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Different roles of two types of endothelin receptors in partial ablation-induced chronic renal failure in rats

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Abstract

Recent work has drawn attention to endothelin as a likely contributor to renal pathogenesis. To elucidate the mechanism of progressive renal disease, we investigated the mRNA expression of endothelin and endothelin receptors, and the effect of endothelin ET_A, and/or ET_B receptor antagonists on disease progression in the remnant kidney model. Proteinuria progressively increased in rats subjected to 5/6 nephrectomy (Nx) after 8 weeks (from 25 ± 3 to 221 ± 28 μg min^{-1} kg^{-1}). Creatinine clearance (Ccr) after renal ablation gradually decreased by 8 weeks (from 5.04 ± 0.42 to 2.68 ± 0.26 ml min^{-1} kg^{-1}). Together with maximal proteinuria and decreased renal function, there was an increase in cortical mRNA expression of prepro endothelin-1 and endothelin ET_A receptor expression, but a decrease in endothelin ET_B receptor expression and in urinary excretion of endothelin-1. Administration (1–3 mg/day) of S-0139, (+)-disodium 27-[(*E*)-3-[2-[(*E*)-3-carboxylatoacryloylamino]-5-hydroxyphenyl]acrylayloxy]-3-oxoolean-12-en-28-oate, an endothelin ET_A receptor-specific antagonist, had a beneficial effect on the evolution of the disease, preventing the appearance of intense proteinuria (113 ± 11) and decreased Ccr (3.97 ± 0.33). High blood pressure was observed in rats with 5/6 Nx and was decreased by S-0139 administration. To examine whether treatment modalities that decrease endothelin ET_B receptor signaling have a deleterious effect on the kidney remnant, the effect of 97-618, an endothelin ET_B receptor-specific antagonist, 4-*tert*-butyl-*N*-[5-(2-methoxyphenoxy)-6-(4-oxobutoxy)pyromidine-4-yl]benzenesulfonamide, was also examined on the action of S-0139. Concomitant administration of S-0139 and 97-618 reversed the beneficial effect of S-0139 alone in the remnant kidney on proteinuria and renal functional impairment. These findings indicate that endothelin participates in the pathogenesis of proteinuria and glomerular injury and that an endothelin ET_A receptor-specific antagonist could be useful in the treatment of some forms of human nephritis. The loss of endothelin ET_B receptor seems to be important in the progression of renal disease.

Keywords: Endothelin; Endothelin receptor; Endothelin receptor antagonist; Renal failure, chronic; Renal function; Proteinuria; RT-PCR (reverse transcription-polymerase chain reaction)

1. Introduction

Endothelin-1, a potent vasoconstrictor peptide of vascular endothelial origin, has been shown to cause a variety of biological effects in nonvascular tissue, including induction of cell hyperplasia and/or hypertrophy, and regulation of renal tubular reabsorption, as well as modulation of other hormone and cytokine production (Brenner et al., 1989; Kohan, 1993). The kidney is a primary target for the biological actions of endothelin. Many recent studies have provided reliable evidence that endothelin-1 may be a contributing factor in the process of progressive glomerulosclerosis that characterizes most experimental nephropathies (Badr et al., 1989; Kon and Badr, 1991). This is consistent with human data indicating that urinary excretion of endothelin is increased in chronic renal failure (Ohota et al., 1991) and that the endothelin concentration is higher than normal in uremic plasma, particularly in hemodialysis patients (Koyama et al., 1989; Suzuki et al., 1990). There also is compelling evidence that endothelin-1 transgenic mice do not become hypertensive, but develop glomerulosclerosis and interstitial fibrosis (Hocher et al., 1997), confirming the contribution of the renal endothelin system to the pathogenesis of glomerulosclerosis, apart from its vasoconstrictive action. However, our understanding of endothelin functions and effect is far from complete.

These effects are mediated through at least two receptor subtypes, endothelin ET_A (which is highly selective for ET-1) and ET_B (nonisopeptide selective) receptors, both of

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which are localized in various structures of the kidney (Jones et al., 1989; Kohzuki et al., 1989; Dean et al., 1994). The wide distribution of endothelin receptors is paralleled by a similarly extensive and overlapping localization of endothelin production, emphasizing the potential importance of paracrine and/or autocrine mechanisms for the action of endothelin (Simonson, 1993; Simonson and Dunn, 1992; Marsen et al., 1994). Although the contribution of two receptor subtypes in the mediation of the renal effect of endothelin in kidney disease is less well defined, endothelin receptor antagonists with different selectivities are crucial tools for elucidating the pathophysiological role of endothelin.

The remnant kidney model induced by 5/6 nephrectomy (Nx) is probably the most extensively studied model of progressive glomerular disease. Rats from this experimental model have been used to evaluate numerous pharmacological agents as to their effectiveness in the treatment of human progressive nephropathies. The finding that administration of an endothelin receptor antagonist in this model reduces glomerular injury and prevents renal function deterioration (Benigni et al., 1993) led us to suggest that the remnant kidney is a suitable model for testing this class of compounds to evaluate them for possible clinical use. Considering the findings that renal endothelin-1 gene expression increased as the disease progressed to renal insufficiency (Orisio et al., 1993), in the present study, we tried to further understand the potential role of endothelin in renal physiology and in pathophysiological states of renal disease, by confirming these findings and analyzing the gene expression of endothelin receptor subtypes in the remnant kidney using our original quantitative reverse transcription-polymerase chain reaction (RT-PCR) method.

Recent work has also led to a consensus that endothelin ET_A receptor antagonism is beneficial not only in renal, but in cardiovascular disease. However, the effectiveness of endothelin ET_B receptor antagonism is not fully justified at present. Lack of a practical endothelin ET_B receptor-selective antagonist seems to be one of the most likely reasons for the scarcity of information about endothelin ET_B receptor antagonism. Another important aim of this study was to prove the value of the endothelin ET_A receptor antagonist for chronic renal failure by direct comparisons of endothelin ET_A and ET_A/ET_B receptor blockades. In the current study, to investigate the effect of dual antagonism which corresponded to the effect of the nonselective endothelin ET_A/ET_B receptor antagonists, we cotreated with each of the specific antagonists.

