Potential Contribution of Endothelin to Renal Abnormalities in Glycerol-Induced Acute Renal Failure in Rats

TOSHIKATSU SHIMIZU, TAKAYUKI KURODA, MINORU IKEDA, SATOSHI HATA and MASAFUMI FUJIMOTO
Shionogi Research Laboratories, Shionogi & Co., Ltd., Futaba-cho, Toyonaka, Osaka 561, Japan
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ABSTRACT

We studied the possible participation of endothelin-1 (ET-1) in the pathogenesis of renal damage in glycerol-induced acute renal failure (ARF). Cortical mRNA expression of ET-1 increased, peaking at 10 hr postinjury, but this did not occur in the medulla, plasma concentration and urinary excretion of ET-1 also increased in this model. There was no change in ETA receptor mRNA, whereas the ETB receptor tended to be downregulated in the kidney after glycerol administration. In situ hybridization study demonstrated that elevated expression of prepro ET-1 was predominantly localized in cells in the proximal tubules of the nephritic kidney. The administration (30–3 mg/kg) of S-0139, (+)-disodium 27-[(E)-3-[2-[(E)-3-carboxyacryloylamino]-5-hydroxyphenyl]acrylayloxy]-3-oxoolean-12-en-28-oate, to prevent and reverse 27-[(E)-3-[2-[(E)-3-carboxylatoacryloylamino]-5-hydroxyphenyl]acrylayloxy]-3-oxoolean-12-en-28-oate, in the ARF kidney using quantitative RT-PCR and in situ hybridization. Another important aim of our study was to evaluate the ability of a novel ETAR antagonist, S-0139: (+)-disodium 27-[(E)-3-[(E)-3-carboxyacryloylamino]-5-hydroxyphenyl]acrylayloxy]-3-oxoolean-12-en-28-oate, to prevent and reverse renal dysfunction in experimental renal failure. S-0139 effectively inhibited specific [125I]ET-1 binding to ETAR with the Ki value of 1.0 nM. S-0139 was less effective in inhibiting specific [125I]labeled ET-1 or ET-3 to ETBR (K_i: 1000 nM) (Mihara et al., 1993).

Methods

Animal study. All experiments used male Sprague-Dawley rats with an initial age of 8 wk. The rats were weighed and deprived of

ABBRVIATIONS: ET, endothelin; ETAR, ET A receptor; ETBR, ET B receptor; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; dNTPs, mixture of dCTP, dGTP, dATP and dTTP; dCTP, deoxyctosinethiphosphate; ppET-1, prepro ET-1; DIG, digoxigenin; PBS, phosphate-buffered saline; TE, tris-EDTA; DTT, dithiothreitol; SDS, sodium dodecyl.
water overnight. At 16 hr later, on the day of the experiment, the rats were lightly anesthetized with ether and a 1:1 (v/v) solution of glycerol and saline was injected (10 ml/kg) into the hind limb muscle, such that each limb received one-half of the required dose. Rats in the non-ARF group were administered saline (1 ml/kg i.p.). The doses of S-0139 chosen have preliminary tested and can be predicted to give a pharmacological effect because S-0139 at 30 mg/kg single i.v. injection caused a significant hypotensive effect to normotensive rats (Ninomiya et al., unpublished data). At 24 hr after glycerol injection, spontaneously voided 5-hr urine was collected using an individual metabolism cage, as previously described (Shimizu et al., unpublished data). At 24 hr after glycerol injection, voided 5-hr urine was collected using an individual metabolism cage, as previously described (Shimizu et al., unpublished data). At 24 hr after glycerol injection, voided 5-hr urine was collected using an individual metabolism cage, as previously described (Shimizu et al., unpublished data). At 24 hr after glycerol injection, voided 5-hr urine was collected using an individual metabolism cage, as previously described (Shimizu et al., unpublished data). At 24 hr after glycerol injection, voided 5-hr urine was collected using an individual metabolism cage, as previously described (Shimizu et al., unpublished data).

