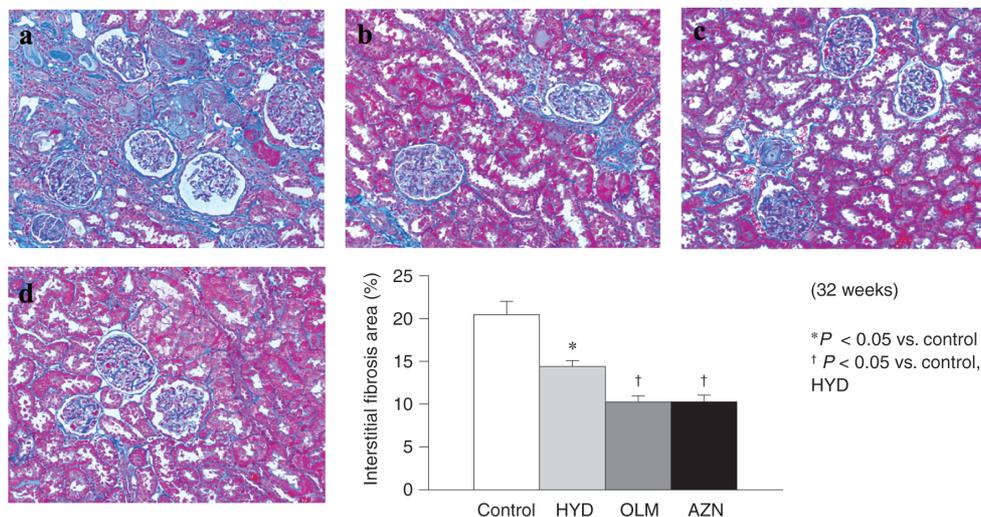


Fig. 5. Masson Trichrome Staining

Representative photographs of Masson trichrome staining in the experimental groups and semiquantitative analysis. Interstitial fibrosis was observed in SHRsp rats treated with vehicle (water) (a). Significantly decreased fibrotic areas were noted in rats treated with HYD (b), OLM (c), and AZN (d). Furthermore, significantly less interstitial fibrosis was in AZN- and OLM-treated than in HYD-treated animals.

stitial fibrosis increased from 16 weeks and expanded through 30 weeks of age compared with WKY (data not shown). In the control group, marked expansion of matrix in glomeruli and interstitial fibrosis were observed (Fig. 5a). In the HYD group (Fig. 5b), on the other hand, fibrosis was locally inhibited and in the OLM (Fig. 5c) and AZN (Fig. 5d) groups marked inhibition of fibrosis was observed. Semi-quantitative analysis revealed that the percentage area of fibrosis was significantly decreased in the HYD-, AZN-, and OLM-treated groups compared with control. Moreover, fibrosis was significantly lower in the AZN and OLM groups *versus* HYD group.

Expression of HSP-47 and Type III Collagen The results of immunohistochemical analyses for HSP-47 and type III collagen in 32-week-old rats are shown in Figs. 6 and 7. In control animals (Fig. 6a) marked expression of HSP-47 was observed in glomeruli and tubular epithelial cells, whereas in contrast, in the OLM (Fig. 7c) and AZN (Fig. 7d) groups HSP-47 expression was significantly decreased. HSP-

47 expression in the AZN- and OLM-treated groups was also lower than that in the HYD group (Fig. 7b), although the difference was not significant. Regarding interstitial expression of type III collagen, in the control group this parameter was markedly observed (Fig. 6a), whereas in the HYD (Fig. 6b), OLM (Fig. 6c), and AZN (Fig. 6d) groups significantly less type III collagen was detected. Moreover, type III collagen expression was significantly lower in the OLM and AZN groups than in the HYD group.

Expression of ED1 In the control group (Fig. 8a), the number of ED1⁺ macrophages in glomeruli and interstitial fibrosis areas was markedly increased *versus* in rats treated with HYD (Fig. 8b), OLM (Fig. 8c), and AZN (Fig. 8d). There was no significant difference of this parameter among AZN-, OLM-, and HYD-treated animals.