2. Materials and methods

2.1. Experimental design

The study consisted of three series of experiments. Experiment I: dose–response and time course of the effect of an endothelin ET_A receptor-specific antagonist, S-0139, (+)-disodium 27-[(*E*)-3-[2-[(*E*)-3-carboxylatoacryloylamino]-5-hydroxyphenyl]acrylayloxy]-3-oxoolean-12-en-28-oate, K_i value ET_A: 1.0 nM, ET_B: > 1000 nM (Mihara et al., 1993), on renal function and measurement of mRNA levels of endothelin and endothelin receptors. Experiment II: antihypertensive effect of S-0139. Experiment III: effect of 97-618, 4-*tert*-butyl-*N*-[5-(2-methoxyphenoxy)-6-(4-oxobutoxy)pyromidine-4-yl]benzenesulfonamide, a newly synthesized selective endothelin ET_B receptor antagonist, K_i value ET_A: 910 nM, ET_B: 0.15 nM (Kawanishi et al., 1998) in rats given S-0139 to evaluate the dual antagonism of endothelin receptors.

2.2. Animal study

All experiments utilized male Wistar rats with an initial age of 8 weeks. All rats had free access to standard laboratory chow and water. The remnant kidney model was induced by surgical renal reduction (5/6 Nx) in two stages. The rats were anesthetized with pentobarbital (50 mg/kg i.p.) for all surgical procedures. Initially, a left midflank incision was made and the left kidney was exteriorized. The renal vessel was temporarily occluded with a hemostatic clamp and both poles of the kidney (two-thirds of the functioning kidney mass) were excised with scissors. Bleeding was controlled with thrombin (Mochida, Tokyo, Japan) administered onto the cut surface. The kidney stump was returned to the abdominal cavity and the incision was closed. Rats were allowed 1 week of recovery before the right kidney was exposed, the renal vessel and ureter were ligated with a silk suture, and the total kidney was removed. As the nonablated control, both stages of the sham operation with manipulation of the renal pedicles involved exteriorizing the kidney and subsequently replacing the intact kidney back into the abdominal cavity. The rats were then given drug vehicle or endothelin antagonists, S-0139 (3 and 1 mg/day) and/or 97-618 (0.5 mg/day), by continuous infusion from a subcutaneous pump (Alzet; osmotic pump, Alza, Palo Alto, CA). S-0139 and 97-618 were dissolved in sterile saline and polyethylene glycol #300, respectively. Because the longest acting pumps are only effective for 4 weeks, they were changed to new ones in the middle of the study. The doses of S-0139 chosen had been tested and predicted to give a pharmacological effect because S-0139 given as a 30 mg/kg single intravenous injection caused a significant hypotensive effect in normotensive rats (Ninomiya et al., unpublished data) and i.p. administration of S-0139 at ≥ 10 mg/kg offered prevention against acute renal failure (Shimizu et al., 1998). In the present study, the 3 mg/rat corresponds to 10 mg/kg if 300 g is the assumed for body weight of the animal. The dose of 97-618, 0.5 mg/rat corresponded to 1.7 mg/kg, was chosen based on the relative potency for the antagonism of S-0139 in vitro which was used to predict the equivalent efficacy for

S-0139. In a preliminary study of the pharmacological potency of 97-618 using anesthetized normotensive rats ($n = 4$), we confirmed that 97-618 (0.03 mg/kg i.v.) significantly inhibits the transient decrease in mean arterial pressure (MAP) seen initially after the injection of endothelin-1 (0.3 nmol/kg i.v.), and significantly enhances the subsequent, sustained pressor responses to endothelin-1 (base; 95 ± 3, $-\Delta$; 15 ± 1 and Δ; 8 ± 1 mm Hg in endothelin-1 alone vs. base; 91 ± 3, $-\Delta$; 5 ± 1 and Δ; 35 ± 3 mm Hg in endothelin-1 with 97-618 pretreated). The effect was dose-related and the minimum effective dose was 0.01 mg/kg of 97-618. In Experiment III during the final week of the study, blood samples from the tail vein of the antagonist-treated rats were taken to determine the plasma S-0139 and 97-618 concentrations by radio receptor assay for each receptor as described previously (Mihara et al., 1993).

2.3. Urine collection

After renal ablation, 5-h urine samples (9:00 a.m.–2:00 p.m.) were collected from individual rats in metabolic cages every 2 weeks in Experiment I, after 4 and 8 weeks in Experiment II and after 8 weeks only in Experiment III, as previously described (Shimizu et al., 1988) for determination of protein and creatinine excretion. Blood was withdrawn from the tail vein with the rat under ether anesthesia for creatinine determination at the end of the urine collection period. The rats were killed 8 weeks after ablation, the kidneys were excised and the cortical tissue was processed for RNA extraction in Experiment I.

2.4. Measurement of MAP

On the morning of the experiment in Experiment II, catheters were surgically implanted in the femoral artery under pentobarbital anesthesia. The left and right femoral artery were used for measurements at 4 and 8 weeks, respectively, after completion of the reduction in renal mass. One end of the catheters was tunneled subcutaneously to the nape of the neck, where the end was sealed by heating. Arterial lines were filled with a heparin–saline solution and occluded when not in use. The animals were allowed at least 5 h of recovery after anesthesia before measurements were begun. To minimize pain on awakening, Xylocaine® (surface anesthetic) was sprayed onto the wound at the neck and groin. MAP was recorded by connecting the catheter to a pressure transducer (TP-400T, Nihon Kohden, Tokyo) attached to a carrier amplifier (AP-601G, Nihon Kohden) and a recorder (WT-645G, Nihon Kohden) and was continuously monitored for 10–20 min until it became stable.

2.5. Biochemical study

Proteinuria was determined by the pyrogallol red method (Micro TP-Test Wako, Wako, Osaka, Japan). Creatinine in urine and plasma was measured by the alkaline picrate method (Creatinine-Test Wako). Endogenous creatinine clearance (Ccr), as an index of changes in renal function, was calculated by employing a standard formula. Immunoreactive endothelin-1 levels in plasma and urine were measured by radioimmunoassay as described in a previous paper (Shimizu et al., 1998) using a commercially available kit (Amersham, Buckinghamshire, UK).

