Mineralocorticoid receptor blockade and calcium channel blockade have different renoprotective effects on glomerular and interstitial injury in rats

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Eplerenone, a selective mineralocorticoid receptor (MR) antagonist, has been used as an antihypertensive drug. We and others have demonstrated that aldosterone induces cell proliferation and deformability in rat mesangial cells (21) and increases oxidative stress in murine podocytes (24); these changes were suppressed by eplerenone. Interestingly, eplerenone suppressed the development of glomerular injury in salt-fed Dahl salt-sensitive (DS) rats due to its blood pressure-lowering effect (32). However, several animal studies reported that amlodipine had no effect on the development of glomerular sclerotic changes in experimental hypertensive model rats (5, 35). Conversely, calcium channel blockade was shown to be effective in the prevention of tubulointerstitial damage (30, 35). Thus the targets of CCBs should be further explored in view of renoprotection; however, this approach to CCBs has yet to be widely investigated.

Chronic hypoxia in the tubulointerstitium is proposed as a major pathway to end-stage renal failure (6, 19, 33). Even if total renal blood flow is well regulated, peritubular capillary loss induces spot hypoxia in the kidney and the degree of the loss is strongly correlated with the progression of tubulointerstitial injury (19, 20, 22). Indeed, reduced perfusion of peritubular capillaries and interstitial hypoxia were observed before tubulointerstitial damage (30, 35). Thus the targets of CCBs remain unclear.

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HYPERTENSION IS THE PRIMARY risk factor for the progression of renal disease and one of the major causes of chronic kidney disease (1, 9). Although there are effective drugs available for hypertension, preventing the development and progression of concomitant diminished renal function remains paramount.

Eplerenone, a selective mineralocorticoid receptor (MR) antagonist, has been used as an antihypertensive drug. We and others have demonstrated that aldosterone induces cell proliferation and deformability in rat mesangial cells (21) and increases oxidative stress in murine podocytes (24); these changes were suppressed by eplerenone. Interestingly, eplerenone suppressed the development of glomerular injury in salt-fed Dahl salt-sensitive (DS) rats despite low plasma aldosterone levels (17). Moreover, clinical studies reported that treatment with eplerenone reduced albuminuria to a greater extent than treatment with an angiotensin-converting enzyme inhibitor, enalapril, with a similar degree of hypotensive effect in hypertensive patients (34). These studies demonstrated that eplerenone is effective in protecting the kidney, particularly the glomeruli.

Calcium channel blockers (CCBs) are widely used in the treatment of hypertension. However, the renoprotective effect of CCBs remains controversial. In a recent clinical study, amlodipine, a dihydropyridine CCB, attenuated the proteinuria in hypertensive patients, although this effect may have been due to its blood pressure-lowering effect (32). However, several animal studies reported that amlodipine had no effect on the development of glomerular sclerotic changes in experimental hypertensive model rats (5, 35). Conversely, calcium channel blockade was shown to be effective in the prevention of tubulointerstitial damage (30, 35). Thus the targets of CCBs should be further explored in view of renoprotection; however, this approach to CCBs has yet to be widely investigated.

Chronic hypoxia in the tubulointerstitium is proposed as a major pathway to end-stage renal failure (6, 19, 33). Even if total renal blood flow is well regulated, peritubular capillary loss induces spot hypoxia in the kidney and the degree of the loss is strongly correlated with the progression of tubulointerstitial injury (19, 20, 22). Indeed, reduced perfusion of peritubular capillaries and interstitial hypoxia were observed before the development of structural tubulointerstitial damage in remnant kidney hypertension model rats (15). We previously reported that a dihydropyridine CCB, azelnidipine, attenuated the reduction in peritubular capillary flow induced by angiotensin II (14), suggesting that calcium channel blockade is useful in preventing peritubular ischemia.

In the present study, we hypothesized that additional treatment with MR antagonists with CCBs elicits better renoprotective effects than monotherapy with either drug, with effects elicited via different mechanisms, including protection of glomeruli by eplerenone and protection of tubulointerstitium by amlodipine. We also investigated whether their renoprotective effects are involved in the improvement of renal hypoxia.

