Calcium Antagonist Inhibits Glomerular Cell Apoptosis and Injuries of L-NAME Exacerbated Nephrosclerosis in SHR

Shigeko WATANABE, Hidehiko ONO*, Toshihiko ISHIMITSU*, Hiroaki MATSUOKA*, Yuko ONO, and Takahiro FUJIMORI

Increased apoptosis of glomerular cells, with progression of glomerulosclerosis, overactivity of the renin-angiotensin system and elevation of glomerular pressure, follows chronic nitric oxide synthase (NOS) inhibition in spontaneously hypertensive rats (SHR). To gain insight into the regulation of glomerular cell apoptosis in severe nephrosclerosis, we investigated apoptosis, the expression of proliferative cell nuclear antigen (PCNA) in glomeruli, and glomerular morphometric changes in 20-week-old SHR, SHR treated with NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME; 80 mg/l in drinking water), and SHR treated with L-NAME and the calcium antagonist, efonidipine (20 mg/kg per day), for 3 weeks. Apoptosis in non-sclerotic glomeruli was quantified by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling. The increase in systolic blood pressure and the severe proteinuria with severe nephrosclerosis induced by chronic NOS inhibition were completely prevented by efonidipine. Furthermore, the glomerular area and capillary tuft area were markedly increased in rats treated with efonidipine compared with both control rats (+30 and +42%, respectively, p<0.01) and rats treated with L-NAME (+35 and +56%, respectively, p<0.01)-treated rats. This calcium antagonist also significantly inhibited the both increases of the glomerular cell apoptosis index (-72%) and the PCNA index (+44%), therefore the alteration between apoptosis and proliferation slightly increased the number of glomerular cells (subcapsular, +22%, p<0.01; juxtamedullary, +2%, not significant). Thus, the calcium antagonist efonidipine seems to play an important role in the regulation of apoptosis and proliferation of glomerular cells and may be effective in preventing nephrosclerosis exacerbated by NOS inhibition. (Hypertens Res 2000; 23: 683-691)

Key Words: apoptosis, glomerular area, nephrosclerosis, NOS, rats-inbred-SHR, calcium antagonist

Introduction

With widespread use of antihypertensive therapy, the morbidity and mortality related to hypertension in major cardiovascular diseases such as stroke, ischemic heart disease, and malignant hypertension have significantly decreased. However, end-stage renal disease with hypertension continues to increase (1) without adequate explanation.

Increased apoptosis in glomerulosclerosis in uninephrectomized (2) and hypertensive rats (3) has been reported recently. Although the available evidence suggests that apoptosis can be induced in glomerular cells by a...
variety of insults, including an elevation of glomerular pressure, it appears that glomerular cell apoptosis results from an exaggerated local production of angiotensin II (Ang II) (3). This possibility is further supported by the finding in hypertensive cardiac hypertrophy that Ang II induces apoptosis of the myocardium in vitro through a mechanism triggered by the interaction of the peptide with AT1 receptors (4, 5). We have previously reported that, in the hearts of spontaneously hypertensive rats (SHR), prolonged (three-week) nitric oxide synthase (NOS) blockade produced marked apoptosis of coronary arterial smooth muscular cells and epicardial myocardial infarction (6). Furthermore, it has been reported recently that calcium antagonists, as well as AT1 receptor antagonists, induced apoptosis in the early phase after treatment in ventricular myocytes of SHR (7).

Recently, in studies of renal hemodynamics, the long-acting dihydropyridine calcium antagonist, efonidipine, was shown to inhibit glomerular hypertension and to exert a diuretic effect (8-11). These effects were associated with the dilatation of efferent arterioles and the reduction of glomerular hydrostatic pressure like an angiotensin-converting enzyme inhibitor (8). We previously reported the beneficial effects of 2 types of calcium antagonist, L-(long-lasting) type amlodipine and T-(transient) type mibefradil (12) and another calcium antagonist, felodipine (13), on systemic and glomerular hemodynamics in SHR with nephrosclerosis caused by the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME). The present study, therefore, was designed to determine whether the calcium antagonist, efonidipine, could alter the pathophysiologic course of severe nephrosclerosis in 20-week-old SHR caused by L-NAME. Furthermore, the effects of the calcium antagonist on glomerular cell apoptosis were also analyzed in L-NAME rats treated with efonidipine.

