**Preliminary Report**

**Effect of a specific endothelin receptor A antagonist and an angiotensin-converting enzyme inhibitor on glomerular mRNA levels for extracellular matrix components, metalloproteinases (MMP) and a tissue inhibitor of MMP in aminonucleoside nephrosis**

I. Ebihara¹, T. Nakamura², Y. Tomino² and H. Koide¹

¹Division of Nephrology, Department of Medicine, Koto Hospital, Tokyo; ²Juntendo University School of Medicine, Tokyo, Japan

**Abstract**

**Background.** We previously reported that mRNA levels for extracellular matrix (ECM) components and endothelin (ET-1) are upregulated in glomeruli of puromycin aminonucleoside (PAN) nephrosis. Angiotensin-converting enzyme (ACE) inhibitors are effective in experimental models of renal injury, including PAN nephrosis. This study was designed to assess whether the glomerular expression of mRNA for ECM components, ET-1, metalloproteinases (MMP), and a tissue inhibitor of metalloproteinases (TIMP) is modulated by a specific endothelin receptor A antagonist (FR139317) or angiotensin-converting enzyme inhibitor (enalapril) in PAN-injected rats.

**Methods.** Animals were divided into six groups. Group 1 consisted of PAN-injected rats given no treatment. In group 2, PAN-injected rats were given enalapril 35 mg/l. In group 3, PAN-injected rats were given an intraperitoneal injection of FR139317. Group 4 consisted of saline-injected rats given no treatment. In group 5, saline-injected rats were given enalapril. In group 6, saline-injected rats were given FR139317. We prepared glomerular RNA and performed Northern blot analysis in all groups.

**Results.** In PAN nephrosis, glomerular mRNA levels for α1(IV) collagen chain, laminin B1 and B2 chains, ET-1, MMP-2 and TIMP-1 increased at the peak of proteinuria on day 8 and then decreased to the control level by day 20, whereas those for α1 (I) and α1 (III) collagen chains, MMP-1, MMP-3 and GAPDH showed little change in PAN nephrosis throughout the experimental periods. In contrast, mRNA levels for heparan sulphate proteoglycan (HSPG) decreased on day 8 and then increased to the control level by day 20. Both enalapril and FR139317 attenuated the increases in mRNA levels for α1(IV) collagen chain (P<0.01), laminin chains (P<0.01), and ET-1 (P<0.01), and attenuated the decreases in mRNA levels for HSPG (P<0.01) in glomeruli of PAN-injected rats. Enalapril had little effect on increased glomerular mRNA levels for MMP-2 and TIMP-1 in PAN nephrosis, whereas FR139317 attenuated the increases in glomerular mRNA levels for MMP-2 (P<0.01) and TIMP-1 (P<0.01).

**Conclusions.** These data suggest that the beneficial effects of enalapril and FR139317 may be related to modulation of glomerular mRNA expression of ECM components and ET-1 and that these agents may follow a different mechanism in regulating the glomerular mRNA expression for MMP-2 and TIMP-1 in PAN nephrosis.

**Key words:** endothelin receptor; angiotensin-converting enzyme; extracellular matrix; metalloproteinase

**Introduction**

In addition to preventing systemic hypertension, angiotensin-converting enzyme (ACE) inhibitors lower urinary protein excretion and preserve glomerular structure in various models of renal disease [1,2]. The mechanisms by which ACE inhibitors retard the progression of renal disease has been examined extensively but not fully clarified. Recently, Remuzzi et al. [2] demonstrated that ACE inhibition enhanced hydraulic permeability and prevented the size-selective defect in the glomerular barrier function by reducing both the fraction of large unselective pores and the peak pore radius. Previously we reported that enalapril effectively reduces the upregulated mRNA expression of endothelin (ET)-1 and extracellular matrix (ECM) components in diabetic glomeruli [3,4]. Recently a strong argument has been made in favour of ET-1 as a mediator of renal injury [5], and the results of studies performed with drugs that bind specifically to ET receptors have become more meaningful. Some
investigators have reported that an ET receptor A antagonist, FR139317, attenuated renal injuries in a remnant kidney model or lupus mice [6,7].

