Preventive Effect of GGSTop, a Novel and Selective γ-Glutamyl Transpeptidase Inhibitor, on Ischemia/Reperfusion-Induced Renal Injury in Rats

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ABSTRACT

GGSTop [2-amino-4-[(3-carboxymethyl)phenyl](methyl)phosphono]butanoic acid, is a novel, highly selective, and irreversible γ-glutamyl transpeptidase (GGT) inhibitor with no inhibitory activity on glutamine amidotransferases. In this study, we investigated the effects of treatment with GGSTop on ischemia/reperfusion-induced renal injury in uninephrectomized rats. Ischemic acute kidney injury (AKI) 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 vehicle-treated AKI rats markedly decreased at 1 day after reperfusion. Treatment with GGSTop (1 and 10 mg/kg i.v.) 5 min before ischemia attenuated the ischemia/reperfusion-induced renal dysfunction in a dose-dependent manner. Histopathological examination of the kidney of AKI rats revealed severe renal damage, which was significantly suppressed by the GGSTop treatment. In renal tissues exposed to ischemia/reperfusion, GGT activity was markedly increased immediately after reperfusion, whereas renal superoxide production and malondialdehyde level were significantly increased 6 h after reperfusion. These alterations were abolished by the treatment with GGSTop. In addition, renal glutathione content was decreased by the 45-min ischemia, but its level was markedly elevated by the GGSTop treatment. Our results demonstrate that the novel and highly selective GGT inhibitor GGSTop prevents ischemia/reperfusion-induced AKI. The renoprotective effect of GGSTop seems to be attributed to the suppression of oxidative stress by inhibiting GGT activation, thereby preventing the degradation of glutathione.

Introduction

γ-Glutamyl transpeptidase (GGT), also named γ-glutamyltransferase or γ-glutamyl peptidyltransferase, is a heterodimeric enzyme found widely in organisms ranging from bacteria to mammals (Hiratake et al., 2004). The mammalian enzymes are anchored to the outside surface of plasma membrane and play a central role in the metabolism of plasma glutathione (GSH) and its S-conjugates via the cleavage of the γ-glutamyl amide bond by hydrolysis and/or transpeptidation (Allison, 1985; Tate and Meister, 1985; Taniguchi and Ikeda, 1998; Han et al., 2007). Serum GGT has been widely used as an index of liver dysfunction and a marker of alcohol intake (Whitfield, 2001). GGT catalyzes the initial step of GSH degradation and transfers the γ-glutamyl moiety of GSH to water (hydrolysis) and amino acids or peptides (transpeptidation) into glutamate and γ-glutamyl-amino acids or peptides, respectively, with a byproduct cysteinyl-glycine (Perry et al., 1998). This cysteinyl-glycine is one of the most reactive thiol compounds that possess very high physiological activity, and it has been reported that this particular thiol can reduce oxygen under normal physiological conditions by reducing ferrie iron Fe3+ into ferrous Fe2+ (Stark et al., 1993). This process is generally known as the iron redox-cycling process and, as a consequence, produces reactive oxygen species (ROS) that subsequently facilitate an oxidative reaction (Dominici et al., 2003, 2005). The highest level of GGT activity is the outer surface of the microvillus membrane (brush border) in the proximal tubule of the kidney (Meister and Tate, 1976; Marathe et al., 1979).

Acivicin [[L-(S,S)-α-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid (AT-125; produced by Streptomyces suiceus)] has been widely used as a GGT inhibitor (Capraro and Hughey, 1985). Previous studies have demonstrated that acivicin has a protective effect on cisplatin-induced nephrotoxicity (Hanigan et al., 1994) and suppresses GGT-dependent oxidative damage in ischemic rat kidney (Cutrin et al., 1993). The protective effect of acivicin on ischemic damage is due to the GGT-inhibitory activity of this compound, which prevents the generation of cysteinyl-glycine from GSH and deranges the functions of the thiol compound.

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2000). However, acivicin is known to inhibit irreversibly various glutamine amidotransferases, including imidazole glycolyl phosphate synthase and guanine monophosphate synthetase, and inactivate a number of biosynthetic enzymes for purine and pyrimidine, amino acids, and amino sugars, which results in a potent cytotoxicity (Earhart and Neil, 1985; Chittur et al., 2001). These findings indicate that GGT is not a natural target of acivicin, but is inhibited fortuitously by acivicin.