Methods

Male 17-week-old SHRs (Charles River Laboratories, Tokyo, Japan) were housed in plastic cages and maintained in a temperature- and light-controlled room. Throughout the study, the rats had free access to standard rat chow. All experiments were approved by the animal care committee of our institution.

The rats were divided into three experimental groups: group 1 (control group, n=13) were administered only tap water for 3 weeks; group 2 (L-NAME group, n=9) were given L-NAME (Sigma Chemical Co., St. Louis, Missouri, USA) in their drinking water (80 mg/l; dose of 14.3±0.7 mg/kg per day for 3 weeks); and group 3 (L-NAME/efonidipine group, n=12) were administered the same dose of L-NAME and the calcium antagonist, efonidipine (20 mg/kg per day by gavage for 3 weeks). The drinking water containing L-NAME was changed daily to ensure a precise dose of L-NAME. During the final week of treatment, all rats were placed in metabolic cages for three days to measure 24-h urinary protein (14). Urinary volume, sodium excretion and uric acid concentrations were measured in all rats as described previously (12, 13, 15). Plasma and urine creatinine levels were measured by an autoanalyzer (Creatinine Analyzer 2; Beckmann Instruments Inc., Fullerton, California, USA). Excretion of urinary nitric oxide of nitrite and nitrate, NO₂+NO₃ (NOₓ), was measured on antibiotics/antimycotics (16).

Histological Studies: Proliferative Cell Nuclear Antigen and Apoptosis Index

Light microscopic examinations were performed after tail-cuff pressure measurements and serum and urinary examinations. The kidneys of each rat were fixed by perfusing with 10% paraformaldehyde, and then organs were removed and weighed. The fixation pressure was the mean arterial pressure of the rats, approximately 230, 190, 150 mmHg for the L-NAME, control, and L-NAME/efonidipine groups, as previously described (6). Using periodic acid-Schiff (PAS) staining, the glomerular injury scores (GIS) of the juxtamedullary and subcapsular layers were assessed on a scale from 0 to 3+: 0 represented no injuries, 1+ was glomerular injury of up to one-third, 2+ was one-third to two-thirds injury, and 3+ was more than two-thirds injured, as previously described (12, 13, 17). The frequency of glomerular lesions was determined by examination of 50 glomerular profiles at two renal depths, each obtained by serial sections. Whole-kidney GIS was obtained by the total scores of the subcapsular GIS and juxtamedullary GIS. The afferent arteriolar injury score (AIS) was also graded from 0 to 3+: 0 was no injury; 1+ was hyalinosis of the arteriolar wall up to 50% of its circumference; 2+ was hyalinosis of the wall between 50% to 100% of its circumference but without luminal narrowing; and 3+ was complete hyalinosis of the arteriolar wall with luminal encroachment. The frequency of arteriolar lesions was determined by examination of each of the 50 arteriolar profiles at all cortical layers.

For each renal specimen, several 5-(m sections were used for in situ determination of apoptotic cells (terminal deoxynucleotidil transferase-mediated dUTP nick-end labeling of fragmented DNA, TUNEL) in the glomerulus, using the in situ Apoptosis Detection Kit (Oncor, Gathersburg, MD). Immunostaining of the glomerular sections was carried out with the streptavidin/biotin immunoperoxidase method (LSAB kit; DAKO) after deparaffinization, as previously described (6). Antiproliferative cell nuclear antigen (anti-PCNA; DAKO) was used to detect nonquiescent cells. For quantification of TUNEL and PCNA immunohistochemistry, at least 30 glomerular sections were examined for each rat and the number of positively stained nuclei per intraglomerular cell number (NGC) were determined. The averages for the rats in...
Table 1. Assessment of Absolute and Relative Renal Mass Index and Blood Pressure

