Protective Effect of Benidipine against the Development of Glomerular Sclerosis in Experimental Nephrotic Syndrome

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ABSTRACT—An experimental focal segmental glomerular sclerosis (FSGS) was induced by the combined administration of puromycin aminonucleoside (PAN) and protamine sulfate (PS). Blood collections were made on days 0, 37, 70 and 94. Urine collections were made on days 0, 24, 80 and 94. Vehicle-treated rats showed severe proteinuria and an increase in serum total cholesterol (sTC). Benidipine (1 or 3 mg/kg, p.o.)-treated rats exhibited less proteinuria and lower sTC than the vehicle-treated rats. On days 70 and 94, both blood urea nitrogen (BUN) and serum creatinine (sCR) values in the vehicle-treated rats were significantly higher than those in normal rats (without treatment with PAN and PS). On the other hand, the treatment with benidipine (1 or 3 mg/kg, p.o.) attenuated the increases in BUN and sCR. On day 94, vehicle-treated rats showed a significant decrease in creatinine clearance as compared with normal rats, but benidipine (1 or 3 mg/kg, p.o.)-treated rats did not. The histology was examined on day 94. Vehicle-treated rats demonstrated a significantly greater percentage of glomeruli with segmental areas of glomerulosclerosis/hyalinosis, mesangial cell proliferation, and mesangial foam cell. Benidipine (3 mg/kg, p.o.) ameliorated the development of renal regeneration as estimated by histological examination. These results suggest that the Ca-channel blocker benidipine is a favorable drug for preventing the progression of glomerular sclerosis.

Keywords: Benidipine, Puromycin aminonucleoside, Nephrotic syndrome

Focal segmental glomerular sclerosis (FSGS) is a condition in which some glomeruli develop segmental areas of sclerosis. However, the mechanisms underlying the progression of renal disease are complex and not fully elucidated. In various nephrosis models, hypertension, elevation of glomerular capillary pressure and renal hypertrophy are suggested as important risk factors which accelerate the development of renal disease (1–4). Some authors reported that antihypertensive agents such as Ca-channel blockers and angiotensin-converting-enzyme (ACE) inhibitors normalized the glomerular capillary hydraulic pressure and attenuated the progression of renal disease in a variety of experimental renal diseases (2–4). Thus, some antihypertensive drugs are now clinically used for renal diseases (5–7).

Mesangial cell proliferation has been observed in a variety of glomerular diseases such as FSGS (8, 9). Furthermore, proliferation of mesangial cells decreases renal mass and induce a reduction in the glomerular filtration rate (GFR). Recently, it was reported that Ca-channel blockers inhibit human mesangial cell proliferation in vitro (10). These observations suggest that Ca-channel blockers might have protective effects against renal injuries.

The rat FSGS model induced by the administration of puromycin aminonucleoside (PAN) and protamine sulfate (PS) is characterized by persistent proteinuria and development of severe renal impairment with histological changes similar to human FSGS (11, 12). However, the effect of any drugs has never been reported prior to the present study.

Benidipine is a potent Ca-channel blocker with long-lasting antihypertensive and antianginal activities (13, 14). The present study was conducted to determine whether the Ca-channel blocker benidipine could prevent nephrotic syndrome in a rat model of FSGS induced by PAN and PS.
MATERIALS AND METHODS

Animals
Male Sprague-Dawley (SD) rats weighing 200–250 g (Charles River Japan, Inc.) were used in this experiment. Diet and water were given ad libitum.

Induction of the nephrotic syndrome
The nephrotic syndrome was elicited with a slight modification of the method of Saito et al. (15). A left unilateral nephrectomy through a flank incision was performed on each rat under pentobarbital (50 mg/kg, i.p.) anesthesia. Three weeks was allowed for recovery. Then the nephrotic syndrome was induced (this was designated day 0) by injections of 10 mg/kg body weight (BW) of PAN administered subcutaneously (s.c.) and 25 mg/kg of PS administered intravenously (i.v.) in two divided doses. This was repeated daily for 4 days. This 4-day course was repeated at intervals of 10 days until a total of four courses had been given. The dose of PAN was reduced to 5 mg/kg BW following the first 4-day course (Fig. 1).

Diuretic studies
Eighteen-hour urine samples were collected from all rats in metabolic cages before injection of PAN and PS for initial control data, and then every two weeks during the course of the experiment. During the collection period, the rats were allowed free access to water but no food. Blood was obtained from the tail vein of each rat. The serum was separated by centrifuging the blood at 1600 × g for 15 min at room temperature. Determinations of urinary protein, albumin, urea nitrogen and creatinine, and of serum albumin, serum total cholesterol (sTC), blood urea nitrogen (BUN) and serum creatinine (sCR) were carried out using an autoanalyzer (AU-510, Olympus, Japan).

