Renoprotective Effects of Benidipine in Combination with Angiotensin II Type 1 Receptor Blocker in Hypertensive Dahl Rats

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We examined the effects of the angiotensin II type 1 receptor blocker candesartan, the calcium channel blockers benidipine and amlodipine, hydralazine, and the combination of candesartan and benidipine or amlodipine on blood pressure and renal function in Dahl salt-sensitive (DS) hypertensive rats. Male DS rats (5 weeks of age) were fed a high-salt (8% NaCl) diet, resulting in hypertension accompanied by glomerular sclerosis and an increased urinary albumin excretion. Drugs were orally administered from 2 to 6 weeks after the start of the feeding. Although candesartan (1 or 10 mg/kg) had little effect on the blood pressure, benidipine (4 mg/kg), amlodipine (4 mg/kg) and hydralazine (5 mg/kg) had similar hypotensive effects. Benidipine, but not amlodipine, hydralazine, or candesartan, significantly inhibited the increase in the albuminuria and glomerular sclerosis. The combination of candesartan (1 mg/kg) and benidipine (4 mg/kg) lowered the levels of blood pressure and albuminuria more effectively than the combination of candesartan (1 mg/kg) and amlodipine (4 mg/kg). These results indicate that benidipine is effective in preventing the impairment of renal function in DS hypertensive rats, and suggest that additional benefits can be expected by combination therapy with benidipine and an angiotensin II type 1 receptor blocker. (Hypertens Res 2003; 26: 635–641)

Key Words: calcium channel blocker, angiotensin II type 1 receptor blocker, combination therapy, hypertension, Dahl salt-sensitive rat

Introduction

Calcium channel blockers (CCBs) have a variety of applications, but are most often used as antihypertensive drugs. Long-acting CCBs in particular have been reported to have favorable effects on hypertension and other cardiovascular diseases. The potent vasodilatory and natriuretic properties of the CCBs are generally recognized as their antihypertensive mechanisms (1). On the other hand, numerous animal studies have demonstrated that the various CCBs differ in their effects on kidney function and glomerular pathology. Some dihydropyridine CCBs preferentially dilate the preglomerular blood vessels (2). The preferential dilation of preglomerular afferent arterioles might induce the elevation of intraglomerular pressure and hyperfiltration, resulting in an increase in the risk of the glomerular injury (3, 4). Yue et al. (5) recently demonstrated that the long-acting dihydropyridine CCB benidipine (6) dilated both the afferent and efferent arterioles, but amlodipine preferentially dilated the afferent arterioles. In addition, Morikawa et al. (7) reported that benidipine lowered the glomerular pressure by decreasing the resistance of efferent arterioles in patients with nondiabetic nephropathy.

Angiotensin II type 1 receptor blockers (ARBs) are seeing increasing use as a first line treatment of hypertension. The effectiveness of ARBs depends on the activity of the renin-angiotensin system (RAS). In fact, the hypotensive effects of ARBs have been shown to be weak in salt-sensitive hypertensive models with low RAS activity (8). In contrast, CCBs...
lower the blood pressure in a RAS-independent manner (9, 10). In recent years, combination therapy with different types of antihypertensive drugs has been increasingly used in an effort to improve on the clinical benefits of monotherapy. In Dahl salt-sensitive (DS) hypertensive rats, the combination of benidipine and the ARB candesartan improved the renal function more effectively than either drug alone (11). In this study, we examined the effects of an ARB (candesartan) and CCBs (benidipine and amlodipine) on the blood pressure and renal function in DS hypertensive rats. In addition, we evaluated the therapeutic efficacy of the combination of candesartan and either benidipine or amlodipine in DS hypertensive rats.