2.6. Total RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from the renal cortex or glomerulus, which was isolated from the cortex by a sieving method in some experiments, using the acid–guanidinium thiocyanate–phenol–chloroform method (Shomczynski et al., 1987). Tissue weighing 50–100 mg was dispersed at 4°C in 4 ml of 4 M guanidine thiocyanate, containing 0.5% sodium sarcosyl and 0.7% 2-mercaptoethanol, with a Polytron homogenizer. The homogenate was mixed with 0.4 ml of 2 M sodium acetate, pH 4.0, 4 ml of phenol, and 0.8 ml of chloroform–isoamylalcohol (49:1, v/v) and kept on ice for 20 min. The sample was centrifuged at $10{,}000 \times g$ for 20 min, and the aqueous phase was extracted with phenol–chloroform. RNA in the aqueous phase was precipitated with isopropanol, collected by centrifugation at $15{,}000 \times g$ for 20 min, and washed with 75% ethanol. The RNA pellet was dissolved in 0.1% diethylpyrocarbonate-treated water and stored at −70°C until use. The concentration of RNA isolated was calculated on the basis of absorbance at 260 nm.

The samples (2 μg of total RNA/30 μl) were heated to 70°C for 10 min and cooled on ice for physical extension of the mRNA. Twenty microliters of the RT reaction mixture containing reaction buffer, 10 mM dithiothreitol, 1 mM dNTP, 50 ng of oligo-dT12-18 primer, 40 units of RNase inhibitor, and 200 units of Super Script™ reverse transcriptase (GIBCO/BRL, Gaitherburg, MD) was added. The reaction mixture (50 μl) was incubated at 37°C for 60 min. At the end of the incubation, the reaction mixture was heated to 95°C for 5 min to inactivate the reverse transcriptase and to denature RNA–cDNA hybrids. The samples were treated with 30 units of RNase H (Takara Shuzo, Kyoto, Japan) at 37°C for 30 min.

Five microliters of the RT reaction mixture was used for PCR amplification with prepro endothelin-1 and endothelin ET_A and ET_B receptors-specific oligonucleotide primers. The expression of β-actin mRNA was also determined as an internal control. PCR primers were selected from published cDNA sequences (Krapf and Solioz, 1991; Lin et al., 1991; Sakurai et al., 1990, 1991) and commercially synthesized (Takara Shuzo). Prepro endothelin-1 primer 1 (forward) was defined by bases 157–176, sequence 5′-TGCTCCTGCTCCTCCTTGAT-3′; primer 2 (reverse), bases 608–627, sequence 5′-CACCACGGGGCTCTGTAGTC-3′. The cDNA amplification product was

predicted to be 471 bp in length. Endothelin ET_A receptor primer 1 (forward) was defined by bases 671–690, sequence 5′-TCGTCATGGTACCCTTCGAA-3′; primer 2 (reverse), bases 1282–1301, sequence 5′-CTCGGTGCT-

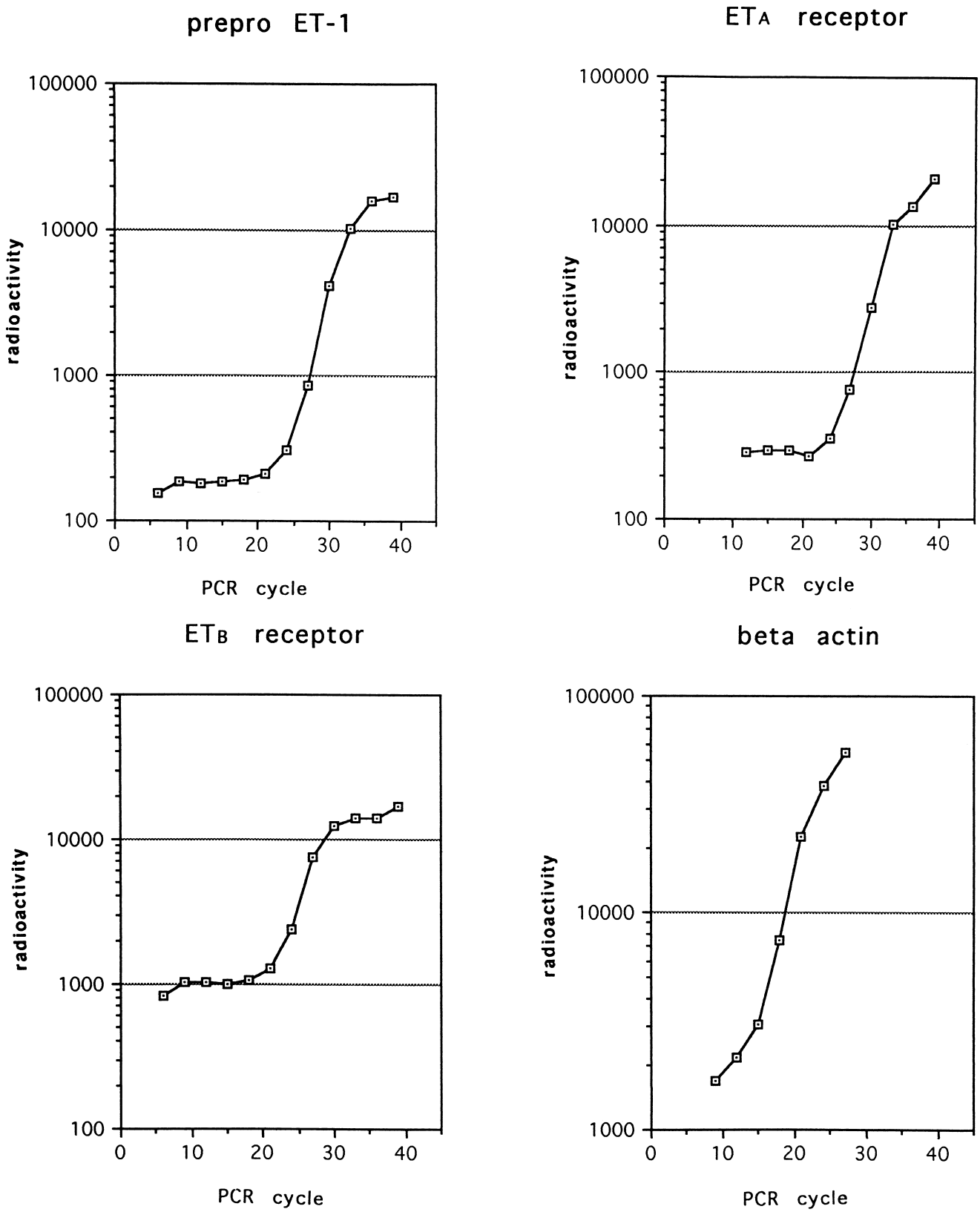

Fig. 1. PCR amplification curve of prepro endothelin-1, endothelin ET_A receptor, endothelin ET_B receptor and β-actin.