GGsTop, a novel phosphate-based and mechanism-based irreversible inhibitor of GGT, exhibits its activity toward human GGT more than 100 times higher than that of acivicin, inhibits only GGT, and does not inhibit glutamine amidotransferases (Han et al., 2007) (Fig. 1). GGsTop is an electrophilic phosphate phenyl ester, but it is nontoxic and chemically stable enough to be used for in vivo studies; less than 4% of the compound is hydrolyzed in neutral water for a month (L. Han, A. Tago, A. Kojima-Yuasa, I. Matsui-Yuasa, and J. Hiratake, unpublished observations).

Indeed, when we administered GGsTop intravenously to rats at a single dose of 30 or 100 mg/kg, no abnormalities were observed at 2 weeks postadministration in general symptoms, body weight, and the amount of food intake. The biopsy performed after the completion of the observation period also showed no abnormalities in major organs and tissues, thereby indicating that GGsTop has no acute toxicities. In addition, a mutagenicity assay (Ames test) using microorganisms showed a negative result (L. Han, A. Tago, A. Kojima-Yuasa, I. Matsui-Yuasa, and J. Hiratake, unpublished observations). Thus, it is apparent that GGsTop is a useful compound as a safe and selective GGT inactivator that can be used as a chemical knockout reagent for in vivo studies.

Ischemia/reperfusion injury is a leading cause of acute kidney injury (AKI), which is a frequent clinical syndrome with high morbidity and mortality (Uchino et al., 2005). Unfortunately, more than half of the hospitalized patients in intensive-care units for the treatment of AKI result in death, despite the installation of new approaches such as advanced monitoring (Yampa et al., 2005). Structural changes in post-ischemic kidneys are characterized by vasoconstriction or necrosis with desquamation of tubular epithelial cells of the tubular lumen. The molecular mechanisms underlying this ischemia/reperfusion injury have not been fully understood, although several causal factors, such as ROS (Vinás et al., 2000), neutrophil infiltration (Teraishi et al., 2004), vasoconstriction (Jerkic et al., 2004), and ATP depletion (Trifillis et al., 1984) have been thought to be involved in the pathogenesis of ischemia/reperfusion-induced AKI.

In this study, we examined the effect of treatment with GGsTop in the uninephrectomized ischemia/reperfusion rat model of AKI and evaluated the possible involvement of the GGT activity and superoxide (O₂⁻) generation in GGsTop-induced actions.

Materials and Methods

Animals and Experimental Designs. Male Sprague-Dawley rats (10 weeks of age; Japan SLC, Shizuoka, Japan) were used. Rats were housed in a light-controlled room with a 12-h light/dark cycle and allowed ad libitum access to food and water. Experimental protocols and animal care methods for this study 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 (50 mg/kg i.p.). After 2-week recovery period, uninephrectomized rats were divided into sham-operated control, vehicle-treated ischemic AKI, and drug-treated ischemic AKI groups. To induce ischemic AKI, the rats were anesthetized with pentobarbital (50 mg/kg i.p.), and then the left kidney was exposed for a small flank incision. The left renal artery and vein were occluded with a nontraumatic clamp for 45 min, and then the clamp was removed for blood reperfusion. GGsTop (1 and 10 mg/kg) or its vehicle (0.9% saline) was given into the left external jugular vein 5 min before the start of ischemia by using a 26-gauge needle. In sham-operated control rats, their left kidneys were treated in the same way as those in the AKI groups, except for the clamping procedure. All of these surgical procedures were carefully performed under a rectal temperature-controlled condition using a heater. The rats exposed to 45 min of ischemia treatment were housed in metabolic cages for 24 h after reperfusion. 5-h urine samples were then taken, and blood samples were drawn from the thoracic aorta at the end of the urine collection period. Plasma was separated by centrifugation and used for the measurement of renal function parameters. The excised kidneys were also used for histological examination with a light microscope.