In next protocol, the estimation of mortality was assessed over 7 days in the postnephritic period. For preparation of periodic RNA samples of the kidney, other animals were killed under pentobarbital anesthesia at 0, 1, 3, 6 10 and 24 hr after glycerol injection.

Further two additional experiments were performed to assess whether the drug treatment had any effect on a more moderate level of failure. First, water was allowed ad libitum, then the rats were subjected without hydropenia before the study. Second, 5 ml/kg of 50% glycerol/saline, which was a half dose as described above, was injected intramuscularly into only one hind limb following a 16-hr period of water deprivation.

Biochemical study. The creatinine concentrations of urine and plasma were determined by the alkaline picrate method using commercial kits (Wako Pure Chemical Industries Ltd., Osaka Japan). The renal function was estimated from the endogenous creatinine clearance, which was calculated by employing a standard formula.

Immunoreactive ET-1 levels in plasma and urine were measured according to the procedure of Suzuki et al. (1990) with slight modification. Samples of 2 ml each of plasma and urine were mixed with 6 ml of 8% acetic acid and boiled for 15 min to abolish intrinsic proteolytic activity. The mixtures were centrifuged at 10,000 g for 15 min. The supernatants were extracted on Sep-pac C18 cartridges (Waters Chromatography Division, Millipore Corp., Milford, MA) preactivated with 6 ml of acetonitrile and 12 ml of water. The columns were washed with 6 ml water and then ET was eluted with 3% acetonitrile. The solvent was evaporated with a centrifugal evaporator and the dry residue was dissolved with 1 ml of radioimmunoassay buffer. ET-1 levels were determined using an ET-1 assay system (Amersham, Buckinghamshire, UK).

Total RNA extraction and quantitative RT-PCR analysis. Total RNA was extracted from the renal cortex and medulla by the acid-quinolinium triacetic-phenoxy-chloroform method (Shomoszynski and Sacchi, 1987). Tissue weighing 50 to 100 mg was dispersed at 4°C in 4 ml of 0.5 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-isomylalcohol (49:1, v/v) and kept on ice for 20 min. The sample was centrifuged at 10,000 × g for 20 min, and the aqueous phase was subjected to phenol-chloroform extraction. RNA in the aqueous phase was precipitated with isopropanol, collected by centrifugation at 15,000 × 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 DTT, 1 mM dNTP, 50 ng of oligo-dT12–18 primer, 40 units of RNase inhibitor and 200 U 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 RTase activity and to denature RNA-cDNA hybrids. The samples were treated with 30 U of RNaseH (Takara Shuzo Corp., Kyoto, Japan) at 37°C for 30 min.

Five microliters of the RT-reaction mixture was used for PCR amplification with ppET-1, ETAR, ETBR and β-actin specific oligonucleotide primers. PCR primers were selected from published cDNA sequences (Sakurai et al., 1991, Lin et al., 1991, Sakurai et al., 1990, Krapf and Solozob, 1991) and commercially synthesized (Takara Shuzo Corp.). Prepro ET-1 primer 1 (forward) was defined by bases 157–176, sequence 5'-TGTCTCGTTGCTTCTCCTGAT-3'; primer 2 (reverse), bases 608–627, sequence 5'-CACCCAGGGGTCTGGTAGTCG-3'. The cDNA amplification product was predicted to be 471 bp in length. ETAR primer 1 (forward) was defined by bases 671–690, sequence 5'-TGTCTAGGTGTTACCTGGA-3'; primer 2 (reverse), bases 1282–1301, sequence 5'-CTCGGTGTCTTGTGCAGCA-3'. The cDNA amplification product was predicted to be 631 bp in length. ETBR primer 1 (forward) was defined by bases 801–820, sequence 5'-TTACAAGACAGCAGAAGACT-3'; primer 2 (reverse), bases 1346–1365, sequence 5'-CACCTGATGAGCAATGAGATG-3'. The cDNA amplification product was predicted to be 565 bp in length. Beta-actin primer 1 (forward) was defined by bases 2170–2194, sequence 5'-CTGATCCATCATCGTCTGGAACTG-3'; primer 2 (reverse), bases 3055–3079, sequence 5'-ACCTTCAACACCCAGCCATGAGC-3'. Beta-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 ET-1, ETAR, ETBR and β-actin. Five units of Taq DNA polymerase (Takara Shuzo Corp.), reaction buffer and 2 mM dNTP were used for each PCR amplification. One micromol of [α-32P]dCTP (10 µCi), 370 Kβq/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. The tubes were placed in a Program Temp Control System (ASTEC, PC-800, Fukuoka, Japan) programed as follows: 27 cycles (pp ET-1), 27 cycles (ETAR), 24 cycles (ETBR) and 18 cycles (β-actin) of the sequential steps of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (extension).