TCTGCACAGGG-3′. The cDNA amplification product was predicted to be 631 bp in length. Endothelin ET_B receptor primer 1 (forward) was defined by bases 801–820, sequence 5′-TTACAAGACAGCCAAAGACT-3′; primer 2 (reverse), bases 1346–1365, sequence 5′-CACGATGAG-GACAATGAGAT-3′. The cDNA amplification product was predicted to be 565 bp in length. β-Actin primer 1 (forward) was defined by bases 2170–2194, sequence 5′-CTGATCCACATCTGCTGGAAGGTGG-3′; primer 2 (reverse), bases 3055–3079, sequence 5′-ACCTTCAA-

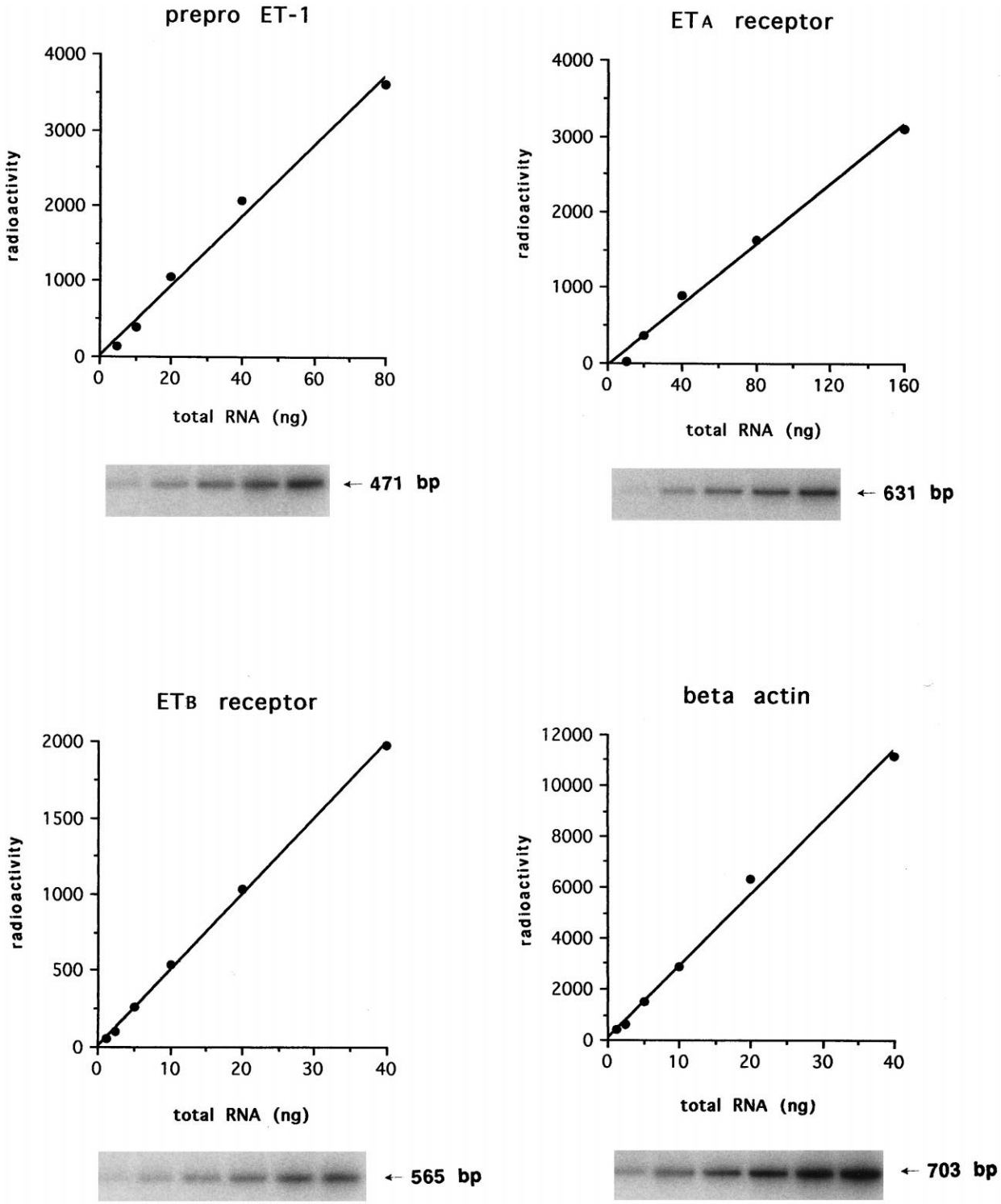

Fig. 2. Analysis of the relationship between the quantity of input total RNA in dilution and the resulting amplified products (upper) and the scanning image of an autoradiograph of the individual gel (lower) showing prepro endothelin-1 (27 cycles), endothelin ET_A receptor (27 cycles), endothelin ET_B receptor (24 cycles) and β-actin (18 cycles) by RT-PCR.

CACCCCAGCCATGTACG-3′. β-Actin primers spanned two introns and resulted in a 703-bp product. Fifty picomoles each of primers 1 and 2 were used for each reaction for prepro endothelin-1, endothelin ET_A receptor, endothelin ET_B receptor and β-actin. Five units of Taq DNA polymerase (Takara Shuzo), reaction buffer and 2 mM dNTP were used for each PCR amplification. One microliter of [α-^{32}P]dCTP (10 μCi, 370 kBq/μl, Amersham) was added to the reaction mixture to label the PCR products. The reaction mixture (50 μl) was overlaid with 50 μl of mineral oil. PCR was carried out using a PCR thermal cycler (ASTEC, PC-800, Fukuoka, Japan). The cycle profile included denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 1 min at 72°C. The reception cycles for each amplification were set as described below.

Five microliters of the PCR products was size-fractionated by 3% agarose gel electrophoresis and the labeled DNA bands were blotted onto a nylon membrane with a Gel Drying Processor (Atto, AE-3700, Tokyo, Japan). Autoradiography with an imaging plate was performed at room temperature for 5 min. The radioactivity of the labeled cDNA bands on the imaging plate was measured using a Bioimage Analyzer (Fujix, BAS2000II, Fuji Film, Tokyo, Japan). The radioactivity of prepro endothelin-1 and endothelin ET_A and ET_B receptors was normalized to that of β-actin.