In separate experiments, rats were sacrificed at various time points after the start of reperfusion for evaluation of renal O₂⁻ production, malondialdehyde (MDA) levels, GGT activities, and GSH content.

Renal Functional Parameters. Blood urea nitrogen (BUN) and creatinine concentration in plasma (Pcr) or urine were determined using a commercial assay kit, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Urine and plasma sodium concentrations were determined using a flame photometer (205D; Hitachi, Hitachinaka, Japan). The fractional excretion of sodium (FENa) percentage was calculated from the following formula: \[ \text{FENa} = \frac{\text{U}_{\text{Na}} \times \text{P}_{\text{Na}}}{\text{C}_{\text{r}} \times \text{P}_{\text{cr}}} \times 100 \], where \( \text{U}_{\text{Na}} \) is the urinary excretion of sodium, \( \text{P}_{\text{Na}} \) is the plasma sodium concentration, and \( \text{C}_{\text{r}} \) is the creatinine clearance.

Histological Studies. Excised left kidneys were processed for light microscopic observation, according to standard procedures. The kidneys were then fixed in phosphate-buffered 10% formalin, pH 7.4, after which they were chopped into small pieces, embedded in paraffin wax, cut at 4 μm, and stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous cast, and medullary congestion, as described by Caramelo et al. (1996). Tubular necrosis and proteinaceous casts were graded as follows: no congestion (severity score 0), mild (1, unicellular, patchy isolated damage), moderate (2, damage less than 25%), severe (3, damage between 25 and 50%), and very severe (4, more than 50% damage). The degree of medullary congestion was defined as: no congestion (severity score 0), mild (1, vascular congestion with identification of erythrocytes by 400× magnification), moderate (2, vascular congestion with identification of erythrocytes by 200× magnification), severe (3, vascular congestion with identification of erythrocytes by 100× magnification), and very severe (4, vascular congestion with identification of erythrocytes by 40× magnification).

Fig. 1. Chemical structure of GGsTop.
The scoring of the histological data were performed by an observer who was blind to the treatment origin of the tissue.

**Biochemical Determinations.** Homogenates of the left kidney were made at 10% in 0.1 M Tris-HCl buffer, pH 7.4, and MDA steady-state levels and GGT activities were determined. MDA was measured according to the method described by Poli et al. (1985) with minor modification. The enzymatic activity of GGT in the homogenates was measured by the method of Tate and Meister (1985). Renal O$_2$ production was measured using a lucigenin-enhanced chemiluminescence assay as described previously (Nakajima et al., 2006). The whole kidney was removed from rats and cut into strips (2-mm pieces). Immediately afterward, renal tissue segments were placed in test tubes containing modified Krebs-HEPES buffer (pH 7.4, 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl$_2$, 1.20 mM MgSO$_4$, 1.03 mM K$_2$HPO$_4$, 25 mM Na-HEPES, and 11.1 mM glucose) and allowed to equilibrate in the dark for 15 min at 37°C before measurements. After the equilibration, lucigenin (5 μM) was added to the tube, and then the luminescence was measured using a luminometer (Sirius-2; Berthold Technologies, Bad Wildbad, Germany). The relative light unit was integrated every 3 s for 15 min and averaged. The renal O$_2$ production was expressed as relative light unit per minute per milligram of dry tissue weight. The renal GSH contents were determined using a commercial assay kit (Cayman Chemical, Ann Arbor, MI).

**Drugs.** GGsTop was synthesized at the Institute for Chemical Research, Kyoto University according to the literature method (Han et al., 2007). This compound is now commercially available from Wako Pure Chemicals under the name of GGSitop. It was dissolved in saline (0.9%). Other chemicals were purchased from Sigma (St. Louis, MO), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemicals.

**Statistical Analysis.** All values were expressed as the mean ± S.E.M. Relevant data were processed by InStat (GraphPad Software Inc., San Diego, CA). For statistical analysis, we used the unpaired Student’s t test for two-group comparison and one-way analysis of variance followed by Dunnett’s tests for multiple comparisons. Differences were considered significant at $P < 0.05$.

