Dual Effects of Lisinopril on Puromycin Aminonucleoside Nephrosis in Unilaterally Nephrectomized Rats

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ABSTRACT—The therapeutic effects of angiotensin converting enzyme inhibitor, lisinopril, on puromycin aminonucleoside (PAN)-induced nephrosis were investigated using unilaterally nephrectomized rats. Lisinopril showed potent dual effects on PAN nephrosis. Lisinopril treatment (50 mg/l in drinking water) from day 5 or day 9 reduced urinary protein excretion and suppressed the development of glomerular sclerosis at 8 weeks after PAN injection (150 mg/kg, i.p.), indicating a therapeutic effect on the nephrosis. Recovery of decreased anionic charge sites on the glomerular basement membrane was involved, at least in part, in the therapeutic action of lisinopril against proteinuria. On the other hand, oliguria and progressive azotemia derived from continuous deterioration of the renal function was induced if the treatment of lisinopril was started on the same day as PAN injection. The renal dysfunction induced by simultaneous administration of lisinopril with PAN could be abolished by combination dosing with sarcosine, an angiotensin II (AII)-receptor agonist. These results indicate that lisinopril treatment attenuates proteinuria by ameliorating the anionic charge barrier on the glomerular basement membrane and that it also protects against the development of chronic renal disease with segmental glomerular sclerosis, although AII depletion during the acute nephrotic stage exacerbates the renal damage in PAN nephrosis of unilaterally nephrectomized rats.

Keywords: Puromycin aminonucleoside nephrosis, Glomerular sclerosis, Lisinopril, Anionic charge barrier

A single or repeated administration of puromycin aminonucleoside (PAN) to rats results in severe proteinuria and marked changes in glomerular ultrastructure that mimic the minimal change nephrotic syndrome in humans (1, 2). In acute PAN nephrosis, the major alteration in glomerular morphology involves the loss of foot processes and detachment of the epithelium from the glomerular basement membrane (GBM) (3). Moreover, the study of the chronic effect of PAN treatment demonstrated that the glomerular injury following a single administration of PAN progresses to focal and segmental glomerulosclerosis (FSGS) (4).

Since Brenner et al. demonstrated the hyperfiltration theory (5), the contribution of hemodynamics to the progression of glomerular disorder has been pointed out. In particular, the possible involvement of endogenous angiotensin II (AII) action in the progression of renal damage has been confirmed by studies using angiotensin converting enzyme (ACE) inhibitor or an AII-receptor antagonist. However, the effects of these drugs on PAN nephrosis are somewhat controversial, varying with the study. Yayama et al. (6) reported that Dup 753, an AII-receptor antagonist, showed a potent effective profile against proteinuria in the early progressing stage of PAN nephrosis. On the other hand, ameliorative effects of L-158809, AII receptor antagonist, on proteinuria could be observed during the late stage, 28 weeks after PAN injection (7). As most reported studies were performed to examine the prophylactic effects of simultaneous administration with PAN injection or of premedication, it remains uncertain whether AII inhibitors show a therapeutic effect on PAN nephrosis.

Proteinuria is known to be caused by a change of glomerular permselectivity such as the disappearance of anionic charge particles in the GBM or dysfunction of size-selectivity of the GBM. Cartwright and Jaenke (8) demonstrated that the beneficial effects of captopril on proteinuria were derived from preventing increased glomerular capillary wall porosity and decreased anionic binding sites on the GBM using unilaterally nephrectomized rats treated with a high protein diet. In PAN nephrosis, however, the influence of ACE inhibitors on
glomerular permselectivity has not been reported so far, in spite of observations of alteration in both the size and charge barrier in glomeruli during marked proteinuria (9, 10).

Hence, the present study was designed to examine the therapeutic effects of lisinopril, a long-acting ACE inhibitor, on the progression of glomerulosclerosis and proteinuria in rats unilaterally nephrectomized with PAN. Also, discussed here is the positive profile of this drug against charge selectivity on the GBM.