As PCR amplification generally lacks quantitative reliance, we first tried to optimize the quantitative PCR. The optimum number of amplification cycles was chosen within the exponential phase that demonstrated a linear correlation between the amount of cDNA and yield of PCR products on the basis of pilot experiments (Fig. 1). From these findings, we decided to estimate the amount of amplified product for prepro endothelin-1, endothelin ET_A receptor, endothelin ET_B receptor and β-actin at 27, 27, 24 and 18 cycles, respectively. To establish the quantitative analysis of mRNA levels with the use of these settings, we confirmed the linearity between the quantity of starting material (total RNA) and that of the amplified product (cDNA). Quantitative analysis was first performed by serial dilution of total RNA isolated from normal kidney cortex as the starting material. A linear regression relationship was obtained for prepro endothelin-1, endothelin ET_A receptor, endothelin ET_B receptor and β-actin within 80, 160, 40 and 40 ng of total RNA, respectively (Fig. 2). Our use of total RNA in RT-PCR amplification was 20 ng: the initial total RNA (2 μg) was finally diluted a hundred times as described above.

The applicability of this method was subjected to preliminary testing in another experiment using similar samples, indicating that the basal level of prepro endothelin-1 and endothelin ET_B receptor mRNA was much higher (> 2-fold, ET_A was comparable) in the medulla than in the cortex which were prepared from the same kidney without disease. These data are consistent with the general observation that endothelin-1 and endothelin ET_B receptor are abundant in the medulla, supporting the reliability of this PCR amplification for quantitation.

2.7. Statistical analysis

All results are expressed as means ± S.E. Comparisons between two groups were analyzed by Student's unpaired *t*-test. Comparisons among three or more groups were analyzed by one-way analysis of variance followed by Dunnett's test to evaluate statistical significance of differences between any two groups. All tests were conducted at the 5% two-tailed probability levels.

3. Results

3.1. Experiment I

3.1.1. Endothelin excretion and plasma endothelin concentration

To confirm the participation of endothelin in this model, urine and plasma endothelin-1 levels were measured in both sham-operated and 5/6 nephrectomized groups. Urinary endothelin-1 excretion (amol min^{-1} kg^{-1}) was significantly higher in 5/6 nephrectomized rats (236 ± 8, $n = 7$, $P > 0.05$ at 4 weeks and 273 ± 25, $n = 9$, $P > 0.05$ at 8 weeks) than in sham-operated rats (159 ± 10, $n = 5$ at 4 weeks and 112 ± 4, $n = 8$ at 8 weeks after ablation). Treatment with S-0139 had no significant effect on the urinary endothelin-1 excretion (274 ± 14, $n = 8$ at 4 weeks and 305 ± 36, $n = 8$ at 8 weeks after ablation). No significant change was observed in the plasma endothelin-1 level (fmol/ml) between sham-operated (5.3 ± 0.5, $n = 8$) and untreated 5/6 nephrectomized rats (6.2 ± 0.2, $n = 6$) at 8 weeks after ablation.

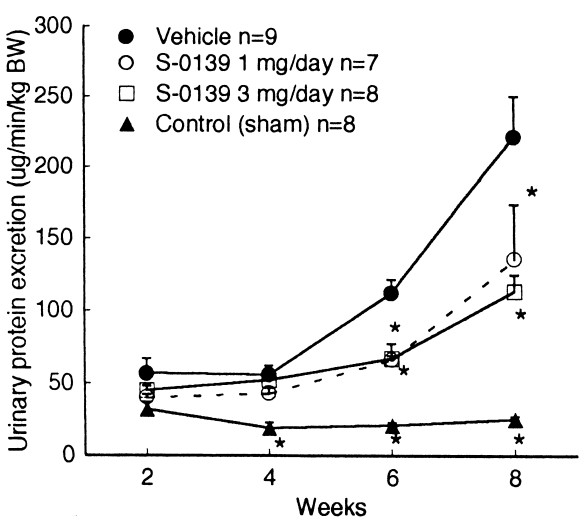

Fig. 3. Time course of urinary protein excretion in 5/6 nephrectomized or sham-operated rats. Values are means ± S.E., $^*P < 0.05$ vs. 5/6 nephrectomized vehicle at each period.

Table 1
Effect of S-0139 on creatinine clearance (ml min^{-1} kg^{-1}) in rats with 5/6 Nx receiving or not receiving S-0139 or in sham-operated rats
Values are means ± S.E.

	2 weeks	4 weeks	6 weeks	8 weeks
Sham-operated, $n = 8$	4.73 ± 0.19	4.42 ± 0.48	5.04 ± 0.35	5.04 ± 0.42
5/6 Nx vehicle, $n = 9$	2.98 ± 0.25[a]	3.43 ± 0.48	3.14 ± 0.19[a]	2.68 ± 0.26[a]
5/6 Nx S-0139 (1 mg/day), $n = 7$	3.40 ± 0.20[a]	4.47 ± 0.65	3.98 ± 0.30[ab]	3.08 ± 0.40[a]
5/6 Nx S-0139 (3 mg/day), $n = 8$	3.23 ± 0.28[a]	3.96 ± 0.26	3.73 ± 0.29[a]	3.97 ± 0.33[b]

[a] $P < 0.05$ compared to sham-operated.
[b] $P < 0.05$ compared to 5/6 Nx vehicle.

3.1.2. Effect of S-0139 on urinary protein excretion (UproV)

We next examined whether S-0139 could ameliorate the proteinuria induced by 5/6 Nx. As illustrated in Fig. 3, a progressive increase was observed in groups of 5/6 nephrectomized rats by 8 weeks. These groups showed significantly higher protein excretion from 4 weeks when compared with the sham-operated group. Protein excretion in the untreated 5/6 nephrectomized group reached 221 ± 28 μg min^{-1} kg^{-1} at 8 weeks (sham-operated group; 25 ± 3). However, animals given S-0139 showed only a modest increase. From 6 weeks, protein excretion in the S-0139-treated group was significantly lower than that in the untreated group. The proteinuria reduction was significantly effective at 6 and 8 weeks in the S-0139-treated groups. The values at 8 weeks for the 3 and 1 mg/day group of animals with 5/6 Nx were 113 ± 11 ($P < 0.01$) and 135 ± 38 ($P < 0.05$), respectively. These results indicate that administration of S-0139 attenuates the progression of proteinuria in renal ablation with 5/6 Nx.