The rat model of puromycin aminonucleoside (PAN) nephrosis is an established model of minimal-change glomerular disease. Previously we reported that the glomerular mRNA for ECM components is altered in PAN nephrosis, and that methylprednisolone (MPSL) has marked effects on the amelioration of abnormal gene expression in glomeruli of rats with PAN nephrosis [8]. However, to our knowledge there are no reports concerning the effects of either FR139317 or enalapril on gene expression for the ECM components, metalloproteinases (MMP), tissue inhibitor of metalloproteinase (TIMP)-1 or ET-1. In this study we examined the effects of FR139317 and enalapril on the gene expression of the ECM components, MMP-1, -2 and -3, TIMP-1 and ET-1 in the glomeruli of PAN-injected rats.

Subjects and methods

Animals

The subjects consisted of 90 male Sprague–Dawley rats (weighing 150–200 g) that were housed in individual metabolic cages, and daily urine volume, water intake, body weight, blood pressure, and urinary protein were measured throughout the experiments. The diet included 22.0% protein, 3.5% fat, 0.4% sodium, 1.0% calcium, and 0.88% phosphate. A single dose (15 mg/100 g body weight) of puromycin aminonucleoside (Sigma Chemical Co., St Louis, MO) was injected intraperitoneally into 45 rats to induce a nephrotic state; the remaining 45 rats were used as controls. The animals were divided into six groups (15 rats in each group). Group 1 consisted of PAN-injected rats given no treatment. In group 2, PAN-injected rats were given enalapril 35 mg/l (Merck, Rahway, NJ) in their drinking water. In group 3, PAN-injected rats were given an intraperitoneal injection of the specific ET receptor A antagonist, FR139317 (Fujisawa, Osaka, Japan) [6,7] at a dose of 3.2 mg/100 g every day for 20 days. Group 4 consisted of saline-injected rats given no treatment. In group 5, saline-injected rats were given enalapril in their drinking water. In group 6, saline-injected rats were given FR139317 for 20 days. All drugs were administered from the time of disease induction (on day 0). The food ingested by PAN-treated rats was weighed daily and the same amount of food was given to control rats.

Five rats from each group were killed on days 0, 8, and 20. The kidneys were removed immediately, and the glomeruli were isolated by sieving through size 60, 150, and 200 meshes. Decapsulated glomeruli were collected from the top of the 200-mesh sieve. The purity of the preparation of decapsulated glomeruli was greater than 95% and contained very few tubular fragments.

During the course of the experiments, 24-h urine specimens were obtained from all rats in metabolic cages daily; urinary protein was measured using the biuret method. Serum total protein, creatinine, and urea were measured by standard laboratory methods. Blood pressure was measured in unanaesthetized rats by indirect tail cuff plethysmography.

Preparation and quantification of glomerular RNA

Total cellular RNA was extracted from the isolated glomeruli of each rat with RNA zol B® (Cinna/Biotecx Laboratories, Inc., Houston, TX). Total glomerular RNA was denatured with formamide and formaldehyde, electrophoresed through a 1% agarose gel (20 µg RNA/lane) containing 2.2 M formaldehyde/0.2 M MOPS (pH 7.0) transferred to a nylon membrane (Gene Screen, New England Nuclear, Boston, MA) overnight by capillary blotting in 10 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M NaH2PO4, 0.001 M EDTA), and baked at 80°C for 2 h. Examination of the membrane under ultraviolet light in the presence of ethidium bromide demonstrated good resolution and integrity of the 28S and 18S ribosomal bands.

cDNA probes and hybridization conditions

The cDNA probes used for Northern blot analysis were as follows: clones for the α1 (IV) collagen chain (pFAC) and laminin B1 (p24) and B2 (p7) chains were isolated from a cDNA library constructed from differentiated F9 teratocarcinoma cell mRNA, as reported previously [9].

Rat α1 (I) (pSR1) and α3 (III) (1.8 Kb EcoRI fragment) collagen and heparan sulphate proteoglycan (HSPG) (clone 7) cDNA probes were provided by Dr Yoshihiko Yamada of the National Institute of Dental Research (Bethesda, MD) [10,11]. Rat MMP-1 and MMP-3 cDNA probes were provided by Dr Lynn M. Matrisian of Vanderbilt University (Nashville, TN) [12]. Human MMP-2 cDNA was provided by Dr Gregory I. Goldberg of Washington University School of Medicine (St Louis, MO) [13]. Human TIMP-1 cDNA was provided by Dr Masanobu Naruto of Basic Research Laboratories, Toray Industries (Kanagawa, Japan). Rat prepro-ET-1 cDNA was provided by Dr Tomoh Masaki of Kyoto University (Kyoto, Japan) [14]. Rat GAPDH cDNA was obtained from the American Tissue Type Culture collection (Rockville, MD). All probes were labelled with [α-32P]-deoxycytidine 5'-triphosphate (3000 Ci/mmol) from NEN Research Products (Tokyo, Japan) by random primer extension.

