Protective Effect of SM-19712, a Novel and Potent Endothelin Converting Enzyme Inhibitor, on Ischemic Acute Renal Failure in Rats

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ABSTRACT—Effects of SM-19712 {4-chloro-*N*-[(4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-yl)amino]carbonyl]benzenesulfonamide, monosodium salt}, a novel endothelin converting enzyme (ECE) inhibitor, on ischemic acute renal failure (ARF) in rats were examined in comparison with those of phosphoramidon, a conventional ECE inhibitor. ARF was induced by occlusion of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. Renal function in ARF rats markedly decreased at 24 h after reperfusion. Intravenous bolus injection of SM-19712 (3, 10, 30 mg/kg) prior to the occlusion attenuated dose-dependently the ischemia/reperfusion-induced renal dysfunction. Histopathological examination of the kidney of ARF rats revealed severe renal damages such as tubular necrosis, proteinaceous casts in tubuli and medullary congestion, all of which were dose-dependently attenuated by SM-19712. Protective effects of phosphoramidon (10 mg/kg) on ARF-induced functional and tissue damages were less potent than that of the same dose of SM-19712. Endothelin-1 (ET-1) content in the kidney after the ischemia/reperfusion was significantly increased, being the maximum level at 6 h after reperfusion, and this elevation was completely suppressed by the higher dose of SM-19712. Our findings support the view that renal ET-1 plays an important role in the development of ischemia/reperfusion-induced renal injury. SM-19712 may be useful in the treatment of ischemic ARF.

Keywords: Endothelin-1, Endothelin converting enzyme, Ischemia, Acute renal failure, Renal function

Endothelin-1 (ET-1) has been considered to be involved in the pathophysiology of cardiac, vascular and renal diseases associated with regional and systemic vasoconstriction (1). The kidney is well known to synthesize ET-1 and to express both endothelin type A (ET_A) and endothelin type B (ET_B) receptors (2, 3). Studies on the localization of ET-receptor mRNA in the rat kidney using microdissection method demonstrated that ET_A receptor is present only in the vascular tissues, in which this subtype mediates the vasoconstrictor and growth effects, and that ET_B receptor distributes abundantly in the collecting tubules (4, 5). In this tubular site, ET-1 inhibits vasopressin-induced increases in water permeability and cyclic-AMP accumulation (6). Several studies indicate that the stimulation of ET_B receptors in the rat results in renal vasoconstriction, as well as renal vasodilatory responses (7–9).

There is accumulating evidence that ET-1 is closely involved in the development and progression of the ischemia/reperfusion-induced renal injury. Renal ET-1 mRNA expression (10, 11), tissue levels of ET-1 peptide (12) and its

affinity for renal ET receptor (13) were known to elevate in the post-ischemic kidney. In the same ischemic acute renal failure (ARF) model in rats, both ET_A-selective and non-selective ET_A/ET_B receptor antagonists have been indicated to attenuate the ischemia/reperfusion-induced renal dysfunction and histological damage (14–17). Moreover, using phosphoramidon, a prototype of endothelin converting enzyme (ECE) inhibitor (18–21), similar renoprotective effects on the ischemic ARF-induced renal injury have been observed (22, 23). However, phosphoramidon is not a specific ECE inhibitor and can also potentially inhibit the activity of neutral endopeptidase (24).

The purpose of the present study is to evaluate the effectiveness of selective inhibition of ECE on the ischemia/reperfusion-induced renal dysfunction and histological damage. We used a sulfonylureid-pyrazole derivative SM-19712 {4-chloro-*N*-[(4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-yl)amino]carbonyl]benzenesulfonamide, monosodium salt}, a newly developed, highly selective and potent ECE inhibitor (25). We examined the effect of SM-19712 on renal dys-

function, histological damage and renal ET-1 overproduction in ischemic ARF, and effects of the drug were compared with those seen with phosphoramidon.