3.1.3. Effect of S-0139 on Ccr

Table 1 summarizes the changes in Ccr after renal ablation with or without S-0139 treatment for four groups of animals. 5/6 Nephrectomized rats gradually exhibited decreased renal function with time. At the end of the experimental period, Ccr in 5/6 nephrectomized rats decreased by half compared with that in the sham-operated ones. This decrease could be significantly attenuated by S-0139 (1 mg/day at 6 weeks and 3 mg/day at 8 weeks) treatment.

3.1.4. mRNA level of prepro endothelin-1 and endothelin ET_A and ET_B receptors in the kidney

RT-PCR analysis for prepro endothelin-1 and endothelin ET_A and ET_B receptors was performed on total RNA

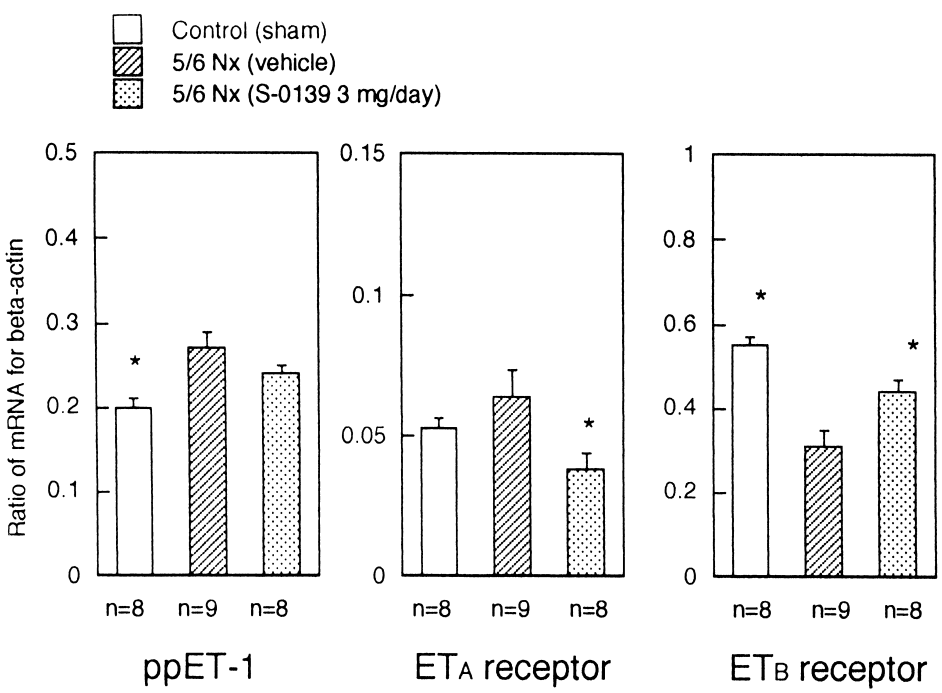

Fig. 4. mRNA levels of prepro endothelin-1 and endothelin ET_A and ET_B receptors in kidney cortex measured in rats with 5/6 nephrectomized or sham-operated rats at the end of the experimental period (8 weeks). Values are means ± S.E., * $P < 0.05$ vs. 5/6 nephrectomized vehicle.

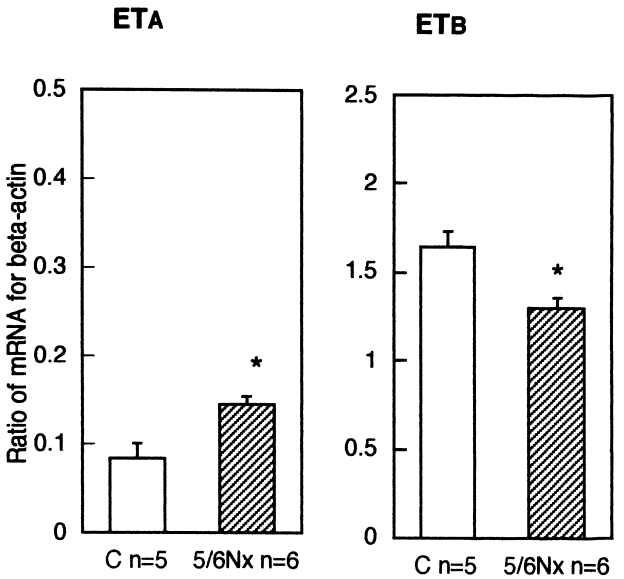

Fig. 5. Glomerular mRNA levels of endothelin ET_A and ET_B receptors measured in rats with 5/6 nephrectomized (hatched bars) or sham-operated rats (open bars) at the end of the experimental period (8 weeks). Values are means ± S.E., $^*P < 0.05$ vs. 5/6 nephrectomized vehicle.

prepared from the renal cortex after 8 weeks following renal ablation and the data are summarized in Fig. 4. There was a significant increase in prepro endothelin-1 mRNA expression of the cortex in the untreated 5/6 nephrectomized rats (prepro endothelin-1: β-actin ratio for sham-operated, 0.20 ± 0.01 vs. untreated 5/6 nephrectomized, 0.27 ± 0.02, $P < 0.05$). S-0139 had no effect upon prepro endothelin-1 mRNA expression (0.24 ± 0.01) in 5/6 nephrectomized rats. The change in expression of endothelin ET_A receptor was not statistically significant, but there was a favorable up-regulation in 5/6 nephrectomized rats. There was a significant amelioration to an up-regulated mRNA level of endothelin ET_A receptor on S-0139 administration. In contrast, the expression of endothelin ET_B receptor was significantly down-regulated in 5/6 nephrectomized rats (endothelin ET_B receptor: β-actin ratio for sham-operated, 0.55 ± 0.02 vs. untreated 5/6 nephrectomized, 0.31 ± 0.04, $P < 0.001$). Treatment with S-0139 produced a significant increase in endothelin ET_B receptor mRNA expression in the cortex (0.44 ± 0.03, $P < 0.05$).