Five microliters of the PCR products were size-fractionated with 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 Inc. Tokyo, Japan). The radio activity of ppET-1, ETAR and ETBR was normalized to that of β-actin. The levels expressed as a percent of the values at the basal level.

As PCR amplification generally lacks quantitative reliance, we optimized the quantitative PCR in advance. The optimum number of amplification cycles was chosen within the exponential phase on the basis of pilot experiments. We decided to estimate the amount of amplified product for ppET-1, ERAR, ETBR 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 ppET-1, ETAR, ETBR and β-actin within 80, 160, 40 and 40 ng of total RNA, respectively. Our practical use of total RNA in RT-PCR amplification was 20 ng; the initial total RNA (2 µg) was finally diluted 100 times as described above.

In situ hybridization study. Antisense and sense single-strand cRNAs were synthesized from cDNA fragments encoding ppET-1 constructed using RT-PCR as mentioned above and subcloned into

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pCR II vector (Invitrogen, San Diego, CA). The template was linearized with the restriction enzyme BamHI (antisense probe) or XhoI (sense probe) and labeled RNA probes were synthesized with T7 (antisense probe) and Sp6 (sense probe) RNA polymerase in the presence of DIG-labeled UTP (DIG Labeling Kit, Boehringer Mannheim, Indianapolis, IN). The probes were precipitated and DIG incorporation was assessed by dot blotting as previously described (Panoskalis-Mortari and Par Bucy, 1995).

Under pentobarbital anesthesia, rats were flushed with PBS through the abdominal aorta followed by perfusion with 4% paraformaldehyde buffered with 1 M PBS (pH 7.4) at 10 hr after glycerol or saline injection after dehydration overnight. The kidneys were swiftly removed and cut into small pieces. The renal cortex tissue was immediately dissected and immersed into a fresh portion of the same fixative at 4°C overnight. All steps were carried out with care to avoid contamination with RNase. Diethylenepropionate-treated water was used at 0.1% to prepare each buffer. Fixed samples were thoroughly rinsed with 0.1 M PBS (pH 7.4) and subsequently dehydrated by passage through an alcohol series diluted with diethylenepropionate-treated water (30, 50, 70 and 90%, for 60 min in each), followed by another alcohol series (95%, 99.5%, and absolute, for 90 min in each) and alcohol-xylene mixed series (25, 50 and 75%, for 90 min in each) and 100% xylene (for 16 hr). The preparations were then passed into paraffin (Paraffin pistilles m.p. 52°C, Merck Co., Rahway, NJ) at 56°C. In situ hybridization was performed on paraffin-embedded sections as described by Angerer et al. (1987) with some modifications. Transversal tissue sections (4 μm thick) were cut from embedded blocks and mounted on slides freshly coated with 3-aminodiaminobenzidine hydrochloride substrate dissolved in 0.2X SSC for 10 min at 50°C. Free labeled cRNA was digested by 10 ng/ml of RNase A in 10 mM Tris-HCl (pH 7.6) containing 0.5 M NaCl and 1 mM EDTA (pH 8.0), and the target mRNA signals were visualized. The slides were examined under bright field microscopy with particular attention paid to evaluation of the glomerular cross views.