Clearance studies
Creatinine clearance (Cle-Cre) was calculated as follows:

\[
\text{Cle-Cre} \left( l/kg/18 \text{ hr} \right) = \frac{\text{uCR} \ (\text{mg/dl}) \times \text{UV} \ (l/18 \text{ hr})}{\text{sCR} \ (\text{mg/dl}) \times \text{BW} \ (\text{kg})}
\]

uCR: urine creatinine, UV: urine volume

Measurement of blood pressure
At day 90, arterial blood pressure in the awake state was measured with the plethysmographic tail method (USM-105-R, Ueda, Japan.)

Histological examination
To assess glomerular and tubulointerstitial lesions, light histological examination was performed by an investigator unaware of the treatment given a particular animal. At sacrifice, the kidneys were removed and fix ed in 10% buffered formalin. Paraffin sections were cut at 3 μm thickness and stained with hematoxylin and eosin, periodic acid-Schiff and Azan. Fifty cortical glomeruli from each specimen were examined. The degree of sclerosis in each glomerulus was graded from 1 to 4, according to the method of Harris et al. (16), with grade 1 being assigned to normal or very slight change and grade 4 being assigned to the most severe sclerosis. The number of glomeruli counted was expressed as a percentage of the total number of glomeruli counted for each rat. The tubulointerstitial lesions including tubular dilatation, tubular regeneration, tubular atrophy, interstitial infiltration, interstitial fibrosis and the presence of protein casts, were assessed semiquantitatively as fol-
lows: −, no change or very focal lesion present; +, 1/5 area of whole coronal section; + +, lesion was observed in 1/5 to 1/2 area of whole section; + + +, lesion was observed in more than 1/2 area of whole section.

**Drugs used**
PAN and PS were purchased from Sigma Chemical Co. Benidipine (hydrochloride, KW-3049) was synthesized in our laboratories. Benidipine was suspended in distilled water so as to make 1 ml suspension per 100 g of animal's BW and was orally administered once a day from day 0. The same volume of vehicle was administered to normal and vehicle-treated (control) animals.

**Statistical method**
Data were expressed as means ± S.E.M. Statistical analyses were performed using Scheffe's test following analysis of variance (ANOVA). Histological data were evaluated by Mann-Whitney's U-test. P values of 0.05 or less were considered statistically significant.

**RESULTS**

BW, UV and urinary excretion of protein and albumin in normal, vehicle-treated and benidipine-treated rats on days 0, 24, 80 and 94 are shown in Table 1. The BW in vehicle-treated rats were lower than those in the benidipine (1 or 3 mg/kg, p.o.)-treated rats during the course of the study. There was no difference in UV among all groups at the times examined. Vehicle-treated rats showed marked excretion of protein and albumin during the course of the experiment. Benidipine (3 mg/kg, p.o.)-treated rats did not show significant increases in urinary excretion of protein and albumin as compared with normal rats.

The results for sTC, albumin, BUN and sCR are shown in Table 2. The elevation in sTC continued throughout 94 days of the study, although the sTC tended to decrease toward the end of the study. This pattern was similar to the changes in urinary excretion of albumin. The administration of benidipine (1 or 3 mg/kg, p.o.) inhibited the increase in sTC. On days 37, 70 and 94, the values for serum albumin in vehicle-treated rats were significantly lower than those in normal rats. Benidipine (1 or 3 mg/kg, p.o.) inhibited the reduction of serum albumin. On day 37, the values for sCR in the vehicle- or benidipine (1 or 3 mg/kg, p.o.)-treated rats were almost the same as that in normal rats, although the values were elevated above the baseline value. However, on days 70 and 94, the group receiving the vehicle alone showed significant increases in sCR and BUN as compared with the normal group. These elevations of BUN and sCR were prevented by

| Table 1. Effects of benidipine on body weight, urine volume, urinary excretions of protein and albumin following the nephrotic syndrome |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | 0                          | 24                          | 80                          |
| Body weight (g) Normal     | 365.5 ± 12.7                | 395.7 ± 15.1                | 553.3 ± 22.5                |
|                            | Control                     | 351.4 ± 13.1                | 395.7 ± 25.6**              |
| Benidipine 1 mg/kg         | 362.0 ± 10.1                | 473.0 ± 16.2                |
| 3 mg/kg                    | 361.4 ± 15.9                | 465.7 ± 24.8                |
| Urine volume (ml/kg) Normal| 35.60 ± 2.50                | 40.68 ± 3.99                |
|                            | Control                     | 45.45 ± 11.79               |
| Benidipine 1 mg/kg         | 31.38 ± 4.65                | 29.18 ± 3.75                |
| 3 mg/kg                    | 45.03 ± 5.91                | 26.05 ± 2.79                |
| Protein (mg/kg) Normal     | 0.04 ± 0.002                | 0.04 ± 0.003                |
|                            | Control                     | 0.90 ± 0.18**               |
| Benidipine 1 mg/kg         | 0.63 ± 0.15                 | 0.35 ± 0.09*                |
| 3 mg/kg                    | 0.27 ± 0.18                 | 0.30 ± 0.06                 |
| Albumin (mg/kg) Normal     | 0.01 ± 0.002                | 0.01 ± 0.002                |
|                            | Control                     | 0.36 ± 0.09*                |
| Benidipine 1 mg/kg         | 0.27 ± 0.07*                | 0.19 ± 0.06*                |
| 3 mg/kg                    | 0.23 ± 0.06*                |