Expression of p22^{phox}, p47^{phox}, and 4-HNE In the control group, p22^{phox} was markedly expressed in glomeruli and interstitium (Fig. 9a), whereas in the OLM (Fig. 9c) and AZN (Fig. 9d) groups this parameter was significantly de-

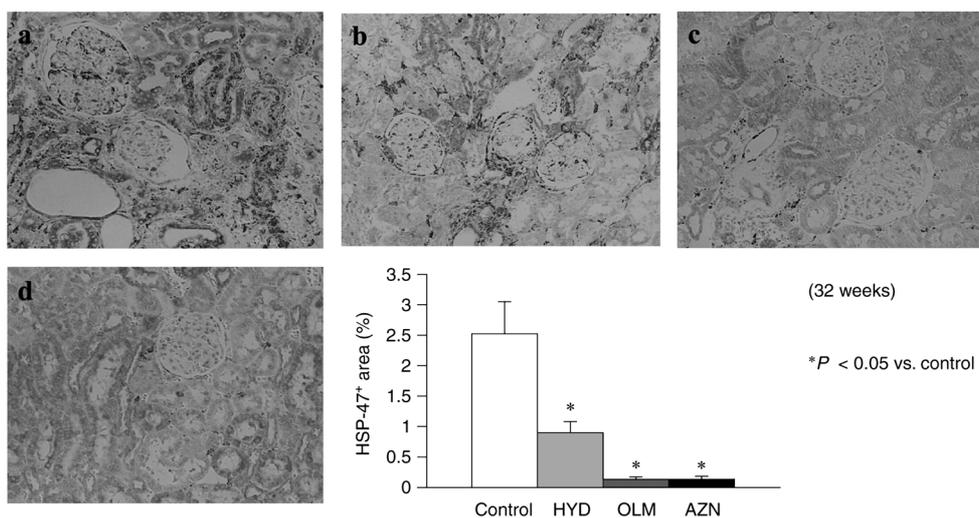


Fig. 6. HSP-47 Staining

Marked expression of HSP-47 was observed in SHRsp treated with vehicle (water) (a) and was significantly reduced in animals treated with HYD (b), OLM, (c), and AZN (d). Furthermore, treatment with OLM and AZN significantly reduced HSP-47 expression compared with in HYD-treated SHRsp.

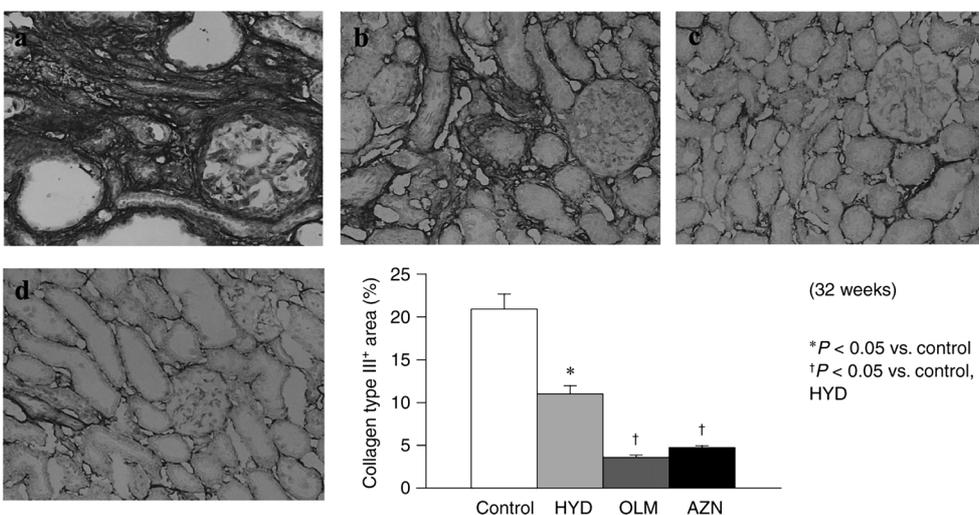


Fig. 7. Collagen Type III Staining

Compared with control (a) and HYD (b)-treated SHRsp, marked reduction of type III collagen expression was observed in OLM- (c) and AZN (d)-treated animals.