Membranes were prehybridized in a solution containing 50% formamide, 5 × SSPE, 5 × Denhards, 0.1% SDS and 100 µg of salmon sperm DNA per ml. Hybridization was performed (using the same solution to which 1 × 106 c.p.m./ml of 32P-labelled cDNA was added) for 24 h at 65°C. Blots were then washed three times in 2 × SSPE, 0.1% SDS at 65°C for 10 min and then in 0.1 × SSPE, 0.1% SDS for cDNA probes at 65°C for 10 min. After drying, membranes were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) at −80°C with intensifying screens for periods of 12 h to 7 days. The relative intensities of bands on the autoradiograms were quantified on a Scanning Shimadzu CS-9000 densitometer (Shimadzu Manufacturing, Kyoto, Japan). Membranes were rehybridized with additional probes.

Quantitation of mRNA

The RNA from five rats was randomly pooled and three Northern blots were performed per group (≈ 15 animals). Densitometry readings were normalized for equivalent amounts of GAPDH per lane. Values were expressed as optical density units relative to the specific mRNA levels observed in glomerular RNA from untreated control rats (group 4).
Statistical analysis

Values were expressed as means ± standard error. Comparisons between two time points in the same animals or between groups paired were made using the paired t test for parametric data or Wilcoxon signed-ranks test for non-parametric data. Non-parametric data from multiple groups were tested using the Kruskall–Wallis test followed by Mann–Whitney U test. Parametric data from multiple groups were compared using one-way analysis of variance (ANOVA).

Results

Biochemical data

Food intake was 10.2 ± 2.2 g, 11.4 ± 1.4 g and 11.6 ± 1.2 g on days 0, 8, 20 respectively in PAN-treated rats (group 1). Food consumption was almost identical in all groups of rats since the same amount of food ingested by nephrotic rats was given to control rats. Biochemical data and renal functions on day 8 are shown in Table 1. The body weights of control and nephrotic rats were not different on any day. Both enalapril and FR139317 did not influence body weight in nephrotic and control rats. Blood pressure of control and nephrotic rats was not different on any day. Both enalapril and FR139317 did not influence blood pressure in both groups. There was a significant rise in serum creatinine and urea in PAN-treated rats. This rise was reduced by either enalapril or FR139317. Because nephrotic rats drink 1.5 times more water than control rats, nephrotic rats received 1.5 times enalapril (real dose: group 2, 1 mg/24 h, group 5, 0.7 mg/24 h).

Urinary protein excretion

In group 1, rats developed proteinuria within 4 days after PAN injection. Proteinuria was highest on day 8. In group 2, rats developed proteinuria within 4 days after PAN injection. Proteinuria was highest on day 8. In group 3, FR139317 suppressed urinary protein (110 ± 14 mg/day on day 8). In group 4, FR139317 suppressed urinary protein (110 ± 14 mg/day on day 8) (P < 0.001). In group 5, urinary protein remained less than 20 mg/day throughout the experimental period (20 days). Neither enalapril nor FR139317 affected urinary protein excretion in control rats (groups 5 and 6). (Table 2). The diminution of the proteinuria was observed in the first 5 days of the treatment with enalapril or FR139317.

Glomerular mRNA analysis

In group 1, mRNA levels for z1 (IV) collagen chain, laminin B1 and B2 chains, MMP-2, TIMP-1 and ET-1 increased significantly when compared with control rats (group 4) on day 8 and then declined to the control level by day 20 (on day 8: z1 (IV), 2.6-fold (P < 0.01); laminin B1, 2.4-fold (P < 0.01); laminin B2, 2.6-fold (P < 0.01); MMP-2, 2.2-fold (P < 0.01); TIMP-1, 2.6-fold (P < 0.01) and ET-1, 2.3-fold (P < 0.01). However, mRNA levels for z1 (I) and z1 (III) collagen chains, MMP-1 and MMP-3 showed little change throughout the experimental period, whereas those for heparan sulphate proteoglycan (HSPG) decreased significantly when compared with control rats (group 4) on day 8 (0.5-fold, P < 0.01) and then increased to the control level by day 20. Enalapril attenuated the increased mRNA levels for z1 (IV) collagen chain, laminin B1 and B2 chains and ET-1 in glomeruli of PAN-injected rats (on day 8; z1 (IV), 0.5-fold (P < 0.01); laminin B1, 0.6-fold (P < 0.01); laminin B2, 0.5-fold (P < 0.01); and ET-1, 0.5-fold (P < 0.01)) when compared with mRNA levels in group 1. In addition, enalapril attenuated the decreased mRNA levels for HSPG (1.5-fold, P < 0.01) on day 8 in glomeruli of PAN-injected rats. However, enalapril did not alter mRNA levels of MMP-2 and TIMP-1 in glomeruli of PAN-injected rats. Enalapril