MATERIALS AND METHODS

Animals and experimental design

Male Sprague-Dawley rats (280–300 g, 10 weeks of age; Charles River Japan, Shizuoka) were used. The animals were housed in a light controlled room with a 12-h light/dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (40 mg/kg, i.p.). To induce ischemic ARF, the rats were anesthetized with pentobarbital, and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a non-traumatic clamp for 45 min. At the end of the ischemic period, the clamp was released to allow reperfusion. In sham-operated control animals, kidneys were treated identically, except for the clamping.

When effects of SM-19712 or phosphoramidon on ischemic ARF were examined, SM-19712 (3, 10 and 30 mg/kg), phosphoramidon (10 mg/kg) or their vehicle (10% polyethylene glycol 400) was administered as a slow bolus injection (volume, 1 ml/kg; duration, 2 min) into the external jugular vein, 5 min before the occlusion. Doses of drugs were determined by examining the inhibitory actions on the big ET-1-induced pressor effect (dose dependent 35–80% inhibition by SM-19712 and 80–90% inhibition by phosphoramidon, respectively).

Animals exposed to 45-min ischemia were placed in metabolic cages at 24 h after the ischemia, and 5-h urine samples were taken. The animals were then anesthetized with sodium pentobarbital (40 mg/kg, i.p.), arterial blood samples obtained and left kidneys were removed. The plasma was separated by centrifugation. The kidneys were used for the determination of ET-1 content and for light microscopic observation, according to standard procedures. Plasma and urine were used for measurements of renal functional parameters and for ET-1 assay (plasma).

In separate experiments, arterial blood samples and left kidneys were obtained from animals, at 2 and 6 h after the 45-min ischemia. These samples were utilized for determinations of plasma ET-1 levels and renal ET-1 contents.

Histological studies

The kidneys were preserved in phosphate-buffered 10% formalin, after which the kidneys were chopped into small

pieces, embedded in paraffin wax, and cut at 4 μ m and stained with hematoxylin and eosin. Histopathological changes were graded as no changes (– or 0), mild ($\pm$ or 1), moderate (+ or 2), severe (++ or 3) and very severe (+++ or 4) based on the microscopical observations of each section. The evaluations were made by an observer who was blind to the treatment origin of the tissue.

Analytical procedures

Blood urea nitrogen (BUN) and creatinine levels in plasma (P_{cr}) or urine were determined using the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemical Industries, Osaka), respectively. Urinary osmolality (U_{osm}) was measured by freezing point depression (Fiske, Uxbridge, MA, USA). Urine and plasma sodium concentrations were determined by using a flame photometer (205D; Hitachi, Hitachinaka). Fractional excretion of sodium (FE_{Na}) was calculated from the following formula: $FE_{Na} = U_{Na}V / (P_{Na} \times C_{cr}) \times 100$, where $U_{Na}V$ is urinary excretion of sodium, P_{Na} is plasma sodium concentration and C_{cr} is creatinine clearance.

Renal and plasma ET-1 assay

ET-1 was extracted from the kidney, according to our method described elsewhere (26). Briefly, kidneys were weighed and homogenized for 60 s in 8 vol ice-cold organic solution (chloroform/methanol, 2:1, including 1 mM *N*-ethylmaleimide). The homogenates were left overnight at 4°C, and then 0.4 vol distilled water was added to the homogenates. Homogenates were then centrifuged at 3000 rpm for 30 min and the supernatant was stored until use. Aliquots of the supernatant were diluted 1/10 with a 0.09% trifluoroacetic acid (TFA) solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% TFA. Eluates were dried in a centrifugal concentrator, and the dried residue was reconstituted in assay buffer for radioimmunoassay (RIA). The clear solution was subjected to RIA. Recoveries of ET-1 from renal tissue by this extraction procedure is approximately 80%. RIA for tissue ET-1 was done, as described elsewhere (27), using ET-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA, USA) that does not cross-react with big ET-1 (28). ET-1 concentrations in plasma were determined by a direct ELISA method without the extraction step, using a commercial assay kit (Bio-medica Gesellschaft mbH, Vienna, Austria). The manufacturer reports that cross-reactivities of the antibodies used are <1% with big ET-1 (1–38) and big ET-1 (22–38), <5% with ET-3, and 100% with ET-2. The intraassay and interassay variabilities of the t are 4.4% and 6.9%, respectively.