The rats in the normal group were not treated with puromycin aminonucleoside and protamine sulfate. Values are expressed as means ± S.E.M. of 5–7 rats. * and **: P < 0.05 and P < 0.01 vs. normal group, respectively.
the administration of benidipine (1 or 3 mg/kg, p.o.). The result for Cle-Cre is shown in Fig. 2. On day 94, Cle-Cre in the vehicle-treated rats was 2.75 ± 0.92 1/kg/18 hr, this value being significantly smaller than that in normal rats in which the corresponding value was 7.37 ± 0.46 1/kg/18 hr. The values in benidipine (1 or 3 mg/kg, p.o.)-treated rats were 4.95 ± 0.95 and 5.67 ± 0.78 1/kg/18 hr, respectively, being higher than that of the vehicle-treated rats.

On day 94, arterial blood pressure was similar in both normal and vehicle-treated rats. While 3 mg/kg (p.o.) of benidipine significantly reduced the arterial blood pressure, 1 mg/kg (p.o.) did not (Fig. 3).

Table 2. Effects of benidipine on serum cholesterol, blood urea nitrogen, creatinine and albumin following the nephrotic syndrome

|                      | Day     |       |       |       |
|----------------------|---------|-------|-------|-------|
|                      | 0       | 37    | 70    | 94    |
| Total cholesterol (mg/dl) | Normal  | 88.5 ± 5.4 | 98.1 ± 6.1 | 89.1 ± 3.3 | 88.4 ± 4.1 |
|                      | Control | 409.3 ± 79.8** | 343.8 ± 32.8** | 181.8 ± 28.1* |
|                      | Benidipine 1 mg/kg | 205.6 ± 37.2 | 285.1 ± 32.3* | 176.4 ± 34.6* |
|                      | Benidipine 3 mg/kg | 248.1 ± 67.4 | 269.8 ± 62.1 | 129.0 ± 17.4 |
| Blood urea nitrogen (mg/dl) | Normal  | 19.8 ± 0.9 | 21.6 ± 0.7 | 19.4 ± 1.2 | 17.0 ± 0.3 |
|                      | Control | 39.9 ± 5.1* | 74.4 ± 24.9** | 122.2 ± 53.7* |
|                      | Benidipine 1 mg/kg | 34.8 ± 4.6 | 30.3 ± 1.6 | 36.6 ± 9.5 |
|                      | Benidipine 3 mg/kg | 39.0 ± 5.6 | 39.7 ± 8.6 | 41.5 ± 16.6 |
| Creatinine (mg/dl) | Normal  | 0.33 ± 0.02 | 0.32 ± 0.02 | 0.33 ± 0.14 | 0.27 ± 0.01 |
|                      | Control | 0.44 ± 0.05 | 1.59 ± 0.63 | 2.15 ± 1.00** |
|                      | Benidipine 1 mg/kg | 0.42 ± 0.03 | 0.52 ± 0.06 | 0.55 ± 0.16 |
|                      | Benidipine 3 mg/kg | 0.47 ± 0.05 | 0.68 ± 0.16 | 0.66 ± 0.28 |
| Albumin (mg/dl) | Normal  | 3.54 ± 0.04 | 3.41 ± 0.05 | 3.67 ± 0.05 | 3.66 ± 0.10 |
|                      | Control | 2.35 ± 0.15** | 2.21 ± 0.13** | 2.51 ± 0.17** |
|                      | Benidipine 1 mg/kg | 2.92 ± 0.21 | 2.51 ± 0.17** | 2.96 ± 0.16* |
|                      | Benidipine 3 mg/kg | 2.80 ± 0.15 | 2.58 ± 0.16** | 3.10 ± 0.10 |

The rats in the normal group were not treated with puromycin aminonucleoside and protamine sulfate. Values are expressed as means ± S.E.M. of 5–7 rats. * and **: P < 0.05 and P < 0.01 vs. normal group, respectively.