MATERIALS AND METHODS

Animal preparation. All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University. Male DS rats aged 6 wk (Seac Yoshitomi, Fukuoka, Japan), weighing 205–220 g at the beginning of the experiments, were randomly selected to receive rat chow containing high salt (4% NaCl; n = 36; Oriental Yeast, Osaka, Japan) or low salt (0.3% NaCl; n = 9; Oriental Yeast). After 2 wk, high salt-fed (HS)
rats were randomly divided into four groups as follows (9 rats in each group): vehicle (0.5% methylcellulose; Nacalai Tesque, Kyoto, Japan); amloidipine (3 mg·kg⁻¹·day⁻¹ po); eplerenone (50 mg·kg⁻¹·day⁻¹ po); and combination treatment of amloidipine (3 mg·kg⁻¹·day⁻¹) with eplerenone (mg·kg⁻¹·day⁻¹). The treatment doses were based on previous studies (8, 23).

Systolic blood pressure (SBP) was measured five consecutive times in the conscious state by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) after the rats had rested for at least 15 min; the mean value of the middle three readings was recorded. Urine samples were collected in metabolic cages for 24 h before initiation of high salt feeding and at weeks 2, 4, 6, 8, and 10 during the treatment period. All animals were given a 24-h acclimatization period before urine collection. Blood and kidney samples were collected at the end of week 10 under pentobarbital sodium anesthesia (50 mg/kg ip). Kidneys were perfused with chilled saline solution, and then kidney sections were either fixed in 10% paraformaldehyde (pH 7.4) and embedded in paraffin for histological examination or frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) for immunohistochemistry and laser capture microdissection. Remaining renal tissues were snap-frozen in liquid nitrogen and stored at −80°C for PCR analysis.

**Histological examination.** The excised kidneys were fixed with 10% formalin (pH 7.4), embedded in paraffin, sectioned into 4-μm slices, and stained with periodic acid-Schiff (PAS) or Azan reagent. Kidneys were perfused with chilled saline solution, and then kidney sections were either fixed in 10% paraformaldehyde (pH 7.4) and embedded in paraffin for histological examination or frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) for immunohistochemistry and laser capture microdissection. Remaining renal tissues were snap-frozen in liquid nitrogen and stored at −80°C for PCR analysis.

**Immunohistochemistry.** Podocyte injury was examined by immunohistochemistry of desmin (anti-Human Desmin Mouse monoclonal antibody, D33, DAKO Cytomation, Glostrup, Denmark). Peritubular capillary density was evaluated by using an anti-rat endothelial aminopeptidase P monoclonal antibody (Bender Med System, Burlingame, CA) (11). Hypoxia was examined by pimonidazole (HyposyProbe, Pharmacia International, Belmont, MA), which binds to tissues with Po2 levels below 10 mmHg (7). In a separate groups of rats (n = 3 for each group), pimonidazole was intravenously injected (60 mg/kg iv) 1 h before the kidneys were perfusion-fixed. An FITC-conjugated mouse anti-pimonidazole monoclonal antibody (1: 2,000, Hyposyprobe) and horseradish peroxidase-conjugated mouse anti-FITC monoclonal antibody (1:500, Nordic Immunology, Tubigur, The Netherlands) were used as primary and secondary antibodies for pimonidazole, respectively. Deperfused sections were acclimated for antigen retrieval and incubated with 0.3% hydrogen peroxide in methanol for 5 min to block endogenous enzymes. After blocking, sections were incubated with primary antibodies overnight at 4°C. Antibodies were visualized by DAB substrate. Counterstaining was performed with hematoxylin. Antibody-positive areas were calculated from 20 randomly selected microscope fields (×200) in each section. To analyze aminopeptidase immunostaining, we excluded glomeruli from the field because glomeruli have strong immunoreactivity for endothelial capillary density. The above histological analysis was performed using a color image-analyzing system (WinRooF software; Mitani, Tokyo, Japan) in a blind manner to avoid bias (12).

**Western blotting.** We microdissected 100 glomeruli by using the laser capture method to analyze the glomerular MR protein expression by Western blotting analysis (31). The 7.5% SDS-polyacrylamide gels were transferred to nitrocellulose membrane (GE Healthcare Life Science, Buckinghamshire, UK) and incubated with an anti-MR (MR 1–18 ID5) antibody (1:200; provided by Prof. Gomez-Sanchez, University of Mississippi) and then with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the bands were detected by chemiluminescence using the ECL plus Western blotting detection system (GE Healthcare Life Science) following the manufacturer’s instructions.