**Statistical analysis.** All results are expressed as mean ± S.E. Statistical analysis was performed using Student’s t test for unpaired samples. P < .05 was considered statistically significant. Statistical evaluation of the curves in survival studies was conducted using a log-rank test of Kaplan-Meier method.

**Results.** To confirm the contribution of ET in this model, we first examined the urinary ET-1 excretion and plasma ET-1 level after the glycerol injected after 24 hr (fig. 1). Both urinary excretion and the plasma level of ET-1 increased markedly in rats with glycerol injection as compared to saline-injected nonnephritic rats. These levels in glycerol-treated animals given S-0139 showed a tendency of decrease but the effect was not statistically significant compared to glycerol-injected rats receiving the vehicle.

The survival of animals subjected to glycerol treatment was assessed from over 7 days in the postnephritic period (fig. 2). Most of the vehicle-treated nephritic animals (85%) died within 7 days. S-0139 treatment dose-dependently improved the survival of animals injected with glycerol. In rats given S-0139 at 30, 10 and 3 mg/kg, respectively, 65, 60 and 40% were alive on postnephritic day 7. There were significant differences (P < .05) in all doses compared to control group (fig. 2A).

As indicated above, S-0139 provided significant improvement against mortality when given i.p. three times after glycerol injection within 9 hr. In the next protocol, we tried to identify the most effective drug application protocol for three times administrations. Figure 2B shows the mortality over 7 days after administration of single i.p. doses of S-0139 (30 mg/kg i.p.) at 1, 5 or 9 hr after glycerol injection. S-0139 was most effective when given 5 hr postinjury. Interestingly, when S-0139 was administered 1 hr after glycerol injection, no improvement in the mortality was observed as compared with the control group which was not given the drug. These two series of survival studies indicate that S-0139 can be effective for attenuating tubular impairment even after failure had occurred if the lapse is a short-term one.

**Fig. 1.** ET-1 levels in urine and plasma in rats treated with S-0139 or its vehicle after 24 hr after glycerol or saline administration. *P < .05 vs. saline.
level was lower than that seen in the above experiment. All animals of these groups survived the insult (data not shown), confirming these results. The functional impairment was also attenuated with the administration of S-0139. These results indicate that administration of S-0139 attenuates impairment of renal function after injection of glycerol.

To obtain further evidence for the possible involvement of ET in this model, we examined whether increased ET-1 production is associated with stimulation of ET mRNA expression. In addition, we examined the concurrent changes of its receptor subtypes. To characterize renal ppET-1, ETAR and ETBR mRNA expression, kidney were obtained from glycerol-treated rats in which ARF had been induced 1, 3, 6, 10 and 24 hr after injection and from saline-injected non-ARF rats (3, 10 and 24 hr) for 0 hr baseline control (fig. 3). The ppET-1 mRNA level in the renal cortex of glycerol-treated rats increased significantly except for the first few hours. The maximal 4-fold increase for the basal level was observed 10 hr after glycerol injection, then the increase decreased but remained significantly higher after 24 hr as compared to the basal level. However, in the medulla, no significant change was observed in the ppET-1 level in glycerol-treated animals throughout the experiment. There was no significant change in ETAR mRNA expression in both cortex and medulla after glycerol administration. However, the expression of ETBR was down-regulated and the change was significant at 3, 6 and 24 hr after injection of glycerol in the medulla compared to the basal level, whereas the change in the cortex was not significant. As many investigators have established, the level of ppET-1 and ETBR mRNA was much higher in the medulla than cortex. Significant 2.4- and 2.1-fold increases for the basal level of the medulla compared to the cortex observed in the ppET-1 and ETBR, respectively.