![Fig. 2](image1.png) **Fig. 2.** The effect of benidipine on creatinine clearance (Cle-Cre) on day 94 following the induction of the nephrotic syndrome. Each bar represents the mean ± S.E.M. of 5–7 rats. *P < 0.05: vs. normal group.

![Fig. 3](image2.png) **Fig. 3.** The effect of administration of benidipine on systolic blood pressure (sBP) on day 94 following the induction of the nephrotic syndrome. Each bar represents the mean ± S.E.M. of 5–7 rats. *P < 0.05: vs. normal group.
Data from the histological examination of kidneys are shown in Tables 3 and 4. Glomerular sclerosis induced by PAN and PS co-administration are characterized by adhesions of capillaries to Bowman’s capsule, an increase in PAS-positive mesangial substance, hyalinosis and fibrosis. In the analysis of glomerular sclerosis, the rats treated with 3 mg/kg of benidipine had fewer glomeruli of grade 4 than vehicle-treated rats. The degrees of tubulointerstitial lesions including tubular dilatation, tubular regeneration, tubular atrophy, interstitial infiltration, interstitial fibrosis and the presence of protein casts were less prominent in benidipine (3 mg/kg, p.o.)-treated rats as compared with those in vehicle-treated rats.

| Glomerular histology | Percent of glomeruli examined |
|----------------------|------------------------------|
| Grading              | Control                       |
|                      | 1 mg/kg                       |
|                      | 3 mg/kg                       |
| (n = 7)              | (n = 5)                       |
|                     | (n = 7)                       |
| 1                    | 9.1 ± 7.5                     | 9.2 ± 8.7 | 8.9 ± 4.6 |
| 2                    | 17.1 ± 5.5                    | 34.0 ± 8.8 | 32.0 ± 7.8 |
| 3                    | 34.6 ± 9.4                    | 37.2 ± 10.6 | 46.0 ± 8.5 |
| 4                    | 39.1 ± 12.9                   | 23.6 ± 14.9 | 13.1 ± 5.2 |

1: Normal or the presence of minimal mesangial hypercellularity, 2: the presence of focal areas of sclerosis and loop collapse in the glomerulus with or without mesangial thickening, 3: segmental sclerosis with loop collapse, 4: global sclerosis, hyalinosis and fibrosis. a: No. of animals examined.

**Table 3.** Histological analysis of glomerular sclerosis 94 days after induction of the nephrotic syndrome in vehicle- and benidipine (1 or 3 mg/kg, p.o.)-treated rats

**DISCUSSION**

Ca-channel blockers, including benidipine, have ameliorating effects on renal function in various animal models of renal failure (2–4, 17). Therefore, Ca-channel blockers can be expected to be drugs for protecting against the renal injury. However, to date, the effect of benidipine on the chronic renal degeneration such as glomerular sclerosis has still not been examined. Therefore, we evaluated the possible protective effect of benidipine by using a rat model of glomerular sclerosis induced by PAN and PS. Our present study showed that benidipine ameliorates both functional and structural parameters of the kidney deteriorated by the administration of PAN and PS.

Among many factors associated with glomerular sclerosis, systemic hypertension and elevation of glomerular capillary pressure are important risk factors for the progression of renal disease (18, 19). Many authors suggested that antihypertensive agents such as ACE inhibitors, which reduce glomerular capillary hydraulic pressure, improve proteinuria and glomerular sclerosis (18, 20–23). On the other hand, it has been reported that Ca-channel blockers show more selective relaxation of renal afferent arterioles than renal efferent arterioles (24, 25). Thus, Ca-channel blockers may induce glomerular hypertension/hyperfiltration and aggravate renal damage. However, our unpublished data have indicated that benidipine increases renal blood flow without elevation of GFR and decreases renal vascular resistance in rats. In addition, some authors have reported that Ca-channel blockers dilate not only the afferent,
mesangial cells can also result in quantitative or qualitative changes in the mesangial matrix proteins that are secreted by these cells and could contribute to glomerulosclerosis (9). Recently, Shultz et al. (10) reported that Ca-channel blockers inhibited proliferation of human mesangial cells induced by PDGF or thrombin. Thus, the suppression of mesangial cell proliferation might have played a role in the protective effect of benidipine against the nephrotic syndrome. Further studies of Ca-channel blockers are needed to determine their effectiveness on mesangial cell proliferation.

In summary, the administration of benidipine to rats with nephrotic syndrome resulted in a lower BUN and sCr and higher creatinine clearance with less histological damage following the induction of the nephrotic syndrome. These results suggest that benidipine is a favorable drug for preventing progressive renal disease. However, further work is required to ascertain whether these observations in the rats with PAN nephrosis can be extrapolated to other forms of the nephrotic syndrome and, in particular, to human nephrotic disease.

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