In additional experiments, the expression of endothelin ET_A and ET_B receptors mRNA in the glomerulus of the kidney from both sham-operated and 5/6 nephrectomized rats was measured after 8 weeks following ablation (Fig. 5). 5/6 Nx caused a significant increase in the amount of endothelin ET_A receptor mRNA of the glomerulus. In contrast, the level of endothelin ET_B receptor found in the glomerulus of rats after 5/6 Nx was significantly lower than that in sham-operated rats.

3.2. Experiment II

We next determined whether S-0139 had an antihypertensive effect in this model (Table 2). MAP was significantly higher in the rats with 5/6 Nx than in the sham-operated rats. Administration of S-0139 to 5/6 nephrectomized rats had little effect on blood pressure. A significant decrease of MAP was observed at 4 weeks after renal ablation, but the decrease was not significant at 8 weeks. In contrast, S-0139 had a significant effect on inhibition of proteinuria even at 8 weeks, as found in Experiment I.

3.3. Experiment III

The present findings suggested that selective down-regulation of endothelin ET_B receptor, but not endothelin ET_A receptor, occurs in the chronic renal failure. To clarify the possible involvement of endothelin ET_B receptor in this study, we examined the effects of a specific inhibitor of endothelin ET_B receptor, 97-618, on the ability of endothelin ET_A receptor antagonist, S-0139, to ameliorate decreased renal function in 5/6 nephrectomized rats (Table 3). As in both Experiments I and II, treatment with S-0139 alone significantly reduced proteinuria in 5/6 nephrectomized rats after 8 weeks in comparison to that in the untreated animals. In contrast, the combination of 97-618 administration with S-0139 increased the UproV compared with S-0139, thus cancelling the beneficial effect of S-0139 given alone.

Likewise, S-0139 treatment ameliorated the reduced renal function detected by Ccr in comparison to the case without treatment. Addition of 97-618 to the treatment regimen significantly decreased Ccr observed with S-0139 alone. There was no significant difference in the Ccr

Table 2
MAP, UproV and Ccr in rats with 5/6 Nx receiving or not receiving S-0139 or in sham-operated rats
Values are means ± S.E.
Number of animals is shown in parentheses.

	MAP (mm Hg)		UproV (μg min^{-1} kg^{-1})		Ccr (ml min^{-1} kg^{-1})
	4 weeks	8 weeks	4 weeks	8 weeks	8 weeks
Sham-operated	99 ± 2 (5)	98 ± 2 (5)	22 ± 2 (5)	20 ± 2 (5)	5.80 ± 0.68 (5)
5/6 Nx vehicle	118 ± 3[a] (5)	120 ± 6[a] (7)	269 ± 82[a] (7)	361 ± 87[a] (7)	3.25 ± 0.27[a] (7)
5/6 Nx S-0139 (3 mg/day)	105 ± 3[b] (6)	115 ± 2[a] (6)	77 ± 11[ab] (7)	142 ± 30[ab] (6)	4.43 ± 0.27 (6)

[a] $P < 0.05$ compared to sham-operated.
[b] $P < 0.05$ compared to 5/6 Nx vehicle.

Table 3
Effect of S-0139 given concomitantly with 97-618 on urine flow, UproV, Ccr and plasma concentration of both antagonists measured in rats with 5/6 Nx at the end of the experimental period (8 weeks)
Values are means ± S.E.
ND = not determined in this study.

	Urine flow (μl min^{-1} kg^{-1})	UproV (μg min^{-1} kg^{-1})	Ccr (ml min^{-1} kg^{-1})	Plasma concentration (pmol ml^{-1})	
				S-0139	97-618
Control, $n = 8$	59.7 ± 3.1	252 ± 56	2.70 ± 0.26	ND	ND
S-0139 (3 mg/day), $n = 8$	51.5 ± 3.9	101 ± 14[a]	3.32 ± 0.11	201 ± 18	ND
S-0139 (3 mg/day), 97-618 (0.5 mg/day), $n = 8$	45.1 ± 7.0	132 ± 37	2.50 ± 0.13[b]	465 ± 32[b]	52.5 ± 4.9
97-618 (0.5 mg/day), $n = 8$	55.8 ± 6.7	141 ± 20	2.92 ± 0.30	ND	42.7 ± 2.6

[a] $P < 0.05$ compared to control.
[b] $P < 0.05$ compared to S-0139 alone.

between untreated animals and those treated with S-0139 plus 97-618. Administration of 97-618 alone did not have any notable effect on either proteinuria or renal function of 5/6 nephrectomized rats. These results indicate that cotreatment with an endothelin ET_B receptor antagonist and an endothelin ET_A receptor antagonist reversed the beneficial effect of endothelin ET_A receptor antagonism seen on both proteinuria and reduced renal function in 5/6 nephrectomized rats.

Plasma concentrations of S-0139 and 97-618 that were > 100-fold higher than each K_i value were observed in the present study. Addition of 97-618 to S-0139 significantly increased the plasma concentration of S-0139 compared with that found in S-0139 alone, but the exact mechanism of this effect is not known at present.

In another study using the same protocol as for Experiment III, we confirmed that 97-618 either alone or in combination with S-0139 had no effect on blood pressure (data not shown). It was also evident that 97-618 had no effect on blood pressure in our other study, showing that 97-618 at an oral dose of 30 mg/kg does not affect the blood pressure of conscious normotensive rats in 2, 4, 6 and 24 h when measured by the tail cuff method (Kawakami et al., unpublished data). In addition, 97-618 at this dose could be absorbed on oral administration and remained at an effective plasma level, 15-5 nmol/ml, for 1–8 h (Mihara et al., unpublished data).

4. Discussion

In the present study, mRNA isolated from a kidney with 5/6 Nx showed up-regulation for prepro endothelin-1. Coincidentally, glomerular endothelin ET_A receptor was up-regulated, whereas the mRNA for endothelin ET_B receptor was down-regulated. Circulating or local levels of peptide hormones may directly regulate the target tissue receptor content, and plasma concentrations may be negatively correlated with the density of specific receptors (Catt et al., 1979). Based on these considerations, the increased endothelin-1 levels in the kidney may be expected to cause down-regulation of both endothelin receptors. Therefore, our finding of an elevated endothelin ET_A receptor mRNA level in the remnant kidney is surprising. Clozel et al. (1993) demonstrated that endothelin-1 produced by cells in culture can cause down-regulation of both endothelin receptors in the same cells by an autocrine mechanism. The possible mechanisms involved in these phenomena include receptor desensitization or down-regulation of the expression of genes for the receptors (Hirata et al., 1988). Up-regulation of the endothelin receptor associated with increased prepro endothelin-1 mRNA levels has been observed in various kidney diseases (Nakamura et al., 1993, 1995; Shimizu et al., 1998). The current study demonstrated the response of ''normal'' down-regulation on endothelin ET_B receptor of the ablated kidney in the presence of increased local production of endothelin-1. Whether this represents a direct effect on the endothelin receptor genes to alter their transcription and/or the stability of their gene transcripts remains to be determined. Furthermore, additional study is needed to determine whether this alteration in gene expression is translated into alterations in protein levels, as assessed by their receptor density measured by radioligand techniques.