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<th>Table 2. Urinary protein excretion (mg/day)</th>
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<td>6. Controls + FR139317</td>
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Values are means ± SE, n = 5, *P < 0.01, bP < 0.001.

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<th>Table 1. Biochemical data and renal functions in groups 1–6 on day 8</th>
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<td><strong>Groups</strong></td>
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Data are means ± SE, n = 5. Group 1 versus Group 2: <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001. Group 1 versus Group 3: <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, <sup>f</sup>P < 0.001. Group 1 versus Group 4: <sup>P</sup><sup>i</sup> < 0.05, <sup>i</sup>P < 0.01, <sup>j</sup>P < 0.001.
did not affect mRNA levels of $\alpha_1$ (I) and $\alpha_1$ (III) collagen chains, MMP-1, MMP-3 and GAPDH in glomeruli of PAN-injected rats.

FR139317 attenuated the increased mRNA levels for $\alpha_1$ (IV) collagen chain (0.5-fold, $P < 0.01$), laminin B1 (0.6-fold, $P < 0.01$), laminin B2 (0.5-fold, $P < 0.01$), MMP-2 (0.5-fold, $P < 0.01$), TIMP-1 (0.5-fold, $P < 0.01$) and ET-1 (0.5-fold, $P < 0.01$) and the decreased mRNA levels for HSPG (1.6-fold, $P < 0.01$) in glomeruli of PAN-injected rats on day 8. FR139317 did not affect mRNA levels of $\alpha_1$ (I) and $\alpha_1$ (III) collagen chains, MMP-1, MMP-3 and GAPDH in glomeruli of PAN-injected rats. Figure 1 shows the Northern blot analysis of mRNA for all components on day 8 in groups 1–3. Neither enalapril nor FR139317 affected mRNA levels for any of these components in glomeruli of control rats (groups 5 and 6). Table 3 shows the relative changes in these mRNAs in groups 1–6 on day 8 after PAN or saline injection.

Discussion

We previously reported that both enalapril and FR139317 prevent renal injuries in animal models [3,4,7]. The beneficial effect of ACE inhibitors was associated with an improved permeability of the glomerular membrane for water and macromolecules based on a mechanism possibly mediated by a reduction in the mean radius of large and unselective pores that perforate the glomerular capillary. Remuzzi et al. [15] reported that an ACE inhibitor, despite only partially controlling systemic blood pressure, effectively prevented proteinuria and glomerular injury and that a calcium-channel blocker was not associated with renal protection. ACE inhibitors may ameliorate glomerular microcirculation by a mechanism that is not directly related to their antihypertensive action. It was also suggested that ACE inhibitors have a direct effect on the structural/functional properties of the glomerular basement membrane [15]. This is consistent with the present study which found that enalapril modulates the ECM composition. Klahr et al. [16,17] have reported that enalapril reduces the increased synthesis of ECM components in the obstructed kidney and suggested that enalapril may affect the balance between production and degradation of ECM components.

The protective effect of ACE inhibitors on renal function is always associated with a remarkably improved glomerular barrier permeability to proteins [18]. HSPG is thought to play an important role in the permselective properties of the glomerular capillary wall. In the present study we showed that enalapril attenuated the decreased HSPG mRNA in the glomeruli of PAN-injected rats. The mechanisms involved in the enalapril effect could be related to glomerular haemodynamics or to a direct inhibition of the mitogenic effect of angiotensin II. Angiotensin II has a direct hypertrophic and hyperplastic action on several types of cells (including glomerular cells) [19,20] and may cause glomerular hypertrophy, an important factor in the induction of glomerular injury [21]. Angiotensin II also stimulates the synthesis of ECM components in mesangial cells [22]. This may be directly involved in glomerular injury, promoting the modulation of ECM components. Therefore, our data may indicate that enalapril reduces the mRNA expression of $\alpha_1$ (IV) collagen chain and laminin chains via the reduction of angiotensin II activity.