<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n=13)</th>
<th>L-NAME (n=9)</th>
<th>L-NAME/efondipine (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>316±10</td>
<td>266±12**</td>
<td>345±3.8*,##</td>
</tr>
<tr>
<td>Left kidney weight (g)</td>
<td>1.25±0.07</td>
<td>1.05±0.03*</td>
<td>1.42±0.04##</td>
</tr>
<tr>
<td>Left kidney/body weight ratio (mg/g)</td>
<td>4.02±0.31</td>
<td>4.01±0.20</td>
<td>4.10±0.10</td>
</tr>
<tr>
<td>Right kidney weight (g)</td>
<td>1.25±0.07</td>
<td>1.05±0.02*</td>
<td>1.45±0.02##</td>
</tr>
<tr>
<td>Right kidney/body weight ratio (mg/g)</td>
<td>4.17±0.02</td>
<td>4.04±0.23</td>
<td>4.21±0.06</td>
</tr>
<tr>
<td>Tail-cuff pressure (mmHg)</td>
<td>226±5</td>
<td>273±10**</td>
<td>175±12**##</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>461±7</td>
<td>432±15</td>
<td>471±8##</td>
</tr>
</tbody>
</table>

Data are mean±SEM, p value of ANOVA. Control, 20-week-old SHR; L-NAME, SHR treated with L-NAME (80 mg/l in drinking water); L-NAME/efondipine, efondipine (20 mg/kg/day) and L-NAME (80 mg/l in drinking water) treated with SHR. ** p<0.01, * p<0.05 vs. control group. ## p<0.01, # p<0.05 vs. L-NAME group.

Table 2. Renal Functional Parameters and Urinary NOx Excretion

<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n=13)</th>
<th>L-NAME (n=9)</th>
<th>L-NAME/efondipine (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.36±0.01</td>
<td>0.48±0.08*</td>
<td>0.30±0.02##</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min/kg body weight)</td>
<td>7.14±0.47</td>
<td>4.10±0.30**</td>
<td>10.1±0.2**##</td>
</tr>
<tr>
<td>Plasma protein (g/dl)</td>
<td>5.5±0.1</td>
<td>5.9±0.1</td>
<td>5.8±0.1</td>
</tr>
<tr>
<td>Urinary protein excretion (mg/day)</td>
<td>20±6</td>
<td>66±15**</td>
<td>24±2##</td>
</tr>
<tr>
<td>Urinary nitrate + nitrite excretion (μmol/day)</td>
<td>5.97±2.58</td>
<td>0.62±0.30*</td>
<td>0.19±0.01*</td>
</tr>
</tbody>
</table>

Data are mean±SEM, p value of ANOVA. Control, 20-week-old SHR; L-NAME, SHR treated with L-NAME (80 mg/l in drinking water); L-NAME/efondipine, efondipine (20 mg/kg/day) and L-NAME (80 mg/l in drinking water) treated with SHR. ** p<0.01, * p<0.05 vs. control group. ## p<0.01, # p<0.05 vs. L-NAME group.

Fig. 1. Effects of efondipine on blood pressure, urinary protein excretion, glomerular injury score (GIS), number of glomerular cells (NGC), apoptosis index, and PCNA index in L-NAME-treated spontaneously hypertensive rats (SHR). Error bars indicate SEM. * p<0.05 vs. control. + p<0.05 vs. L-NAME.
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Each of the three groups were considered as TUNEL and PCNA labeling indices, respectively. Morphometrical Analysis: AG, AT, Wall Thickness Ratio.

All microscopic slides were examined using a computer analyzer system (Image Quest; Hamamatsu Photonics, Hamamatsu and MacScope, Mitani Co., Fukui, Japan), and calculations were made using stereologic principles (3). The glomerular areas (AG) of the subcapsular and juxtamedullary glomeruli were measured by tracing the outlines of those glomerular capillaries having a vascular pole, and the glomerular capillary tuft area (AT) was measured by tracing the luminal area of all capillaries falling within one high power field (×400), as previously described. The NGC was counted as the total number of three kinds of intraglomerular cells; endothelial, mesangial, and epithelial cells in the AG. In addition, the wall thickness was assessed as the ratio of media thickness to outer radius of afferent arterioles and interlobular arteries, according to our previous report. Inner and outer circumferences were measured by above computer analyzer system (ImageQuest). Values were corrected to the radius of each vessels, which were assumed to be circular, by the following calculation: Wall thickness ratio=(R-r)/R, where R or r is the radius of the outer or inner circumference, and R-r represents medial thickening (3).