Methods

Animals
Male Dahl salt-sensitive rats at 5 weeks of age (Japan Shizuoka Laboratory Animal Center, Inc., Hamamatsu, Japan) were used. The rats were kept at 19–25°C under a 12-h light–dark cycle, and they had free access to tap water and commercial chow (FR-2; Funabashi Farms, Chiba, Japan). All animals received humane care in compliance with the “Guiding Principles for the Care and Use of Laboratory Animals” formulated by the Japanese Pharmacological Society, and the protocol was approved by the Bioethical Committee of the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd.

Drugs
Benidipine hydrochloride (benidipine), candesartan cilexetil (candesartan) and amlodipine besilate (amlodipine) were synthesized at Kyowa Hakko Kogyo (Tokyo, Japan). Hydralazine was purchased from Sigma (St. Louis, USA). These drugs were suspended in 0.5% w/v Methyl Cellulose 400cps (Wako Pure Chemical, Osaka, Japan) in a volume of 5 ml/kg body weight. The dose of benidipine was selected on the basis of the study by Uehara et al. (12). Hydralazine at 5 mg/kg was reported to decrease the systolic blood pressure (SBP) by approximately 50 mmHg in spontaneously hypertensive rats (SHR) (13). Candesartan at 1 mg/kg was reported to inhibit pressor responses to exogenous angiotensin II in conscious normotensive rats (14). Other chemicals were of reagent grade from commercial sources.

Experimental Procedure
DS rats aged 5 weeks were fed a high salt diet (FR-2 containing 8% NaCl; Funabashi Farms). As normal controls, eight DS rats aged 5 weeks were fed a normal salt diet (FR-2 containing 0.19% NaCl; Funabashi Farms). Two weeks later, the rats were placed in metabolic cages for 24 h and urine was collected to determine the urine volume and albuminuria. SBP was measured in awake animals by the tail-cuff method (Ps-600; Riken Development, Tokyo, Japan). Blood was obtained from the tail vein. The DS rats fed the high-salt diet were then allocated to the following 8 groups with similar degrees of albuminuria and hypertension (8 rats in each group): control (vehicle); candesartan 1 mg/kg (C1); candesartan 10 mg/kg (C10); benidipine 4 mg/kg (B); amlodipine 4 mg/kg (A); hydralazine 5 mg/kg (H); candesartan 1 mg/kg combined with benidipine 4 mg/kg (C1 + B); and candesartan 1 mg/kg combined with amlodipine 4 mg/kg (C1 + A). At 4 weeks after the start of the repeated dosing, urine was collected for 24 h and SBP was measured by the tail-cuff method at 3 to 5 h after drug administration. The concentration of albumin in the collected urine was measured using a rat microalbumin assay kit (Testant; BL Co., Ltd., Numazu, Japan) with an autoanalyzer (AU600; Olympus, Tokyo, Japan). The assay has a coefficient of variation of 10% in the range of 2 to 500 µg/ml for a 15 µl urine sample. Urinary albumin excretion (mg/kg/24 h) was calculated from the urinary albumin concentration and urine volume. After collection of the urine, blood was obtained from the tail vein. Serum creatinine was measured with the autoanalyzer. After completion of the final measurements, the rats were weighed and anesthetized with ether, and then the kidneys were removed.

Histological Examination
The left kidneys were immediately cut into transverse sections around the renal pelvis at a thickness of approximately 5 mm and immersed in 10 vol% buffered formalin solution for fixation. After fixation, paraffin sections were made by a standard method. These sections were stained with periodic acid-Schiff (PAS) for light microscopic observation. The specimens were observed under a light microscope (BX60; Olympus). Histopathological scoring was performed according to the method of Uehara et al. (12).

The glomerulosclerosis scores were obtained as follows. A minimum of 100 glomeruli in each specimen were examined. The severity of the lesions was graded from 0 to 4+ according to the percentage of glomerular involvement: 0, no lesions; 1+, less than 25%; 2+, 26 to 50%; 3+, 51 to 75%; 4+, more than 76%. The injury score was obtained by multiplying the degree of damage (0 to 4+) by the percentage of glomeruli displaying the corresponding degree of severity.