Drugs

SM-19712 was provided by Sumitomo Pharmaceuticals Co., Ltd. (Osaka). It was dissolved in 10% polyethylene glycol 400. Phosphoramidon was obtained from Peptide Institute, Inc. (Osaka) and was dissolved in the same solution. Other chemicals were obtained from Nacalai Tesque (Kyoto) and Wako Pure Chemical Industries (Osaka).

Statistical analyses

Values are each a mean $\pm$ S.E.M. For statistical analysis, we used one-way analysis of variance followed by Bonferroni's multiple comparison tests. Histological data were analyzed by the Kruskal-Wallis nonparametric test combined with a Steel-type multiple comparison test. For all comparisons, differences were considered significant at $P < 0.05$.

RESULTS

Renal function after the ischemia/reperfusion and effects of SM19712 and phosphoramidon

As shown in Figs. 1 and 2, renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 24 h after the reperfusion. As compared with

sham-operated rats, untreated ARF rats showed significant increases in BUN (76.3 ± 9.6 vs 26.3 ± 1.7 mg/dl), Pcr (1.57 ± 0.17 vs 0.66 ± 0.04 mg/dl), urine flow (UF) (79.8 ± 11.1 vs 27.9 ± 2.9 μ l/kg per minute) and FE_{Na} ($0.83 \pm 0.20\%$ vs $0.24 \pm 0.04\%$) and significant decreases in C_{cr} (2.30 ± 0.31 vs 4.57 ± 0.42 ml/kg per minute) and Uosm (523 ± 69 vs 1490 ± 124 mOsm/kg). The administration of SM-19712 (3, 10 and 30 mg/kg, i.v.) produced dose-dependent preventive effects against the ischemia-induced deterioration of renal function. When 30 mg/kg of SM-19712 was given, all renal function changes induced by the ischemia/reperfusion were significantly and markedly suppressed (BUN, 42.1 ± 4.4 mg/dl; Pcr, 0.83 ± 0.05 mg/dl; C_{cr} , 4.80 ± 0.37 ml/kg per minute; UF, 44.3 ± 2.9 μ l/kg per minute; U_{osm} , 1088 ± 82 mOsm/kg; FE_{Na} , $0.32 \pm 0.05\%$). Phosphoramidon (10 mg/kg) also attenuated the decreased responses of renal function to the ischemia, although the effects were only partial and less potent than those observed by 10 and 30 mg/kg SM-19712.

Histological renal damage after the ischemia/reperfusion and effects of SM19712 and phosphoramidon

Histopathological examination revealed severe lesions in the kidney of untreated ARF rats (24 h after the 45-min

Fig. 1. Effects of SM-19712 and phosphoramidon on blood urea nitrogen (BUN, A), plasma creatinine (P_{cr} , B) and creatinine clearance (C_{cr} , C) at 1 day after ischemia/reperfusion. Drugs were given intravenously 5 min before the ischemia (45 min). ARF, acute renal failure. Each column and bar represents the mean $\pm$ S.E.M. of 7–8 rats. * $P < 0.05$, ** $P < 0.01$, compared with sham rats; † $P < 0.05$, ‡ $P < 0.01$, compared with untreated ARF rats.