One-way ANOVA, followed by Duncan’s multiple range test (18), was performed to test for between-group significance. All data are expressed as the means±SEM. A probability level of <5% was considered to indicate statistical significance.

Results

The ratios of left and right kidney weights to body weight were not significantly different among the three groups, although there was a significant increase of body weight in L-NAME/efonidipine rats (group 3). Absolute kidney weights were reduced significantly by L-NAME (group 2) and were increased significantly by efonidipine treatment. Tail-cuff pressure was elevated in rats receiving prolonged L-NAME, but L-NAME/efonidipine markedly reduced tail-cuff pressure (p<0.01), compared with pressures for control and L-NAME rats (Table 1, Fig. 1).

Serum creatinine and urinary protein excretion were significantly decreased by efonidipine (group 3). The reduction of 24-h-creatinine clearance by L-NAME (group 2) and were increased significantly by efonidipine treatment. Tail-cuff pressure was elevated in rats receiving prolonged L-NAME, but L-NAME/efonidipine markedly reduced tail-cuff pressure (p<0.01), compared with pressures for control and L-NAME rats (Table 1, Fig. 1).

Renal Morphological Findings

Renal histological examination of the L-NAME rats revealed severe nephrosclerosis and tubulo-interstitial

![Fig. 2. Light micrographs. 20-wk-old SHR (controls, top) with minor abnormalities of glomeruli and tubulo-interstitium. SHR treated with NOS blockade (L-NAME, middle) with histological feature such as malignant nephrosclerosis. Left glomerulus showing hypertrophic global sclerosis and two glomeruli of right with global collapse of glomerular capillary lumen. Severe irregular fibrosis and dilated tubules including periodic acid-Schiff stain positive protein cast. Calcium antagonist, efonidipine, treated with L-NAME/SHR (L-NAME/efonidipine, lower). Glomeruli showing an increase of glomerular volume compared with control (top) and L-NAME (middle). The glomerulus is normal in cellularity and slightly an increase of mesangial matrix. The glomerular capillary lumens are patent, and almostly normal in tubulo-interstitium. (Periodic acid-Schiff stain, original magnifications ×60)
changes, as previously reported (12, 13, 17). In fact, the GIS was increased more in the L-NAME group (group 2) than in the control group (group 1). However, the efonidipine treatment prevented the severe glomerulosclerosis and ischemic changes caused by prolonged NOS inhibi-

Fig. 3. Light micrographs. Glomerular capillary tufts in control rats (top) are patent, and these areas were normal size in minor abnormalities of glomeruli. In L-NAME rats (middle), the tufts area was decreased with hypertensive glomerular collapse. Efonidipine treated L-NAME rats (lower) showed marked dilatation of glomerular tufts compared with those of control and L-NAME rats (Elastica-Masson; magnification ×330).

Fig. 4. TUNEL staining micrographs. A: In control SHR, the TUNEL-positive cells (apoptosis) showing a few of glomerular endothelial cells (arrow) and tubular epithelial cells (arrow). B: Intraglomerular apoptosis in L-NAME SHR were induced in endothelial, mesangial, and epithelial cells of minor abnormalities of glomeruli. C: Apoptosis of sclerotic glomeruli was expressed in glomerular epithelial cells (arrow) with collapse of L-NAME treated SHR. D: Focal tubular epithelial with nuclear irregularities and protein casts were detected as tubular cell apoptosis in L-NAME SHR. E: In L-NAME SHR, focal endothelial cells was detected as apoptosis.
The renal histological appearance of the L-NAME rats revealed severe nephrosclerosis with global sclerosis and glomerular ischemic changes (Fig. 2). Tubular dilatation and cast formation, and irregular fibrosis and infiltration by inflammatory cells, with a marked increase in medial thickness, were observed. Efonidipine treatment (lower photo in Fig. 2) prevented glomerular sclerosis and collapse in L-NAME rats, and the glomerular area of L-NAME/efonidipine rats were larger than those of control and L-NAME rats (Fig. 1, upper and middle photos in Fig. 2).