The severity of arterial injury was graded from 0 to 3+: 0, no lesions; 1+, medial and intimal hyperplasia or necrosis and lumen narrowing; 2+, medial necrosis with infiltration of inflammatory cells; and 3+, medial and intimal hyperplasia and/or medial necrosis accompanied by inflammatory cell infiltration and thrombus formation. The arterial injury score was obtained by multiplying the degree of damage (0 to 3+) by the percentage of arteries displaying the corresponding degree of severity.
Table 1. Baseline Values of Body Weight, Systolic Blood Pressure, Serum Creatinine and Urinary Albumin Excretion in Dahl Salt-Sensitive Hypertensive Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Doses (mg/kg/day)</th>
<th>Body weight (g)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Urinary albumin excretion (mg/kg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>□</td>
<td>198 □ 3</td>
<td>119 □ 2***</td>
<td>0.21 □ 0.00</td>
<td>3 □ 1***</td>
</tr>
<tr>
<td>Control</td>
<td>□</td>
<td>189 □ 4</td>
<td>156 □ 2</td>
<td>0.18 □ 0.00</td>
<td>64 □ 14</td>
</tr>
<tr>
<td>Candesartan (C1)</td>
<td>1</td>
<td>196 □ 5</td>
<td>157 □ 2</td>
<td>0.17 □ 0.00</td>
<td>59 □ 9</td>
</tr>
<tr>
<td>Candesartan (C10)</td>
<td>10</td>
<td>193 □ 3</td>
<td>152 □ 1</td>
<td>0.18 □ 0.00</td>
<td>58 □ 9</td>
</tr>
<tr>
<td>Benidipine (B)</td>
<td>4</td>
<td>192 □ 3</td>
<td>154 □ 1</td>
<td>0.18 □ 0.00</td>
<td>58 □ 10</td>
</tr>
<tr>
<td>Amlodipine (A)</td>
<td>4</td>
<td>192 □ 4</td>
<td>154 □ 1</td>
<td>0.17 □ 0.00</td>
<td>57 □ 8</td>
</tr>
<tr>
<td>Hydralazine (H)</td>
<td>5</td>
<td>196 □ 3</td>
<td>154 □ 1</td>
<td>0.18 □ 0.00</td>
<td>61 □ 12</td>
</tr>
<tr>
<td>Candesartan</td>
<td>1</td>
<td>193 □ 3</td>
<td>154 □ 2</td>
<td>0.18 □ 0.00</td>
<td>57 □ 8</td>
</tr>
<tr>
<td>+ Benidipine (C1 + B)</td>
<td>4</td>
<td>193 □ 3</td>
<td>154 □ 2</td>
<td>0.18 □ 0.00</td>
<td>57 □ 8</td>
</tr>
<tr>
<td>Candesartan</td>
<td>1</td>
<td>190 □ 4</td>
<td>153 □ 1</td>
<td>0.18 □ 0.00</td>
<td>57 □ 8</td>
</tr>
<tr>
<td>+ Amlodipine (C1 + A)</td>
<td>4</td>
<td>190 □ 4</td>
<td>153 □ 1</td>
<td>0.18 □ 0.00</td>
<td>57 □ 8</td>
</tr>
</tbody>
</table>

Each value represents the mean □ SEM of 8 rats. *** p < 0.001 compared with the value in the control group.

The severity of tubular lesions was graded from 0 to 4 +: 0, no lesions; 1 +, very mild focal dilatation of tubules; 2 +, larger number of dilated tubules with widening of the interstitium; 3 +, fairly extensive dilatation of tubules with cystic formation and widening of the interstitium; 4 +, complete atrophy of tubules.

Statistical Analysis

All values are expressed as the means □ SEM. Statistical analysis was performed using statistical analysis software (SAS, release 6.12; SAS Institute, Inc., Cary, USA). Inter-group differences were assessed by Wilcoxon’s rank sum test for a two-group comparison or the Kruskal-Wallis test followed by Steel’s test for multiple group comparison. A difference was considered statistically significant at p < 0.05.