Next is the physiological or pathophysiological significance of the present results. We speculate that the down-regulation of endothelin ET_B receptor and coincidental up-regulation or steady state level of endothelin ET_A receptor is an example of heterogeneous gene expression occurring in the final stages of chronic renal failure with differences in the balance between endothelin ET_A and ET_B receptors in the kidney, especially the glomerulus, as the pathogenic factor. Mesangial proliferation, which is widely accepted as an action mediated through the endothelin ET_A receptor (Ohlstein et al., 1992; Kohono et al., 1994; Nitta et al., 1995), is considered to be a major mechanism involved in glomerulosclerosis (Fukuda et al., 1996). Although the physiological role of endothelin ET_B receptor is still controversial, recent reports have shown that the two receptors have an adverse effect on each other, for example, endothelin ET_A receptor mediates vasoconstrictor responses, whereas endothelin ET_B receptor mediates va-

sodilator effects (Takayanagi et al., 1991) via endothelin-induced release of nitric oxide. In addition, the finding that L-arginine supplementation antagonizes endothelin-1-induced mesangial cell proliferation (Mattana and Singhal, 1995) may suggest a possible pathway in which stimulation of endothelin ET_B receptor inhibits cell growth. Therefore, the absence of endothelin ET_B receptor induction in the glomerulus, which would occur in the mesangial cells, may exert a deleterious effect and further exacerbate endothelin ET_A receptor-mediated mesangial proliferation, leading to the development and maintenance of glomerulosclerosis in the remnant kidney. An alternative interpretation is that up-regulation or unchanged endothelin ET_A receptor, coincidentally occurring with down-regulation of endothelin ET_B receptor, accounts for the enhanced selective renal response to endothelin-1 through endothelin ET_A receptor underlying such kidney disease. Our recent study on acute renal failure also supported this notion, demonstrating that down-regulation of endothelin ET_B receptor associated with the progression of tubular damage is important in the pathogenesis of glycerol-treated rat (Shimizu et al., 1998).

Additional action mediated by endothelin ET_B receptor leads to the suggestion that loss of endothelin ET_B receptor may be deleterious. First, endothelin ET_B receptor mediates vasodilatation and diuretic effects (Warner et al., 1989) because hypertension and antidiuresis have a negative impact on the progression of renal disease. Second, endothelin ET_B receptor helps regulate endothelin levels by clearing endothelin-1 (Fukuroda et al., 1994). Endothelin ET_B receptor blockade potentiates renal endothelin-1 action and results in elevated endothelin levels, due to inhibition of endothelin ET_B receptor-mediated clearance. The clearance role of endothelin ET_B receptor may explain the rebound increase in endothelin concentrations following administration of nonselective endothelin ET_A/ET_B receptor antagonist, but not endothelin ET_A receptor selective antagonist (Hemsen et al., 1995).

If both receptors have a functionally adverse effect on each other, the beneficial effect of endothelin ET_A receptor blockade might be counteracted by endothelin ET_B receptor antagonism, from which a deleterious effect is expected. These notions led us to the hypothesis that the endothelin ET_A receptor selective antagonist may have greater therapeutic potential than nonselective endothelin ET_A/ET_B receptor antagonist in the treatment of kidney disease. Our study demonstrated that treatment modalities, which decrease endothelin ET_B receptor signaling, cancel the beneficial effect of endothelin ET_A receptor antagonism by S-0139. Because 97-618 did not affect S-0139-induced lowering of blood pressure, we can eliminate the involvement of the blood pressure increase in the canceling effect. Furthermore, 97-618 alone had no effect, or a rather beneficial effect, on renal function, indicating that 97-618 itself does not induce renal malfunction, and suggesting that it has a paradoxical effect on the receptor subtypes opposite to that observed with S-0139. Therefore, the present data do not show an endothelin ET_B receptor antagonist per se to be deleterious.

Other recent findings suggest that a nonselective antagonist, Bosentan, has a beneficial effect on the development of nephritis, preventing the appearance of intense proteinuria, morphological lesions and renal functional impairment in experimental immune complex nephritis (Gomez-Garre et al., 1996). However, that study included no direct comparison of the efficacy with that of an endothelin ET_A receptor-specific antagonist. The potential deleterious effects of endothelin ET_B receptor antagonism are still speculative. Additional studies are needed to solve this interesting problem.

The present study contains a suggestive data suspecting a potential role of endothelin in the pathogenesis of renal disease. Our observations with the model used seem paradoxical as effect of S-0139 between amelioration of proteinuria and displayed a hypotensive effect. Namely, the hypotensive effect was significant at 4 weeks after ablation and then declined at 8 weeks when decreased proteinuria was progressing. The antihypertensive effect must be one beneficial factor of S-0139 therapy in 5/6 nephrectomized rats, although it is weak and could not have been sufficient to lessen the damage at the end-point of our study. Together with the finding that normalizing systemic blood pressure per se is not enough to prevent the progression of renal disease in this model (Anderson et al., 1986), the present data led us to suspect another specific effect of endothelin for protection against renal damage. Some effects on the local determinants of renal injury may explain the protective effect of endothelin ET_A receptor antagonism (King and Brenner, 1991).

5. Summary

Our study revealed an overexpression of endothelin in the kidney, with maximum proteinuria in rats after 5/6 renal ablation. S-0139 was effective for lowering the progression of chronic renal failure, indicating that endothelin ET_A receptor plays an important role in the development of the disease. Based on our findings, we also hypothesize that the loss of endothelin ET_B receptors is important in the pathogenesis of chronic renal failure and that the endothelin ET_A receptor-specific antagonist may be more useful than a nonselective antagonist in the treatment of some forms of human nephritis.

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