The localization of ET-1 mRNA in ARF kidney, which was positive for this mRNA at RT-PCR, was also examined by in situ hybridization (fig. 4). In diseased kidney, the brownish-colored signals resulting from diffusion of diaminobenzidine reaction products were clearly present in rat renal cortex region (i.e. the cortical tubules and endothelial cells of blood vessel), but not in the medulla. Intense signals were found mostly within the proximal convoluted tubules surrounding the glomeruli, showing areas of focal lesions of this disease model, whereas most distal convoluted tubules exhibited comparatively weak expression of the mRNA. In contrast, poor signals were seen within the proximal tubules and the endothelial cells of the arcuate artery and vein in the normal kidney. Moreover, there was no staining signal of the mRNA in tissues from normal or diseased kidneys from the controls of this experiment using the sense probe.

### Discussion

Many previous studies have implicated several vasoconstrictive substances as mediators of vasoconstriction in ARF. ET-1 is one of the most potent vasoconstrictors in the renal circulatory bed, strongly suggesting that ET-1 plays a significant pathophysiological role in human ARF. Because a reduced glomerular filtration rate with renal vasoconstriction may be the primary phenomenon and is a common feature in ARF, intervention by inhibition of the renal ET system may be of therapeutic usefulness in ARF. Our results strongly suggest that the ET-1 generated in the kidney may partici-
The present study clearly showed that the over-expressed ppET-1 mRNA was observed mainly in proximal tubules in the kidney with ARF. These results suggest that ET-1 generated in the proximal tubules may participate directly in the generation of tubular damage in ARF. Because intense ppET-1 mRNA showed at least a 4-fold increase of the basal level in the cortex at 10 hr after glycerol-injection. No increase of the ppET-1 expression was observed in the medulla. ETAR expression did not show any marked change, whereas ETBR tended to be down-regulated. The finding of a time-dependent up-regulation of ppET-1 mRNA in the kidney of glycerol-treated rats suggests that ET-1 might play an amplifying modulator role in the development of this ARF.

In this study, we demonstrated that S-0139 treatment after the induction ARF was found to be effective for preventing uremic death and that it was most effective when given 5 hr after glycerol injection. Pharmacokinetics of S-0139 may explain the differences in efficacy to understand the significance with different treatment time at 1, 5 or 9 hr postinjury treatment in this study because the pharmacological half-life of S-0139 is very short by excreted mainly from liver. Total body clearance and mean residence time at 50 mg/kg i.v. of S-0139 in normal rats (n = 3) were 360 ± 96 ml/hr/kg and 0.181 ± 0.035 hr, respectively, suggesting that t1/2 of S-0139 is within 1 hr at a guess (Okabe et al., unpublished data). The inhibitor effect was associated with preventing decreased renal function after injury. ET secretion in ARF condition seems to be affected little after administration of S-0139. Thus, S-0139 administered after ARF could elicit significant improvement damage induced by deleterious effect of ET in the kidney. This suggests that S-0139 has a therapeutic effect and can be clinically useful in patients for ARF.

A previous in situ hybridization study using normal kidney showed the presence of ET-1 in the glomeruli, vasa recta and inner medulla (MacCumber et al., 1989). Ujiie et al. (1992) demonstrated that ET-1 mRNA was detected only in the glomerulus and inner medullary collecting duct among nephron segments, using microdissected tubule fragments that were subsequently subjected to RT-PCR. These studies did not detect ET-1 mRNA in other portions of the nephron such as proximal tubules. These results agree with the results of in situ hybridization studies performed with human kidney (Pupilli et al., 1994), showing that ET mRNA was detected in vasa recta bundles and capillaries and in medullary collecting duct. Based on this evidence, ET-1 mRNA expression in the cortex derives from the glomerulus and the vascular system. In contrast to these findings, we could not detect ppET-1 mRNA in any area of the steady-state kidney. The questions of why and how different mRNA display different distributions remain to the elucidated. However, it is not well known how mRNA levels for ET are regulated in the kidney with renal disease. In situ hybridization in the present study clearly showed that the over-expressed ppET-1 mRNA was observed mainly in proximal tubules in the kidney with ARF. These results suggest that the ET-1 generated in the proximal tubules may participate directly in the generation of tubular damage in ARF. Because intense ppET-1