**RESULTS**

Systolic blood pressure, urinary protein/creatinine, plasma creatinine, and creatinine clearance. Vehicle-treated HS rats developed hypertension, whereas LS rats exhibited normal SBP (Fig. 1). Treatment with eplerenone significantly suppressed high salt-induced blood pressure elevation, although the SBP value was significantly higher compared with LS rats at week 10. Amlodipine treatment or combination treatment normalized SBP to levels seen in LS rats.

As shown in Fig. 2, vehicle-treated HS rats showed a marked elevation in urinary protein excretion compared with LS rats at week 10. Treatment with eplerenone or amloidipine significantly reduced the development of urinary protein excretion compared with vehicle treatment. Combination treatment further reduced the high salt-induced proteinuria in DS rats (P < 0.05 vs. amlodipine or eplerenone).

Plasma creatinine levels were elevated, and creatinine clearance was decreased in vehicle-treated HS rats compared with LS rats. Treatment with eplerenone or amloidipine tended to improve these changes but not significantly so. Combination treatment significantly suppressed both the elevation of plasma...
creatinine levels and the decrease in creatinine clearance by high salt feeding (Table 1).

**Histological findings.** Glomerulosclerosis and hypertrophy were analyzed by PAS staining (Fig. 3A). Tubulointerstitial fibrosis was analyzed by Azan staining (Fig. 3B). The percentages of the positive staining areas are shown in Table 2.

Vehicle-treated HS rats exhibited injured glomeruli characterized by sclerosis (Fig. 3Ab), enlarged glomerular size (13,377 ± 154 μm²), and severe tubulointerstitial fibrosis (Fig. 3Bb) compared with the LS group (Fig. 3Aa and Bb, and Table 2; glomerular size 8,373 ± 142 μm²). Treatment with eplerenone significantly suppressed glomerulosclerosis (Fig. 3Ac) and hypertrophy (10,130 ± 190 μm²) and partially suppressed the development of fibrosis (Fig. 3Bc). Conversely, treatment with amlodipine failed to suppress glomerulosclerosis (Fig. 3Ad) and glomerular hypertrophy (12,596 ± 140 μm²) but markedly improved tubulointerstitial fibrosis (Fig. 3Bd). The combination treatment dramatically reduced both glomerular injury (Fig. 3 Ae, 9,553 ± 219 μm²) and tubulointerstitial fibrosis (Fig. 3Be).

**Podocyte injury.** We estimated podocyte injury by analyzing immunohistochemistry of desmin (Fig. 3C) and mRNA expression of podocin and nephrin, two major proteins that are necessary to maintain podocyte function (Fig. 4). In vehicle-treated HS rats, the immunopositive area for desmin was increased (Fig. 3Cb) and glomerular expression levels of podocin and nephrin were substantially decreased compared with those in LS rats (Fig. 3Ca). Eplerenone monotherapy or combination treatment reversed the increased desmin staining (Fig. 3Cc and Ce) and decreased expression of podocin and nephrin by high salt feeding. Amlodipine monotherapy had no effect on desmin staining (Fig. 3Cd) or glomerular podocin and nephrin mRNA levels.

**Glomerular MR and Sgk-1 gene expression.** As shown in Table 1, plasma aldosterone levels were decreased by high salt feeding (Fig. 5). Although none of the treatment regimens suppressed the increase in glomerular MR mRNA expression, eplerenone monotherapy and combination treatment inhibited the increased transcription of Sgk-1 in high salt-fed DS rats, while amlodipine monotherapy had no effect.

Unfortunately, the band we could detect in the laser-captured glomeruli by using Western blot analysis was not of enough quality, probably because of the small amount of protein we could technically collect from glomeruli dissected by the laser-capture method.

**Renal interstitial hypoxia.** Hypoxia is a potential mechanism for the development of renal interstitial fibrosis in the hypertensive kidney (19). The pimonidazole (a hypoxic marker)-positive area in the renal cortex was markedly increased in vehicle-treated HS rats compared with LS rats (Fig. 6, Aa and Ab). Treatment with eplerenone had no effect on the increased
pimonidazole staining (Fig. 6Ac), while amlodipine attenuated the increased staining (Fig. 6Ad). The combination treatment significantly ameliorated the increased pimonidazole-positive area in the renal cortex (Fig. 6 Ae).