	Sham (n=6)	Untreated ARF (n=7)	ARF + Phosphoramidon 10 mg/kg (n=8)	ARF + SM-19712		
				3 mg/kg (n=7)	10 mg/kg (n=8)	30 mg/kg (n=8)
Histopathological changes/grade	- ± + ++ +++ (0 1 2 3 4)	- ± + ++ +++ (0 1 2 3 4)	- ± + ++ +++ (0 1 2 3 4)	- ± + ++ +++ (0 1 2 3 4)	- ± + ++ +++ (0 1 2 3 4)	- ± + ++ +++ (0 1 2 3 4)
Tubular necrosis	6 0 0 0 0	0 0 0 3 4 (3.57 ± 0.24)	0 0 6 2 0 (2.25 ± 0.16 ^b)	0 1 1 4 1 (2.71 ± 0.22)	1 3 2 2 0 (1.62 ± 0.38 ^b)	1 5 2 0 0 (1.12 ± 0.23 ^b)
Proteinaceous casts in tubuli	6 0 0 0 0	0 0 0 5 2 (3.29 ± 0.18)	0 2 2 3 1 (2.38 ± 0.38)	0 3 1 3 0 (2.00 ± 0.38 ^a)	0 4 1 3 0 (1.88 ± 0.35 ^a)	1 6 1 0 0 (1.00 ± 0.19 ^b)
Medullary congestion	6 0 0 0 0	0 0 1 5 1 (3.00 ± 0.22)	0 3 3 2 0 (1.88 ± 0.30 ^a)	0 3 1 3 0 (2.00 ± 0.38)	1 3 4 0 0 (1.38 ± 0.26 ^b)	4 3 1 0 0 (0.63 ± 0.26 ^b)

To evaluate the contribution of ET-1 in the ischemia/reperfusion-induced renal injury and protective effects of SM-19712 and phosphoramidon, renal ET-1 contents and

Fig. 3. Light microscopy of outer zone outer stripe of medulla of the kidney treated with vehicle (untreated), SM-19712 (3, 10 and 30 mg/kg) or phosphoramidon (10 mg/kg) 1 day after the ischemia/reperfusion, and the kidney of a sham rat. Drugs were given intravenously 5 min before the ischemia (45 min). ARF, acute renal failure. Severe tubular necrosis is observed in the untreated ARF rat. Magnification, $\times 102$.

Fig. 4. Light microscopy of inner zone of medulla of the kidney treated with vehicle (untreated), SM-19712 (3, 10 and 30 mg/kg) or phosphoramidon (10 mg/kg) 1 day after the ischemia/reperfusion, and the kidney of a sham rat. Drugs were given intravenously 5 min before the ischemia (45 min). ARF, acute renal failure. Severe proteinaceous casts in tubuli are observed in the untreated ARF rat. Magnification, $\times 102$.

Fig. 5. Light microscopy of outer zone inner stripe of medulla of the kidney treated with vehicle (untreated), SM-19712 (3, 10 and 30 mg/kg) or phosphoramidon (10 mg/kg) 1 day after the ischemia/reperfusion, and the kidney of a sham rat. Drugs were given intravenously 5 min before the ischemia (45 min). ARF, acute renal failure. Severe congestion and hemorrhage are observed in the untreated ARF rat. Magnification, $\times 102$.

Fig. 6. Effects of SM-19712 and phosphoramidon on plasma ET-1 (A) and renal ET-1 (B) levels at 1 day after ischemia/reperfusion. Drugs were given intravenously 5 min before the ischemia (45 min). ARF, acute renal failure. Each column and bar represents the mean $\pm$ S.E.M. of 7–8 rats. ** $P < 0.01$, compared with sham rats.