MATERIALS AND METHODS

Experimental procedure to induce PAN nephrosis in unilaterally nephrectomized rats

Six-week-old male Wistar strain rats (Japan SLC, Inc., Shizuoka) were subjected to ligation of the left renal artery and renal vein, and then the left kidney was nephrectomized under anesthesia with sodium pentobarbital 2 weeks prior to PAN injection. The animals were allowed to become accustomed to the metabolic cages during sessions conducted over 3 days. The animals were divided into four groups. Group 1 (PAN control) was given no therapy. Group 2 (PAN + lisinopril from day 1) was treated with lisinopril starting from the first day to the end of the study (8 weeks). Group 3 (PAN + lisinopril from day 5) was treated with lisinopril from the 5th day to the end of the study. Group 4 (PAN + lisinopril from day 9) was treated with lisinopril from the 9th day to the end of the study.

PAN nephrosis was induced by single intraperitoneal injection with 150 mg/kg body weight of PAN dissolved in 2 ml of saline on the first day, while lisinopril dissolved in drinking water at 50 mg/l was given ad libitum (1-2 mg/day) from the first day, the 5th day and the 9th day throughout the experiment, respectively. These animals were housed in the metabolic cages during the experimental period and food was freely available for intake.

Urinary protein excretion was measured by the pyrogallol red method with the Micro TP-test (Wako Chemicals Industry, Osaka), while blood urea nitrogen was determined by the urease-indole method with the Urea Nitrogen B-test (Wako Chemicals Industry).

Preparation of kidney tissue for electron microscopy

Rats were flushed with PBS through the abdominal aorta followed by perfusion with 2.5% glutaraldehyde buffered with 0.1 M sodium phosphate (PB) buffer (pH 7.4) at room temperature. The kidneys were removed and cut into small pieces (1 mm³), which were immersed in the same fixative for 1 hr at room temperature and then postfixed with 2% OsO₄ in 0.1 M PB (pH 7.4) for 1 hr at 4°C. The fixed specimens were stained with 2% uranyl acetate for 1 hr at room temperature, dehydrated with graded concentrations of ethanol and embedded in epoxy resin. Ultrathin sections, doubly stained with uranyl acetate and lead citrate, were examined with an electron microscope (100-CX; Jeol, Tokyo).

Preparation of kidney tissue for light microscopy

Kidneys at the follow-up period of the study were processed for light microscopy. The right kidney was removed under anesthesia with pentobarbital, and tissue samples were fixed in 10% formalin and embedded in paraffin. Sections (4-μm-thick) were stained with the periodic acid-Schiff reaction and hematoxylin.

Detection of anionic sites in glomerular basement membranes

To evaluate the number of anionic sites in GBM, the rat with urinary protein excretion closest to the average one for each group was histologically examined using the polyethyleneimine (PEI) binding method of Schurer et al. (11) at 6 weeks following PAN injection. Rats were injected in the tail vein with 0.2 ml of 0.5% PEI (M.W. 50000) solution adjusted to pH 7.4 with HCL. The osmolarity of the PEI solution was brought to 400 mOsm with NaCl and sucrose. The animals were sacrificed 15 min after the injection. The kidney was removed, cut into small pieces and immersed in a mixture of 0.1% glutaraldehyde and 2% phosphotungstic acid buffered with 0.1 M sodium cacodylate buffer (SCB, pH 7.4) for 1 hr at room temperature. The materials were washed three times in SCB and post-fixed in 2% OsO₄ buffered with SCB for 2 hr at 4°C. Tissue pieces were then rinsed, dehydrated in graded ethanol and embedded in epon epoxy resin. Ultrathin sections were contrasted with lead citrate and examined with a Jeol 100-CX electron microscope. Glomerular anionic sites stained with PEI were quantitated by counting the number of dense deposits in the linear GBM on enlarged photographs (×48,000) and expressed as the mean±S.E. of stained anionic sites per 1,000-nm length of GBM.

Protective effects of a Ca²⁺ blocker, atrial natriuretic peptide (ANP) and AII-receptor agonist on lisinopril-induced renal dysfunction in the early nephrotic stage in PAN

PAN nephrosis was induced in the same manner as described above. Lisinopril treatment was started from the same day as PAN injection. Nifedipine (3 mg/kg/day) and nicardipine (10 mg/kg/day) suspended in polyethylene glycol 400 (2 ml/kg) were orally administered simultaneously with PAN injection, while ANP diluted with saline was intravenously injected from the tail vein at the
dose of 3 μg/kg/ml/day. Administration of these drugs continued throughout the duration of the study. Sarco sine diluted with saline was subcutaneously given from an osmotic mini pump (Alzet; Alza Corporation, Palo Alto, CA, USA) at the flow rate of 200 ng/kg/min.