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**Table 2**

*Effect of S-0139 on urine flow, plasma creatinine concentration (PCr) and creatinine clearance (CCr) in two types of moderate acute renal failure by glycerol treatment*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Urine Flow (μl/min/kg)</th>
<th>PCr (μg/ml)</th>
<th>CCr (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhydropenic glycerol 10 ml/kg</td>
<td>90.0 ± 10.3</td>
<td>12.6 ± 0.8</td>
<td>3.26 ± 0.36</td>
</tr>
<tr>
<td>S-0139 30 mg (n = 8)</td>
<td>80.8 ± 4.6</td>
<td>9.3 ± 0.6</td>
<td>4.52 ± 0.39</td>
</tr>
<tr>
<td>Hydropenic glycerol 5 ml/kg</td>
<td>73.9 ± 8.8</td>
<td>13.1 ± 1.6</td>
<td>2.52 ± 0.45</td>
</tr>
<tr>
<td>S-0139 30 mg (n = 6)</td>
<td>62.6 ± 8.3</td>
<td>9.0 ± 0.9</td>
<td>3.53 ± 0.46</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E. Number of rats in parentheses.

* P < .05 significant difference between groups with and without S-0139 administration.

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**Figure 3.** Time course of kidney mRNA levels of prepro ET-1, ETA receptor and ETB receptor in glycerol-treated ARF and saline-treated non-ARF rats. The value from nonglycerol injected rats was indicated at 0 hr as a baseline control *P < .05 vs. baseline (0 hr) values. #P < .05 between cortex and medulla at basal level. Each point consists of four animals in glycerol-treated ARF and three animals in saline-treated non-ARF.
expression may be seen only in the kidney in a severe nephritic condition, studies of the mechanisms underlying the regulation of renal ET production and secretion are difficult to perform with intact kidney using our technique of *in situ* hybridization.

We showed that despite the ETAR remaining unchanged, a
down-regulation of ETBR mRNA expression was observed in damaged kidney with ARF. Because the physiological role of ETBR is not well defined, it is not clear whether its down-regulation has a beneficial or deleterious effect on the setting of ARF. However, there are indirect evidences indicating that loss of ETBR may be deleterious, because ETBR seems to have beneficial function in physiological condition. Because hypertension and anti-diuresis have a negative impact on the progression of renal disease, it is possible that ETBR mediated vasodilatation and diuresis (Warner et al. 1989) bring profitable effect in such abnormal condition. Because ETBR helps regulate ET levels by clearing ET-1 (Fukuroda et al. 1994), loss of ETBR function results in elevated ET levels due to inhibition of ETB-mediated clearance and potentiates renal ET-1 action through ETAR. The clearance role of ETBR may explain the rebound increase in ET concentrations after administration of non-selective ET antagonist but not ETA selective antagonist (Hensen et al. 1995).

More recent findings on a model of glycerol-induced renal failure suggested that the use of the nonselective ETA/ETB receptor antagonist Bosentan is beneficial for decreasing renal damage (Karam et al., 1995). According to our hypothesis, if antagonism of ETBR is deleterious in such cases, because the nonselective antagonist blocked the effects of ET at both ETAR and ETBR, the beneficial effects of ETAR blockade might be counteracted by blockade of ETBR, thus diminishing the therapeutic effect. It is clinically important to find which types of ET receptor antagonist effectively prevent renal disease. Further experiment will be required to elucidate the difference between two types of the antagonist on the therapeutic potency in comparative study under the same experimental conditions.

In summary, our results provide further evidence that ET-1 plays an important role in the pathogenesis in a rat model of ARF. Also, intervention by inhibition of the ETA receptor signal in this model offers beneficial effects as evidenced by improvement of renal function and a drop in mortality.

References


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