Results

Table 1 shows the baseline values of body weight, SBP, serum creatinine and urinary albumin excretion before the drug treatment. SBP and urinary albumin excretion were significantly increased in the DS rats fed the high-salt diet for 2 weeks. The treatment groups were well matched at baseline in terms of body weight, SBP, serum creatinine, and urinary albumin excretion. Each one in the hydralazine-treated and control groups died in the 2nd or 4th week, respectively, after the start of the repeated dosing. No rats in the other groups died during the experimental period. Figures 1–5 show the results after 4 weeks of drug treatment.

Body Weight and Blood Pressure

Body weight in DS rats fed a high salt diet for 6 weeks (control rats) was significantly decreased as compared with rats fed standard chow (normal rats) (Fig. 1). None of the drug treatments affected the weight in DS rats fed a high salt diet. Hypertension developed progressively in the control rats, but did not develop in the normal rats (Fig. 2). The hypotensive effects were comparable among the high salt-fed DS rats of the 3 groups treated with benidipine (B), amlodipine (A), and hydralazine (H). Candesartan (C1 or C10) had little effect on SBP, whereas the combined use of candesartan (1 mg/kg) and benidipine (C1 + B) augmented the hypotensive effects of benidipine. SBP in the candesartan combined with benidipine (C1 + B) group was significantly lower than that in the groups receiving either drug alone (C1 or B), and was also significantly lower than that in the candesartan combined with amlodipine (C1 + A) group.

Serum Creatinine and Albuminuria

Serum creatinine in the control rats was increased as compared with that in the normal rats (Fig. 3). Benidipine, amlodipine, and candesartan combined with benidipine or amlodipine significantly inhibited the increase in serum creatinine. Candesartan alone did not affect serum creatinine.

Urinary albumin excretion in the control rats was significantly increased as compared with that in the normal rats (Fig. 4). Benidipine, and benidipine combined with candesartan significantly inhibited the increased urinary albumin excretion. Amlodipine, candesartan (10 mg/kg), hydralazine, and candesartan combined with amlodipine tended to decrease urinary albumin excretion, but the effects were not significant. The level of albuminuria in the candesartan combined with benidipine group was lower than that in the benidipine alone or amlodipine combined with candesartan group.

Histological Examination

In the control rats, glomerulosclerosis, arterial injuries and
tubular lesions were prominently observed in the kidney (Fig. 5). All the lesions in the hypertensive DS rats treated with benidipine were significantly reduced as compared with those in the control rats. Amlodipine, hydralazine, and candesartan combined with amlodipine (C1 + A) significantly decreased the scores of the tubular and arterial lesions, and tended to reduce the glomerular sclerosis. Candesartan (1 and 10 mg/kg) alone did not affect the lesions. The combination of candesartan and benidipine significantly reduced the scores of glomerulosclerosis and tubular lesions at a level equivalent to that by benidipine alone. The arterial injury score in rats receiving benidipine in combination with candesartan was lower than the score in rats receiving either drug alone.

**Discussion**

Uncontrolled hypertension has been related to increased cardiovascular morbidity and mortality, and progressive renal functional impairment (15). In the present study, significant
hypotensive effects were observed in DS hypertensive rats administered benidipine, amlodipine, or hydralazine, whereas candesartan had only minor effects on blood pressure. The doses of candesartan used in this study were sufficient to exert antihypertensive effects in SHR (16). The low efficacy of candesartan appeared to be due to the low activity of RAS in high salt-fed DS rats, as previously reported (8).