At 7 days after PAN injection, blood samples were collected from the orbital sinus under anesthesia with ether to estimate the concentration of blood urea nitrogen. These samples were immediately centrifuged at 1,600 × g for 10 min at 4°C, and the plasma was kept at −20°C until measurement.

Chemicals

Lisinopril, nifedipine and nicardipine were synthesized in our laboratories, while puromycin aminonucleoside, sarcosine and polyethyleneimine were purchased from Sigma (St. Louis, MO, USA), and ANP was from Peptide Institute, Inc. (Osaka).

Statistics

Data are shown as the mean and standard error. The significance of the difference from the control value was evaluated by Tukey's method (12).

RESULTS

Acute and chronic nephrotic stage induced by PAN

Changes of urinary protein excretion and body weight after intraperitoneal PAN injection in unilaterally nephrectomized rats are shown in Fig. 1. Marked hyperproteinuria was observed from the 4th day, reaching the maximum level on the next week after PAN injection. Although the amount of urinary protein excretion slightly decreased after that, the hyperproteinuria remained during the experimental period. Urinary protein excretion at the end of the follow-up period reached the level of 344 ± 17 mg/day, which was ten times higher than that in control rats subjected to unilateral nephrectomy. As for the changes of body weight, marked reduction was in-

![Fig. 1. Changes of body weight and urinary protein excretion following PAN injection in unilaterally nephrectomized rats. Plots show the mean values and the bars, the standard error. Symbols are: ○, control (N=3); ●, PAN (N=5). * and **: significantly different from the control value at P < 0.05 and P < 0.01, respectively.](image-url)
duced by PAN injection; this lasted for 2 weeks, although there was a transient recovery phase observed from the 6th to the 10th day. Subsequently, body weight gradually increased in the same manner as in the control rats. These results indicated that the physiological conditions fluctuated dramatically day by day during the acute stage in PAN nephrosis.

In the present study, considering the changes of body weight and urinary protein excretion, lisinopril treatment was started from the 5th day and the 9th day, respectively.

The effect of PAN was assessed on the ultrathin sections (Fig. 2). Five days after PAN injection, we observed that a number of foot processes were fused to each other. In addition, the cytoplasm of the podocytes contained a large number of lysosomes. The loss of foot processes and filtration slits was widely observed on day 9 after PAN injection. The foot process areas of the podocytes were replaced by areas of flattened cytoplasm. Podocytes with stress fiber-like microfilament bundles along the basement membrane were observed.

Histological changes of glomeruli and tubules on the final day of the experiment (8 weeks after PAN injection) examined by light microscopy are shown in Fig. 6b. Marked enlargement of glomerular capillaries and thickening of basement membranes beneath Bowman’s capsular epithelial cells were observed. The rest of the area in the glomeruli was occupied by PAS-positive glomerular matrices, indicating the development of typical focal segmental glomerular sclerosis. The tubules were expanded, and interstitial inflammation was observed between them.

Effects of lisinopril on acute and chronic PAN-nephrosis

The responses of lisinopril against PAN-induced hyperproteinuria are shown in Fig. 3. When lisinopril was administered simultaneously with PAN injection (group 2), animals showed oliguria followed by marantic death by the following week, although urinary protein excretion on the 7th day was significantly less than that in PAN-treated rats (group 1). This result means that lisinopril causes deterioration of renal function in the acute nephrotic stage if given simultaneously with PAN injection. On the other hand, when the lisinopril treatment was started from the 5th day (group 3) and the 9th day (group 4), successive hyperproteinuria observed in the chronic nephrotic stage was markedly improved, indicating that lisinopril has a therapeutic effect against PAN nephrosis. However, hyperproteinuria in the acute nephrotic phase was not ameliorated significantly in group 3 or 4. Because there was no difference between groups 3 and 4 as to the antiproteinuric effect of lisinopril, we used group 4 to evaluate the influence of lisinopril on the number of anionic sites in GBM. Figure 4 shows the typical ultrastructure of glomerular capillary walls after PEI perfusion on each group.