To further confirm the hypoxia in the renal cortex, we analyzed the mRNA expression levels of VEGF as a hypoxia-responsive gene (Fig. 7). Consistent with pimonidazole staining, vehicle-treated HS rats showed an increased expression of VEGF that was suppressed by amlodipine monotherapy or the combination treatment but not by eplerenone alone.

It is proposed that peritubular capillary loss is one of the causes of renal hypoxia (3, 10). As shown in Fig. 6B, the area positive for anti-endothelial aminopeptidase P antibody was smaller in the renal cortex of vehicle-treated HS rats than that in LS rats (Fig. 6, Ba and Bb). The decreased aminopeptidase P immunoreactivity was partially restored by amlodipine (Fig. 6Bd) or the combination treatment (Fig. 6Be), whereas eplerenone treatment had no effect on the loss of immunoreactivity by high salt feeding (Fig. 6Bc).

DISCUSSION

In the present study, we demonstrated that a combination therapy of eplerenone with amlodipine elicited additive effects in preventing the development of renal injury in genetically salt-sensitive hypertensive rats. Eplerenone, in particular, showed a strong protective effect on the glomeruli rather than the tubulointerstitium. Conversely, amlodipine substantially protected against tubulointerstitial injury. The distinct targets for renoprotection between eplerenone and amlodipine suggest that their renoprotective effects are additive when combined in hypertension therapy.

Recent studies suggest that the dysregulated aldosterone/MR signaling causes deleterious effects in the glomeruli. Aldosterone/MR signaling activation induced cell proliferation in rat cultured glomerular mesangial cells (21, 28). Aldosterone/MR induces the proliferation of mesangial cells through the up-regulation and the increased activity of an MR transcript, Sgk-1 (29), which is a critical phenomenon during the development of glomerulosclerosis. In the present study, high salt feeding significantly reduced plasma aldosterone levels, whereas MR and Sgk-1 mRNA levels were increased in the glomeruli of HS rats. Thus plasma ligand levels may not play important roles in stimulating MR/Sgk-1 signaling. Rather, upregulation of receptor levels would be responsible for the augmentation of this signaling in the glomeruli of HS rats, although the mechanism by which salt feeding induced MR mRNA upregulation is unclear in the present study. Importantly, the inhibition of glomerular MR/Sgk-1 signaling by eplerenone was accompanied by an improvement in salt-dependent hypertension-induced glomerulosclerosis. Both MR and Sgk-1 are also expressed in podocytes and have been implicated in the development of proteinuria (24, 25). Consistent with earlier studies

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Table 1. Plasma creatinine, creatinine clearance, and plasma aldosterone at week 10

<table>
<thead>
<tr>
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<th>LS + Vehicle (n = 9)</th>
<th>HS + Vehicle (n = 9)</th>
<th>HS + Eplerenone (n = 9)</th>
<th>HS + Amlodipine (n = 9)</th>
<th>HS + Combination (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.47±0.01</td>
<td>0.63±0.02*</td>
<td>0.55±0.01</td>
<td>0.57±0.01</td>
<td>0.52±0.02†</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>2.5±0.1</td>
<td>2.0±0.1*</td>
<td>2.2±0.1</td>
<td>2.3±0.1</td>
<td>2.5±0.2†</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/ml</td>
<td>855±48</td>
<td>294±20*</td>
<td>290±15*</td>
<td>268±15*</td>
<td>334±37*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of rats. LS, low salt; HS, high salt. *P < 0.05 vs. LS + vehicle. †P < 0.05 vs. HS + vehicle.
our present results show that the protective effects of eplerenone on podocyte injury and proteinuria are accompanied by the inhibition of glomerular Sgk-1 transcription, suggesting that MR/Sgk-1 signaling participates in the podocyte injury in DS rats. We found that high salt feeding in DS rats enhanced the pimonidazole staining and VEGF expression in renal interstitium, indicating the presence of hypoxia in the kidney of hypertensive DS rats. There is a growing body of evidence that chronic hypoxia in the tubulointerstitium is a major pathway to end-stage renal failure (6, 19). A number of mechanisms that could induce hypoxia have been suggested, such as a reduction of peritubular capillary flow (3, 10, 15, 22, 36) and an increase in tubular metabolic demand for oxygen (27). We previously reported that an L-type CCB, azelnidipine, attenuated the reduction of peritubular capillary blood flow in response to angiotensin II, suggesting that calcium channel blockade alleviates renal hypoxia via maintenance of the peritubular capillary blood supply (14). Tanaka et al. (27) showed that calcium flux via L-type calcium channels mediated the ATP depletion-induced apoptosis in cultured proximal tubular cells and ischemia-reperfusion-induced apoptosis in the rat kidney, indicating that L-type calcium channels on tubular cells play an important role in hypoxia-induced tubular damage. These findings concur with our current results showing that the inhibition of calcium channels by amlodipine improved oxygen conditions, restored the loss of peritubular capillaries, and prevented the tubulointerstitial fibrosis in the kidney of high salt-fed DS rats. Thus treatment with CCBs may be effective in preventing the hypoxia and subsequent tubulointerstitial injury during the development of salt-sensitive hypertension.