Benidipine prevented the development of albuminuria and renal injuries more effectively than amlodipine or hydralazine. The antihypertensive effect of benidipine may contribute to its renoprotective effect in DS rats. However, the hypotensive effects of hydralazine and amlodipine were comparable to that of benidipine. These results suggest that the beneficial renoprotective effects of benidipine cannot be attributed only to the antihypertensive effects of the drug. In the present study, amlodipine and hydralazine significantly reduced the scores of arterial injuries and tubular lesions, but had no significant effects on albuminuria or glomerulosclerosis. This is in accordance with previous reports (17, 18). The difference in effects of these drugs on albuminuria was related to that observed on glomerular lesions, but not to the vascular or tubular lesions that seem to be determined by blood pressure.

It is of interest that benidipine but not amlodipine effectively ameliorated the renal injury in the DS hypertensive rats. In general, L-type CCBs, such as nifedipine and amlodipine, preferentially dilate preglomerular blood vessels, resulting in hyperfiltration that has been recognized as an important factor of glomerular injury (19). However, benidipine has been reported to dilate both the afferent and efferent arterioles (5). In vivo micropuncture studies have revealed that benidipine decreased glomerular pressure in SHR (20). Furthermore, Morikawa et al. (7) reported that benidipine lowered the glomerular pressure by decreasing the resistance of efferent arterioles in patients with nondiabetic nephropathy. In contrast, amlodipine failed to reduce glomerular pressure or glomerular size and injury in SHR and deoxycorticosterone-salt hypertensive rats (18). Taken together, these results indicate that the unique renal hemodynamic action of benidipine may contribute to the renoprotective effect in DS rats. Transforming growth factor-β (TGF-β) plays a principal role in extracellular matrix accumulation and progression of glomerular injury in DS hypertensive rats (21, 22). Myocardial remodeling and left ventricular hypertrophy in DS hypertensive rats have been shown to be ameliorated by a subdepressor dose of benidipine, and the amelioration was partly due to decreases in the expression of TGF-β in the left ventricle (23). In addition, Nakamura et al. (24) reported that benidipine reduced the glomerular expression of TGF-β and α-smooth muscle actin in glomerulonephritis rats through a hypotension-independent mechanism. The renoprotective effects of benidipine may thus be at least partly mediated by a direct action on the kidney.

There are several lines of evidence that the local RAS activation contributes to the renal injuries (11, 25). Although we did not find a significant renoprotective action of candesartan in this study, some studies have shown that ARBs had renoprotective effects in DS hypertensive rats (26, 27). The discrepancy may be due to the difference in experimental protocols. In one of the former studies, plasma renin activity (PRA) was suppressed in salt-loaded DS rats after 6 weeks of high-salt feeding (26). In the present study, we...
examined the effects of drugs from 2 to 6 weeks after the start of the high-salt diets (low PRA period). This may be the reason for the weak effects of candesartan, although, if so, the details remain to be clarified.

In the treatment of hypertensive patients, combination therapy with different types of antihypertensive drugs is recommended if monotherapy fails to achieve adequate blood-pressure control. In the present study, the combination of candesartan and benidipine produced a greater hypotensive effect than benidipine alone. Furthermore, the combination of candesartan and benidipine inhibited the development of hypertension, albuminuria and renal injury more effectively than the combination of candesartan and amlodipine. These results suggest that, in combination with an ARB, benidipine may be more suitable for the renoprotection in salt-sensitive hypertension than amlodipine. However, in the present study, the effects of the drugs were examined only for 4 weeks. Further studies will be needed to clarify the efficacy of these drug therapies in patients with salt hypertension and renal damage associated with hypertension.

In conclusion, the present study demonstrated that benidipine is more effective than amlodipine or candesartan in preventing the impairment of renal function in DS hypertensive rats, and suggests that the combination of candesartan and benidipine decreases blood pressure and inhibits renal dysfunction more effectively than the combination of candesartan and amlodipine. If monotherapy with an ARB fails to achieve adequate blood-pressure control, combination therapy with an ARB and benidipine may provide better blood pressure control and may be useful in the treatment of the renal dysfunction caused by hypertension.

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