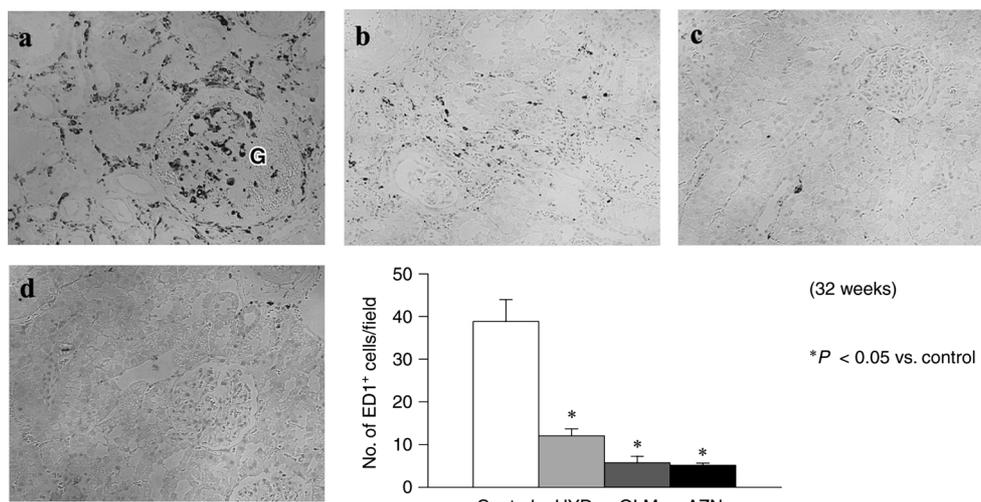


Fig. 8. ED1 Staining

Macrophage infiltration as indicated by ED1 staining was present in glomeruli (G) and tubulointerstitium in control SHRsp rats (a) but markedly diminished in rats treated with HYD (b), OLM (c), and AZN (d).

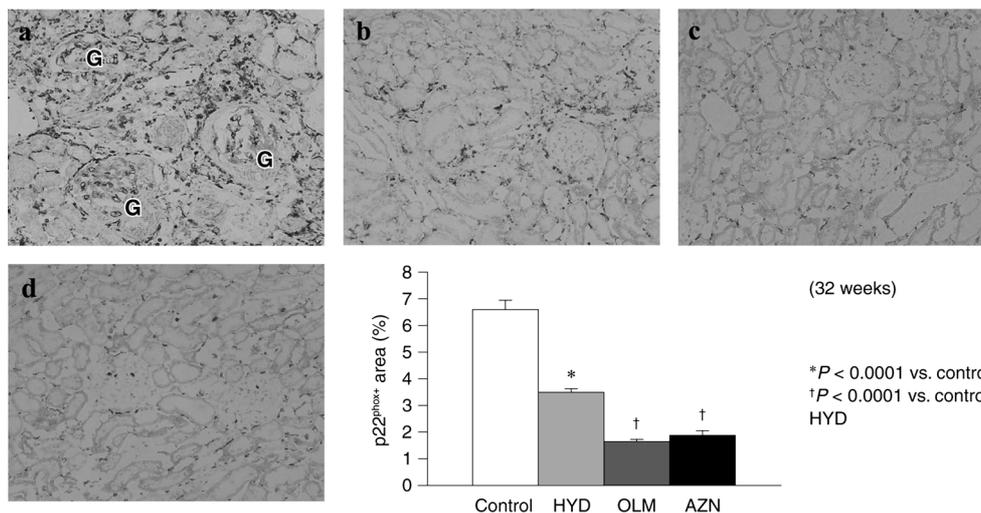


Fig. 9. NADPH Oxidase Isoform p22^{phox} Staining

The p22^{phox} component was markedly expressed in glomeruli (G) and tubulointerstitium in kidneys of SHRsp rats (a). Whereas, treatment with HYD (b), OLM (c), and AZN (d) significantly reduced p22^{phox} staining in these animals. Furthermore, significant reduction of p22^{phox} expression was observed in the OLM and AZN groups vs. HYD group.

creased. Similarly, there was marked p47^{phox} expression in glomeruli (data not shown) accompanied by dilated tubules and hypertrophy of arterial walls in the control group (Fig. 10a) whereas in the OLM (Fig. 10c) and AZN (Fig. 10d) groups p47^{phox} expression was significantly reduced. Moreover, there was less p22^{phox} and p47^{phox} expression in the AZN and OLM groups compared with the HYD group (Figs. 9b, 10b).