Morphometrically, the mean glomerular area was not significantly different between control and L-NAME rats, although the juxtamedullary glomerular area of L-NAME rats was significantly reduced concomitantly with the severe GIS elevation. The efonidipine-treated glomerular area (group 3) was significantly larger than the control and L-NAME glomerular areas (groups 2 and 3). On the other hand, the glomerular capillary tuft area was also reduced by L-NAME (group 2) in subcapsular and juxtamedullary glomeruli. Efonidipine further increased the glomerular capillary tuft area by more than 120% of that of L-NAME rats (Fig. 3), and this was associated with a significantly reduced GIS. The increased glomerular and glomerular tuft growth promoted by efonidipine was supposedly reflected in an increased kidney weight.

### Glomerular Cell Number (NGC) and Apoptosis, PCNA Index (Table 3, Fig. 1 and 4)

The number of intraglomerular cells (NGC) was decreased by chronic NOS inhibition (group 2), although efonidipine treatment inhibited the reduction of NGC by L-NAME. In the subcapsular cortex, the calcium antagonist efonidipine rather increased NGC by approximately 10% compared with control rats. Nevertheless, there were no significant differences in NGC between L-NAME- and efonidipine-treated rats.

Efonidipine treatment inhibited the increase of the apoptosis index by approximately 72%, and increased approximately 30% of PCNA index by chronic L-NAME administration. Thus, the PCNA/apoptosis ratio was higher in the L-NAME/efonidipine rats than in the L-NAME rats (Table 3, Figs. 1 and 4). PCNA-positive cells in the glomeruli of Group 1 corresponded to epithelial and endothelial cells. Efonidipine treatment increased the expression of PCNA in the glomerulus with glomerular enlargement, but efonidipine downregulated the induced apoptosis in mesangial and epithelial cells of sclerotic glomeruli associated with the preventing glomerular scler-
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Wall-to-Lumen Ratio of Afferent and Interlobular Arteries

The wall-to-lumen ratios of afferent arterioles and interlobular arteries were significantly increased in L-NAME (group 2) compared with control rats (p<0.01, Table 4). These elevations of wall-to-lumen ratio were associated with a decrease of inner lumen diameter. However, these increased wall lumen ratios were significantly inhibited by efonidipine treatment.

Discussion

The results of the present study show that efonidipine improves renal function and severe hypertensive nephrosclerosis as well as reducing urinary protein excretion. This renoprotective effect of efonidipine was associated with enlargement of glomeruli and glomerular capillary tufts, and furthermore inhibited the induction of glomerular apoptosis and activated the glomerular cell proliferative index.

These findings are consistent with our previous findings on the renoprotective effects of felodipine and amloidipine, two different types of L-type calcium antagonist, and mibebradil, a T-type calcium-channel receptor antagonist. In those studies, these calcium antagonists not only prevented but also reversed L-NAME-exacerbated nephrosclerosis in SHR (12, 13). Therefore, the enlargement of the glomeruli and capillary tufts in efonidipine-treated rats might be caused by increased single-nephron plasma flow and reduction of afferent arteriolar resistance. Yaoita et al. reported, using isolated glomeruli of New Zealand white rabbits in a microperfusion technique, that efonidipine not only dilated afferent arterioles but also significantly dilated glomerular efferent arterioles, but nifedipine and nicardipine had no effects on efferent arterioles (8). This efferent arteriolar vasodilation with a decrease of efferent arteriolar resistance, and further, the reduction of glomerular pressure by efonidipine, might lead to the prevention of hypertensive glomerular injuries by L-NAME. Then, in this similar hypertensive model, the calcium antagonist, like an endothelin-1 receptor antagonist (19) efonidipine, might prevent severe nephrosclerosis caused by prolonged NOS inhibition with these renal hemodynamic changes, such as an increase of single-nephron plasma flow and the reduction of arteriolar resistance.