Table 2. Changes in plasma ET-1 level and renal ET-1 content at 2 and 6 h after the ischemia/reperfusion

		Sham	Untreated ARF	ARF + SM-19712 (30 mg/kg)
Plasma ET-1 level (pg/ml)	2 h	8.52 ± 1.94 (n = 7)	8.06 ± 1.23 (n = 8)	8.34 ± 1.41 (n = 6)
	6 h	8.26 ± 1.79 (n = 7)	7.96 ± 0.48 (n = 8)	10.31 ± 0.83 (n = 7)
Renal ET-1 content (ng/g tissue)	2 h	0.29 ± 0.03 (n = 7)	0.48 ± 0.06 ^a (n = 8)	0.27 ± 0.04 ^c (n = 6)
	6 h	0.32 ± 0.03 (n = 7)	0.81 ± 0.11 ^b (n = 8)	0.25 ± 0.03 ^c (n = 7)

ARF, acute renal failure. ^a $P < 0.05$, ^b $P < 0.01$, compared with sham rats. ^c $P < 0.01$, compared with untreated ARF rats.

plasma ET-1 levels at 24 h after the ischemia/reperfusion were determined. As shown in Fig. 6A, no significant changes in plasma ET-1 levels were observed between all the experimental groups. In contrast, renal ET-1 contents were significantly increased in animals exposed to the 45-min ischemia, being about twofold over the sham-operated group. SM-19712- or phosphoramidon-pretreatment tended to suppress the increased ET-1 contents, although observed changes were not statistically significant (Fig. 6B). Based on the observations that ET-1 overproduction occurs at an early phase after the ischemia/reperfusion (11, 16) and that serum concentration of SM-19712 (30 mg/kg) administered intravenously decreased with a half-life of about 20 min (25), we further evaluated renal ET-1 contents and plasma ET-1 levels at 2 and 6 h after the ischemia/reperfusion. As shown in Table 2, the increase in renal ET-1 content was more marked at 6 h. In addition, SM-19712 at 30 mg/kg abolished the elevation of renal ET-1 contents at these early phases. There were no significant alterations in plasma ET-1 levels.

DISCUSSION

In the present study, we obtained evidence that SM-19712, a highly selective ECE inhibitor, markedly overcame the ischemia/reperfusion-induced impairment of renal function in rats. Histological renal damage induced by this post-ischemic ARF were also prevented by treatment with SM-19712 and to a lesser extent, decreased with phosphoramidon, a prototype of ECE inhibitor that has been reported to attenuate the post-ischemic ARF (22, 23). In addition, SM-19712 efficiently suppressed the elevation of renal ET-1 level at an early phase after the reperfusion, without changing plasma ET-1 level. Taken together, it seems likely that SM-19712 improved the renal dysfunction and tissue injury induced by the ischemia/reperfusion by inhibiting the local ET-1 production in the kidney.

Previous studies demonstrated that renal ET-1 mRNA expression (10, 11), tissue levels of ET-1 peptide (12) and its affinity for renal ET receptor (13) were elevated in the post-ischemic kidney. Using the same ischemic ARF model in rats, Kon et al. (29) noted that intrarenal arterial injection of an ET antibody ameliorated the decreases in renal blood flow and glomerular filtration rate induced by the ischemia/reperfusion. Moreover, both ET_A-selective and nonselective ET_A/ET_B receptor antagonists have been reported to attenuate the ischemia/reperfusion-induced impairment of renal function (14–17). From these findings, it is evident that the increased production of ET-1 in the kidney and its ET_A-mediated action play an important role for the development of the postischemic ARF.

It remains obscure whether an overproduction of ET-1 in the ischemic kidney occurs in vascular endothelium, in tubular cells or in both. In *in vitro* studies using human proximal tubular cells, ET-1 gene expression and the peptide production were up-regulated under the hypoxic conditions (30). On the other hand, it has been well documented that hypoxia induces ET-1 gene expression and its secretion in cultured vascular endothelial cells (31–33). Most recently, Wilhelm et al. (11) indicated, using an ischemic ARF model, that initial ET-1 gene up-regulation in the kidney occurs secondary to the ischemia, and reperfusion contributes to sustaining this up-regulation. In addition, they observed a marked increase of ET-1 in the endothelium of the peritubular capillary network, suggesting that ET-1-induced peritubular vasoconstriction plays a pathophysiological role in ischemia/reperfusion-induced tubular necrosis. Taken together, it is reasonable to consider that ET-1 production is up-regulated in both vascular endothelium and tubular cells of the ischemic kidney and that this peptide acts in a paracrine or autocrine fashion on target cells. It is difficult to determine whether the increased production of ET-1 under the ischemic condition leads to the impairment of renal function or results from the ischemia/reper-