In the control group (Fig. 11a) strong expression of 4-HNE was observed in glomeruli and interstitium, whereas this parameter was significantly reduced in the OLM (Fig. 11c) and AZN (Fig. 11d) groups. Moreover, 4-HNE expression was reduced in the AZN- and OLM-treated groups compared with in the HYD group (Fig. 11b).

DISCUSSION

In this study the renoprotective effect of AZN was evalu-

ated in SHRsp for 12 weeks after onset of severe hypertension and compared with that exerted by OLM, which has been widely reported renoprotective.^{14,15)}

SHRsp, bred to develop hypertension soon after birth, rapidly present with hypertensive disorders of various organs.^{16–18)} Regarding the kidney, elevation of plasma renin level begins at age 18 weeks¹⁹⁾ and disturbance of renal function from 22–24 weeks.²⁰⁾

Although many attempts have been made at treating SHRsp with ARBs or CCBs, treatment was often performed before the appearance of hypertensive disorders, and it remains unknown whether these drugs exert renoprotective effects when they administered after the onset of kidney disease.

In our experiment using an SHRsp model of hypertensive nephropathy, AZN and OLM administered at dose levels expected to produce similar degrees of hypotensive activity suppressed urinary protein elimination, macrophage invasion

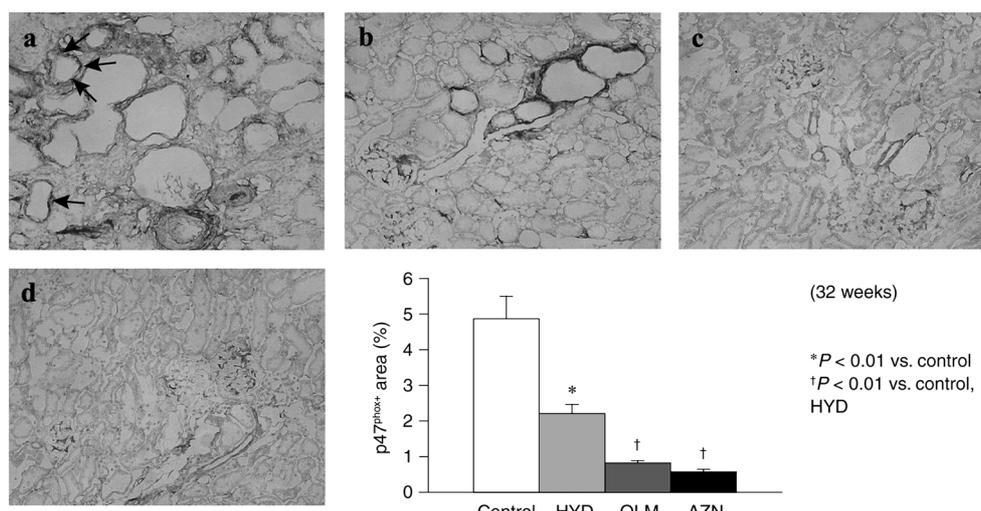


Fig. 10. NADPH Oxidase Isoform p47^{phox} Staining

The p47^{phox} component was markedly expressed in tubular epithelial cells (arrows) in kidneys of SHRsp rats (a). Whereas, treatment with HYD (b) and especially OLM (c) and AZN (d) significantly reduced p47^{phox} staining in these animals.