Fig. 2. Morphologic changes seen in the control and PAN nephrotic rat kidney 5 days or 9 days after PAN injection. The number of foot processes was greatly reduced compared with that of the control (a), and many of the membranes between the adjacent foot processes were in close contact (arrow heads) on day 5 (b). The loss of foot processes was widely seen on day 9 after PAN injection, and the base of the podocytic cell body became flat (c). a, ×9,100; b, ×9,100; c, ×8,000.
Fig. 3. Improving effect of lisinopril on urinary protein excretion in PAN-treated rats. Plots show the mean values and the bars, the standard error. Symbols are: ○, PAN treated (N=5); ●, PAN + lisinopril from day 1 (N=6); □, PAN + lisinopril from day 5 (N=6); ▲, PAN + lisinopril from day 9 (N=7). * and **: significantly different from the PAN-treated value at P < 0.05 and P < 0.01, respectively.

In the control animals, electron-dense deposits consisting of the heparan sulfate-PEI complex were distributed as linear arrays in the lamina rara externa (LRE) of the GBM at intervals of 40–50 nm. Anionic sites similar to those of LRE were also seen in the lamina rara interna (LRI) of the GBM. However, the anionic charge sites in LRI were less in number than those of LRE (Fig. 4a).

A marked reduction in the number of stained anionic sites were observed in the LRE to which flattened glomerular epithelial cells in the PAN nephrotic glomerulus were attached. Interestingly, the deposits within the LRI were also dramatically reduced at the same capillary wall (Fig. 4b), suggesting that PAN treatment affected the production of heparan sulfate proteoglycans in both glomerular epithelial cells and endothelial cells.

In contrast, lisinopril treatment recovered the number of anionic charge sites at the glomerular capillary walls. The morphology of glomerular epithelial cell foot processes in lisinopril-treated nephrotic rats returned to that in the control animals. However, the electron-dense deposits...
deposits in lisinopril-treated glomeruli were larger in size than those seen in control ones (Fig. 4c).

Table 1. Number of anionic charge sites on the glomerular basement membrane at 6 weeks following PAN injection

| Treatment                  | N | Charge sites at 6 weeks |
|----------------------------|---|------------------------|
| Control (uni-nephrectomy)  | 29 | 23.3 ± 0.27^a          |
| PAN-treated (group 1)      | 17 | 17.5 ± 0.65            |
| PAN + lisinopril (group 4) | 35 | 25.7 ± 0.34^a          |

Lisinopril administration was started from day 9. Data represent the mean and standard error. ^aP<0.01 versus PAN-treated value.

Table 1 shows the number of anionic binding sites per 1000 nm of GBM in groups 1 and 4 at 6 weeks following PAN injection. The mean value was calculated from 17 to 35 electron photographs taken from an individual animal whose urinary protein excretion was closest to the average for its group. In sham-operated rats without PAN, the number of anionic sites per 1000 nm of GBM was 26.8 ± 0.63 (N=17, data not shown). In PAN-treated rats, the anionic charge sites significantly decreased at 6 weeks after PAN injection compared with unilaterally nephrectomized ones without PAN. Lisinopril treatment improved PAN-induced reduction of the sites on the GBM. More anionic sites were found in lisinopril-treated rats than in unilaterally nephrectomized rats without PAN.

Figure 5 shows the renal wet weight on the follow-up

Fig. 6. Light microscopy of kidney tissue from PAN-treated rats in the follow-up period of the experiment. a: Normal without PAN, b: PAN-treated (group 1), c: PAN + lisinopril starting from day 9 (group 4). × 250.
period of the study. It was significantly lower in groups 3 and 4 than in PAN-treated rats, indicating that lisinopril has a suppressive effect on renal hypertrophy in the chronic nephrotic stage. No differences were found between groups 3 and 4 as to such therapeutic efficacies.

Representative light micrographs of kidney tissue from groups 1 and 4 are shown in Fig. 6. Lisinopril treatment also ameliorated PAN-induced development of focal segmental glomerular sclerosis (Fig. 6c). In addition, expansion of renal tubules clearly improved.