The early effect of captopril on proteinuria is largely a reflection of its well-known ability to inhibit kinase activity and augment the local level of bradykinin. Tanaka et al. [23] reported that early-phase proteinuria of puromycin aminonucleoside nephrosis is independent of angiotensin II and that the antiproteinuric effect of ACE inhibitors is, at least in part, channelled through the activation of bradykinin. Further investigation is needed to understand the effects of the bradykinin antagonists on the gene expression of ECM components, MMPs and TIMP-1 in the glomeruli of PAN nephrosis.

In the present study we showed that glomerular mRNA levels for MMP-2 and TIMP-1 are increased on day 8 at the peak of proteinuria in PAN nephrosis, whereas those of MMP-1 and MMP-3 are unchanged throughout the experimental period. The relative

![Fig. 1. Northern blot analysis of glomerular mRNAs in groups 1–3.](image-url)
expression of TIMP versus MMP in the glomeruli may be critical in modulating the balance between the maintenance and destruction of the ECM in renal diseases, including PAN nephropathy. We showed that enalapril has no effect on the gene expression of MMP-2 and TIMP-1 in the glomeruli of PAN nephropathy. We previously reported that enalapril has no effect on decreased mRNA levels of MMP-1 and MMP-3 or increased mRNA levels for TIMP-1 in diabetic glomeruli [4].

Endothelin (ET)-1 is a potential mediator of renal damage. Remuzzi et al. [2] reported that an ACE inhibitor, lisinopril, significantly reduced urinary excretion of ET-1 in nephrectomized rats. Firth et al. [24] reported that an ACE inhibitor, ramipril, reduced the elevation of ET-1 mRNA in the clipped kidney. In the present study we showed that enalapril reduced the elevation of ET-1 mRNA in the glomeruli of PAN-injected rats. The mechanisms of the specific suppressing effect of the ACE inhibitors on renal ET-1 formation must remain speculative at the moment.

In addition we showed that an ET receptor A antagonist, FR139317, reduced the elevation of z1 (IV) collagen chain, laminin chains, MMP-2, TIMP-1 and ET-1 in the glomeruli of PAN-injected rats. These data appear to indicate that ET-1-induced renal injury in the rat is exerted through binding with its ET receptor A, since blood pressure remained normal in all animals with or without PAN injection during the period of study, and FR139317 did not modify blood pressure in a significant manner. Our data suggest that the improvement observed with FR139317 may be due to the blockade of direct ET-1 effects on the glomeruli. FR139317 reduced urinary protein excretion and attenuated the decreased mRNA levels for HSPG in the glomeruli of PAN nephropathy. We previously reported that HSPG may play an important role in proteinuria of PAN nephropathy [8]. Therefore the effect of FR139317 on urinary protein excretion may be due in part to the attenuation of reduced HSPG mRNA in glomeruli of PAN nephropathy. On the other hand this is not the only ET type of receptors in the rat kidney. ET receptor B is widely represented, particularly in glomeruli. We previously reported that mRNA levels of ET receptor B increased in the glomeruli of PAN-injected rats [25]. Recently, Iwasaki et al. [26] reported that treatment with a specific receptor B antagonist, but not an ET receptor A antagonist, abolished the increase in ET-1 gene expression in mesangial cells. ET receptor B mediates the autostimulation of ET-1 production and may amplify the adverse effects mediated by this peptide. A non-selective ET antagonist that binds both the ET receptor A and B reduced blood pressure and urinary protein excretion in the models of renal mass ablation [27]. Further studies would be needed to determine whether ET receptor B antagonist modulates the gene expression of ECM components, MMPs and TIMP-1 in the glomeruli of PAN-injected rats.

In summary, our data demonstrated that enalapril attenuates increases in mRNA levels for z1 (IV) collagen chain, laminin chains, ET-1 and decreases in mRNA levels for HSPG in glomeruli of PAN-injected rats but does not affect changes in MMP-2 and TIMP-1 mRNA expression. In addition we demonstrated that FR139317 attenuates the changes of glomerular mRNA for all components mentioned above. The gene regulation of ECM components may be different from that of MMP/TIMP in PAN nephropathy.

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References
3. Fukui M, Nakamura T, Ebihara I et al. Effects of enalapril on


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