fusion-induced injury in endothelial and/or tubular cells. However, findings of the present study as well as recent pharmacological studies using ET receptor antagonists (14–17) support the possibility of ET-1 as a causal factor of ischemic ARF.

In the present study, the improvement of renal dysfunction and tissue injury by the higher dose (30 mg/kg) of SM-19712 was incomplete, in spite of the fact that increases in renal ET-1 content at 2 and 6 h after the ischemia/reperfusion were completely suppressed by this dose of SM-19712. The reason for this discrepancy is unclear, but several possibilities are conceivable. Pathogenesis of ischemia/reperfusion injury is complicated by various cellular events such as adenosine triphosphate depletion, intracellular Ca^{2+} accumulation, phospholipase activation and membrane lipid alterations, cytoskeletal dysfunction, neutrophil infiltration and the generation of reactive oxygen species (34), some of which may be independent of renal ET-1 overproduction. Thus, although the development mechanisms of ischemia/reperfusion-induced renal injury is highly complex, we believe that renal ET-1 overproduction is a major causal factor of this disease.

There is no available evidence about the mechanisms of ET-1 overproduction in the kidney exposed to the ischemia/reperfusion. One possible candidate may be an intracellular Ca^{2+} accumulation, which is also an important mediator for the pathogenesis of ischemia/reperfusion injury of the kidney (35). An increase of calcium entry has been known to induce the expression of ET-1 gene in endothelial cells (36). Most recently, we demonstrated that KB-R7943, a novel $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor, efficiently improves the renal dysfunction and tissue injury induced by the ischemia/reperfusion (37), suggesting that an activation of the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange plays an important role in the pathogenesis of ARF. In addition, we noted that KB-R7943 could markedly suppress the increased level of ET-1 content in the kidney after ischemia/reperfusion (J. Yamashita et al., unpublished data). Taken together, ET-1 overproduction seems to be positioned down-stream to the Ca^{2+} overload, in the cascade of ischemia/reperfusion-induced renal injury, although ET-1 overproduction may result in the further increase of intracellular Ca^{2+} level.

We have originally found that a peptidic and non-selective metalloprotease inhibitor phosphoramidon inhibits the pressor action of big ET-1 in vivo (18) and the secretion of ET-1 from cultured vascular endothelial cells (20), as well as the ET-converting activity in endothelial cells (19). After this, several studies using experimental animals have demonstrated the protective effect of phosphoramidon against the pathogenesis of various cardiovascular disease models such as cerebral vasospasm (38), deoxycorticosterone acetate-salt-induced hypertension (39), ischemic ARF

(22, 23) and neointimal formation after balloon injury (40). However, it cannot be determined that the above protective effect of phosphoramidon is due to the inhibition of ET-1 production, since phosphoramidon is a potent inhibitor of neutral endopeptidase (24) and since the treatment with selective inhibitors of this enzyme is also beneficial to cardiovascular diseases including hypertension (41). In the present study, our data clearly indicated the beneficial effect of SM-19712 with high selectivity to ECE on renal dysfunction and tissue injury induced by the ischemia/reperfusion. We believe that SM-19712 would be a useful tool for clarification of the pathophysiological role of ECE.

In conclusion, our findings strongly support the view that ET-1 overproduction plays an important role in the development of the ischemia/reperfusion-induced renal injury. A selective and potent ECE inhibitor SM-19712 may be useful in the treatment of ischemic ARF.

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