Drug effects on lisinopril-induced deterioration in PAN nephrosis

As mentioned above, lisinopril induced marantic death within two weeks if given simultaneously with PAN. Table 2 shows the concentration of blood urea nitrogen at 7 days after PAN injection. In PAN-treated rats, the concentration of blood urea nitrogen was significantly higher than that in non-treated rats subjected to unilateral nephrectomy. The increment was enhanced by the simultaneous administration of lisinopril with PAN, indicating deterioration of renal function. We tried to abolish the deterioration by combination therapy of lisinopril with a Ca²⁺-receptor antagonist (nifedipine, nicardipine), ANP or an All-receptor agonist. Figure 7 shows the change of mortality after PAN injection and the macrobiotic effects of these drugs when lisinopril treatment was started from day 1. Nifedipine, nicardipine and ANP showed no macrobiotic effects, while sarcosine inhibited marantic death due to renal deterioration observed after combination dosing of PAN and lisinopril starting from day 1. These results indicated that All depletion during the early stage of PAN nephrosis (within 5 days after PAN injection) caused renal dysfunction followed by marantic death.

DISCUSSION

In the present study, we have shown that an All converting enzyme inhibitor (ACEI), lisinopril, ameliorates the glomerulosclerosis induced by PAN. Lisinopril exhibited potent therapeutic effects on proteinuria and progressive glomerular sclerosis when the treatment is started from day 5 or 9. However, no antiproteinuric effects of lisinopril were observed in the acute nephrotic stage within 2 weeks after PAN injection, although they were found in the late chronic one. Moreover, lisinopril inhibited PAN-induced renal hypertrophy and the expansion of renal tubules in that period. These findings indicate that the therapeutic properties of lisinopril against PAN nephrosis are mainly observed during the development of glomerular sclerosis in the late chronic phase.

At the end of the follow-up period, sustained proteinuria was remarkably improved, and the extension of the extracellular matrix in the glomeruli was also ameliorated by lisinopril treatment. PAN treatment caused reduction of anionic sites in the GBM within 4 hr (10). Our data suggest that lisinopril completely improved the reduction of anionic charge sites in GBM, corresponding to the attenuation of urinary protein excretion at 6 weeks following PAN injection. It is known that heparan sulfate proteoglycan, a major component of anionic charge sites, in the lamina rara externa of the GBM is produced by glomerular epithelial cells. In lisinopril-treated rats, morphological improvement of glomerular epithelial cells was observed at 6 weeks following PAN injection (Fig. 4c). Thus, recovery of reduced anionic charge sites in the GBM might be attributable to the amelioration of epithelial cell function. These findings indicate that recovery of the loss of anionic sites from the GBM is involved in the positive action of lisinopril on proteinuria in PAN nephrosis. However, the fact that the amount of
urinary protein excretion of lisinopril-treated rats was significantly higher than that of unilaterally nephrectomized rats without PAN at the follow-up period suggests that glomerular dysfunction other than the disappearance of anionic charge sites in the GBM also contributes to PAN-induced proteinuria. The alteration of glomerular size selectivity in PAN-treated rats has been demonstrated (9). Also, ACEI improves the size selectivity in humans with IgA nephropathy (13). Therefore, ACEI may have improved both charge and size defects in glomerular permeability.

Since Tagma et al. (14) demonstrated the utility of captopril for proteinuria in diabetic patients, numerous attempts have been made to apply ACEIs to other types of nephritis and nephrosis. Initially, the protective effects of ACEIs against other types of nephritis or nephrosis were interpreted as the result of reducing intraglomerular hypertension. Recently, as the accumulation of the extracellular matrix in these diseases has been noticed in the development of glomerular sclerosis (15), the explanation of ACEIs' improvement of nephritis or nephrosis has been altered. Transforming growth factor β (TGF-β1), which is known to inhibit metalloprotease (MMP) and to enhance the tissue inhibitor of metalloprotease (TIMP), is regarded as one of the most important growth factors regulating the accumulation of the extracellular matrix in the kidney. All stimulates extracellular matrix synthesis through induction of TGF-β1 using mesangial cells, suggesting an interaction between endogenous All and TGF-β1 in vivo (16). Although the therapeutic action mechanism of lisinopril in PAN nephrosis was unclear in this study, this drug may also influence the gene expression of TGF-β1. Further investigation is needed.