Because the regulation of apoptosis has been found to be altered in glomeruli with chronic NOS blockade in SHR (3), we investigated the regulation of glomerular cell susceptibility to apoptosis in treatment with a calcium antagonist. Several investigators, including ourselves, have proposed the following mechanisms for the regulation of glomerular cell apoptosis: first, the induction of apoptosis by activation of the renin-angiotensin system (4, 20); second, the inhibition of apoptosis by NOS stimulation (21, 22); and third, shear stress and elevation of glomerular pressure (3). Fortuno et al. reported that the interaction of angiotensin II with its AT1 receptor may induce not only left ventricular growth and fibrosis but also programmed myocardial cell death in animals and humans with arterial hypertension (5). Furthermore, Diep et al. reported that the apoptosis in the media of blood...
vessels was enhanced by both AT1 and AT2 receptors \((20)\). We observed that enhanced glomerular cell apoptosis in L-NAME SHR is normalized by treatment with the calcium antagonist, efonidipine. One possible explanation is that the reduction of glomerular hydrostatic pressure inhibits glomerular cell apoptosis. Unfortunately, we did not observe the glomerular hydrostatic pressure in this study. Our other studies, using the L-type calcium antagonists, felodipine and amlodipine \((12, 13)\), indicated that these calcium antagonists reduced glomerular pressure with a fall of blood pressure when they were compared with the pressures in the L-NAME-treated rats. As regards the induction of apoptosis by the upregulation of the renin-angiotensin system or NOS inhibition, efonidipine did not alter the plasma angiotensin II concentration (data did not shown) or urinary \(\text{NO}_x\) excretion. These data suggested that inhibition of apoptosis by efonidipine occurred without the effects of the renin-angiotensin system or NOS inhibition, but that it might play an important role at least in the reduction of glomerular hydrostatic pressure for regulation of apoptosis of glomerular cells. Recently, in rat renal vasculature, Mattson \textit{et al.} reported that vasa recta, glomeruli, and afferent arterioles contained large amounts of calcium-dependent NOS enzyme activity \((23)\). Taken together, chronic NOS inhibition in this study produced the induction of glomerular-arteriolar apoptosis, and therefore the calcium antagonist might be inhibit glomerular cell apoptosis. Moreover, the intraglomerular cell number changes caused by efonidipine treatment were slightly increased with, independently, the inhibition of apoptosis and the activation of PCNA expression.

The L-type \(\text{Ca}^{2+}\) antagonist efonidipine prevented the induction of glomerular cell apoptosis by chronic NOS inhibition in SHR. Increases in the concentration of free cytoplasmic \(\text{Ca}^{2+}\) \([\text{Ca}^{2+}]_{i}\) have been linked to cell death (including apoptosis) in a number of experimental systems using animals and cultured cells \((24-27)\). Intracellular DNA cleavage by a \(\text{Ca}^{2+}\)-and \(\text{Mg}^{2+}\)-dependent endonuclease, activated by intranuclear \(\text{Ca}^{2+}\) uptake, is a apoptosis marker. Junnti-Berggren reported that the L-type \(\text{Ca}^{2+}\) antagonist, verapamil, prohibit \(\text{Ca}^{2+}\) from entering the cell, DNA fragmentation (apoptosis) was prevented in the RINm5F cells (insulin-producing cell) and normal cell \textit{in vitro} \((28)\). Thus, as one possible explanation, efonidipine in our study might have blocked \(\text{Ca}^{2+}\) influx and inhibited induction of apoptosis in the glomerular cells.

In conclusion, the balance between an increase of the intraglomerular cell number and the induction of apoptosis may play an important role in the development of hypertensive glomerular damage. This balance may be affected \textit{in vivo} by glomerular hemodynamics, intracellular \(\text{Ca}^{2+}\) concentration and \(\text{NOx}\) production, as well as by angiotensin II levels. Ischemic changes in the glomerular lus in L-NAME SHR result in an increase of systolic blood pressure, reduction of the glomerular or tuft area and decrease of the intraglomerular cell number, and are associated with glomerular cell apoptosis, which may modulate the degree of glomerular growth. On the other hand, calcium antagonists produced beneficial changes, with glomerular enlargement and inhibition of apoptosis, and with a decrease in glomerular injuries and urinary protein excretion. Thus, the present results extend our knowledge on the essential role of calcium antagonists in blood pressure control, the histology of nephrosclerosis, and apoptosis, as shown by decreases in blood pressure, glomerular growth, the inhibition of DNA fragmentation, and increases proliferative cell nuclear antigen expression.

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**References**