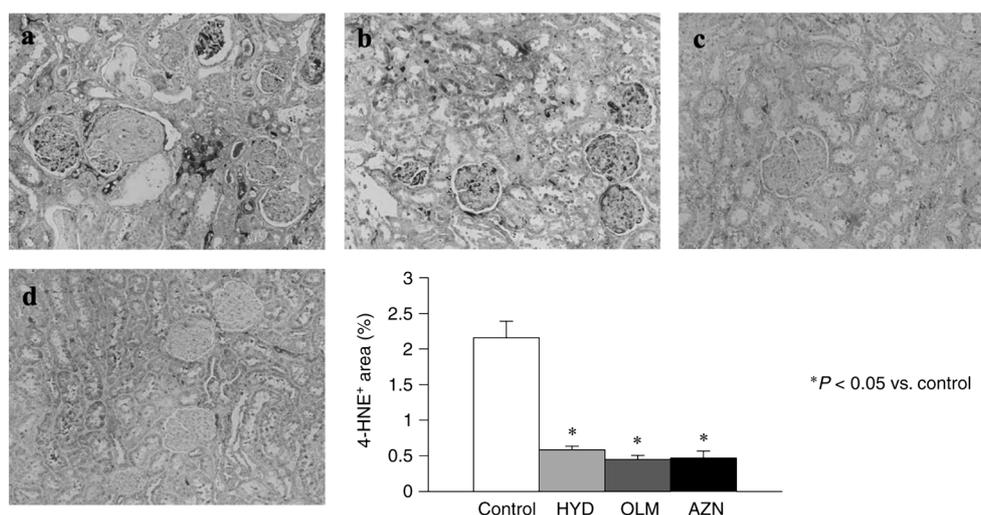


Fig. 11. 4-HNE Staining

Renal deposition of 4-HNE was far more prominent in control SHRsp (a). Whereas, treatment with HYD (b), OLM (c) and AZN (d) reduced 4-HNE staining.

of tissue, and interstitial fibrosis and exerted antioxidative action. In terms of these effects, there was no significant difference detected between the AZN and OLM groups, suggesting that both agents are nephroprotective in SHRsp.

In the present study, HYD was used to evaluate the direct effects of blood pressure reduction on kidney protection. However, although the blood pressure-lowering effects of HYD were similar to those of AZN and OLM, there were significantly less improvement of interstitial fibrosis and antioxidative effects in the former than in the latter two groups. Hence it was suggested that AZN and OLM protect the kidneys by a mechanism other than hypotensive activity alone.

To date, inflammatory reactions in kidneys and interstitial fibrosis of the heart have been reported in SHRsp.^{14,18} Oxidative stress is also commonly observed in tissues of these animals.^{21,22} Reports of studies using other animal models have demonstrated that suppression of oxidative stress results in reductions of inflammation, cellular damage, and subsequent fibrosis.^{23,24}

According to one prevailing view, elevation of intracellular calcium levels stimulated by hydrogen peroxide or hypoxia activates a complex cytotoxic signaling cascade involving calcium-dependent enzymes such as nitric oxide synthase (NOS) and the production of nitric oxide (NO) and superoxide leading to oxidative stress and cellular injury.^{25–27} Other investigators reported that treatment with calcium antagonists alleviates macrophage invasion of renal stroma and kidney fibrosis in rats undergoing unilateral ureteral obstruction (UUO)²⁸ and in SHR.²⁹ It is unknown, however, whether these effects are attributable to the antioxidative activity of calcium antagonists.

On the other hand, there are many reports that in vascular endothelial cells, calcium antagonists suppress inflammatory reactions by reducing intracellular levels of reactive oxygen species (ROS) and inhibit activity of chemoattractant chemokines.^{30–32} Therefore it seems probable that in our animal model AZN suppressed macrophage invasion of rat glomerulus and fibrosis areas.

In the present study, exacerbation of renal dysfunction and tissue damage were suppressed by AZN to a degree similar to that effected by OLM. High renal levels of angiotensin II in SHR are reported involved in the pathogenic mechanism of tubulointerstitial injury.

In summary, the present study in an animal model of hypertensive renal injury suggests that AZN suppresses renal interstitial fibrosis and reduces oxidative stress and thereby exerts renoprotective effects that are independent of its hypotensive action. Because treatment with both ARB and CCB exerted renoprotective effects, it seems probable that combined use of these two drug classes might be especially beneficial in protecting the kidneys in patients with hypertension.

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