Our findings suggest that much attention should be paid to ACEIs in clinical use. We have found that lisinopril showed dual effects between the acute and late phases of PAN nephrosis. It is well-known that PAN nephrosis can be mainly classified into two different nephrotic stages. The acute stage observed within 2 weeks after PAN injection is characterized by morphological changes of podocytes including the loss of foot processes and massive proteinuria. On the contrary, in the chronic stage, increase of extracellular matrix in glomeruli and beneath Bowman's capsular epithelial cells is observed. Lisinopril caused deterioration of renal function in the acute nephrotic stage if its administration was started from day 1. The dose of lisinopril used in this study was 50 mg/l in drinking water, corresponding to a daily administration of 5–10 mg/kg/day, which is sufficient to inhibit angiotensin converting enzyme activity. Clearly, renal dysfunction in the acute nephrotic stage induced by lisinopril was not due to an excessive dose, because the detrimental effects were observed even with daily oral administration of a low dose of lisinopril (0.3 mg/kg/day) (data not shown). Moreover, a similar deteriorative effect was induced in our model by another converting enzyme inhibitor, captopril, indicating that this phenomenon may be common among ACEIs (data not shown).

Although the pharmacological actions of ACEIs or All-receptor antagonists on PAN nephrosis have been reported by several groups (6, 7, 17–19), none of them showed their deteriorative properties. The renal damage in PAN nephrosis differs among experimental protocols. In the present study, experimental nephrosis was induced by a single injection of 150 mg/kg of PAN into unilaterally nephrectomized rats, which seem to show more severe renal damage than that in previously reported PAN-nephrosis. This might be one of the reasons why lisinopril caused deterioration of renal function in the acute nephrotic stage. If administration was started from the 5th or the 9th day, renal dysfunction was not induced in this model. Thus, lisinopril seems to cause renal impairment only when some factors (i.e., unilateral nephrectomy, severe nephrosis induced by high dosage of PAN, and successive administration of ACEI within 5 days after PAN injection) coincided in the acute nephrotic stage. Renal dysfunction did not occur if one of those factors was omitted.

The deterioration of renal function induced by ACEI has been reported in patients with renovascular obstruction or chronic renal failure (20, 21). It is very important to investigate why these adverse effects occur in such patients. Hollenberg (22) has reported that acute renal failure after captopril administration occurred only in patients who were already azotemic before the treatment. Oliguria and progressive azotemia are also observed in the present PAN-induced nephrotic animals. Our model may be useful for investigating the cause of ACEI-induced renal dysfunction and how to avoid drug-induced deterioration. ACEI-induced renal dysfunction is presumed to be caused by the reduction of intraglomerular blood pressure originating from predominant vasodilatation of the renal efferent artery in humans (23). Ca^2+–channel blockers are known to act as vasodilators of the renal efferent artery and to subsequently increase renal blood flow. Moreover, ANP was reported to be effective in patients with acute renal failure by increasing creatinine clearance (24). These actions of the Ca^2+–channel blocker and ANP were expected to cancel lisinopril-induced renal dysfunction in our PAN nephrosis. Thus, we tried to protect against lisinopril-induced renal dysfunction at the acute nephrotic stage by combination therapy with Ca^2+–channel blockers, ANP or saracoline, All-receptor agonist. However, neither Ca^2+–channel blockers (nifedipine, nicardipine) nor ANP showed macrobiotic effects. On the other hand, saracoline showed potent protective
effects on lisinopril-induced renal dysfunction. ACEIs are known to have many effects; they inhibit AII synthesis, enhance the kallikrein-kinin system, affect the cyclooxygenase pathway of arachidonic acid metabolism (25) and prevent mesangial macromolecular deposition (26). Our observations show that AII depletion during the acute nephrotic stage in PAN nephropathy is the major cause of lisinopril-induced renal dysfunction. The role of AII at the early phase of PAN nephropathy remains unknown.

In summary, this is the first report on the dual effect of lisinopril in PAN nephropathy. Beneficial therapeutic properties of lisinopril on marked proteinuria and the progression of glomerular sclerosis were exhibited in the chronic nephrotic stage, while the depletion of AII during the acute nephrotic stage rather exacerbated renal function. The antiproteinuric effect of lisinopril contributed, at least in part, to the improvement of reduced anionic charge sites on the glomerular basement membrane.

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