Table 2. Glomerular PAS-positive area, Azan-positive area, and anti-desmin antibody-positive area at week 10

<table>
<thead>
<tr>
<th></th>
<th>LS+Vehicle (n = 9)</th>
<th>HS+Vehicle (n = 9)</th>
<th>HS+Eplerenone (n = 9)</th>
<th>HS+Amlodipine (n = 9)</th>
<th>HS+Combination (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS, %</td>
<td>5.4±0.5</td>
<td>33.9±1.5*</td>
<td>15.9±0.5*</td>
<td>29.9±1.2†</td>
<td>10.1±0.3+§</td>
</tr>
<tr>
<td>Azan, %</td>
<td>1.03±0.08</td>
<td>9.50±0.63*</td>
<td>6.99±0.45†</td>
<td>1.73±0.18†</td>
<td>1.20±0.09‡</td>
</tr>
<tr>
<td>Desmin, %</td>
<td>0.50±0.04</td>
<td>2.98±0.29*</td>
<td>0.66±0.14*†</td>
<td>3.06±0.11†</td>
<td>0.35±0.08§</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = no. of rats. PAS, periodic acid-Schiff. *P < 0.05 vs. LS+vehicle. †P < 0.05 vs. HS+vehicle. ‡P < 0.05 vs. HS+aletterone. §P < 0.05 vs. HS+amlodipine.
Recently, Dietz et al. (4) have reported that dihydropyridine CCBs compete with aldosterone for binding to MR. Furthermore, nimodipine, a dihydropyridine CCB that has shown the most potent antagonistic effect in their study, inhibited aldosterone-induced expression of epithelial sodium channel-γ, one of the target genes of MR, in vivo. Based on these findings, they concluded that dihydropyridine CCBs have MR antagonist activity. Therefore, amlodipine may also inhibit MR-dependent action and elicit a renoprotective effect similar to that of eplerenone, the glomerular protection in the present study. However, amlodipine failed to show any protective effect on the glomeruli in DS rats. It is important that the IC50 value of amlodipine was the highest in the dihydropyridine CCBs used in the study by Dietz et al. The IC50 value of amlodipine was 50 times higher than that of nimodipine. Therefore, the ineffectiveness of amlodipine as an MR antagonist might be simply due to its weak affinity for MR.

MR blockade failed to show any effect on the high salt-induced renal hypoxia in DS rats, possibly owing to the lack of function of MR in preserving capillary flow. There have been no reports of MR-dependent blood flow regulation in the kidney, and the present study showed that chronic MR blockade with eplerenone did not prevent the peritubular capillary loss. Thus it can be speculated that MR blockade induces neither enhanced blood flow of remaining capillaries nor preventive effects against the reduction in capillary numbers in high salt-fed hypertensive DS rats.

In conclusion, the present study indicates that the inhibition of MR with eplerenone prevents the development of glomer-
ular injury and that calcium channel blockade with amlopidine ameliorates tubulointerstitial fibrosis via improvements in tubulointerstitial oxygen conditions. Therefore, treatment with eplerenone combined with amlopidine provides additive renoprotective effects characterized by reductions in both glomerulosclerosis and tubulointerstitial fibrosis.

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