### Results

**Renal Function after Ischemia/Reperfusion and the Effects of GGSTop Treatment.** As shown in Fig. 2, the renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured at 1 day after reperfusion. Compared with sham-operated (plus vehicle) control rats, vehicle-treated AKI rats showed significant increases in BUN (103.4 ± 7.0 versus 28.9 ± 6.5 mg/dl), Pcr (2.66 ± 0.28 versus 0.90 ± 0.12 mg/dl), and FE$_{Na}$ (20.3 ± 5.7 versus 3.5 ± 0.9%) and significant decreases in Ccr (1.20 ± 0.22 versus 4.35 ± 0.57 ml/min/kg). The intravenous administration of GGSTop (1 and 10 mg/kg) 5 min before ischemia produced a dose-dependent preventive effect against the ischemia/reperfusion-induced renal dysfunction. When a higher dose of GGSTop (10 mg/kg) was given, renal function parameters, including BUN, Pcr, and Ccr, markedly improved. No significant effects were observed when GGsTop at 10 mg/kg was given to sham rats.

**Histological Renal Damage after the Ischemia/Reperfusion and Effects of GGSTop Treatment.** Histopathological examinations revealed severe lesions in the kidneys of vehicle-treated AKI rats (1 day after ischemia/reperfusion). These changes were characterized by tubular necrosis in the outer zone outer stripe of the medulla (Fig. 3B) (severity score, 3.67 ± 0.21; Table 1), medullary congestion and hemorrhage in the outer zone inner stripe of the medulla (Fig. 3F) (severity score, 3.67 ± 0.21; Table 1), and proteinaceous casts in tubuli in the inner zone of medulla (Fig. 3J) (severity score, 3.67 ± 0.21; Table 1). The preischemic treatment with GGSTop at the higher dose (10 mg/kg) produced marked and significant preventive effects against the development of tubular necrosis (Fig. 3D) (severity score, 1.83 ± 0.31; Table 1), medullary congestion and hemorrhage (Fig. 3H) (severity score, 2.33 ± 0.33; Table 1) and proteinaceous casts in tubuli (Fig. 3L) (severity score, 1.50 ± 0.22; Table 1).

**Renal GGT Activities after Ischemia/Reperfusion and the Effects of GGSTop Treatment.** As shown in Fig. 4, renal GGT activity in vehicle-treated AKI rats increased immediately after the start of reperfusion and thereafter gradually decreased. The preischemic treatment with GGsTop...
at 1 or 10 mg/kg markedly suppressed the renal GGT activity increased by the 45-min ischemia.

**Renal O$_2^\cdot$ Production after Ischemia/Reperfusion and the Effects of GGsTop Treatment.** As shown in Fig. 5, renal O$_2^\cdot$ production in rats subjected to 45-min ischemia increased gradually after the reperfusion. At ~6 to 29 h after reperfusion, there were significant increases in the renal MDA level. The preischemic treatment with GGsTop at 1 or 10 mg/kg markedly suppressed the renal MDA level after reperfusion.

**Renal MDA Levels after Ischemia/Reperfusion and the Effects of GGsTop Treatment.** As shown in Fig. 6, renal MDA level in rats subjected to 45-min ischemia increased gradually after the reperfusion. At ~6 to 29 h after reperfusion, there were significant increases in the renal MDA level. The preischemic treatment with GGsTop at 1 or 10 mg/kg markedly suppressed the renal MDA level after reperfusion.

**Renal GSH Content after Ischemia/Reperfusion and the Effects of GGsTop Treatment.** Renal GSH contents were decreased by 45-min ischemia, and the decreased level continued after reperfusion. Preischemic treatment with GGsTop at 10 mg/kg markedly elevated the renal GSH content after reperfusion (Fig. 7).

**Discussion**

Postischemic AKI is a frequent clinical syndrome with high morbidity and mortality (Thadhani et al., 1996). Reperfusion of previously ischemic renal tissue initiates a series of complex cellular events that result in renal injury or possibly death of renal cells caused by the combination of apoptosis and necrosis (Lieberthal and Levine, 1996). Numerous attempts have been made to prevent AKI by using animal
models that represent ischemia/reperfusion-induced renal injury, and various vasodilative agents, including natriuretic peptides, adenosine, dopamine receptor agonists, and N-acetylcysteine, have been considered to be useful for the prevention and management of AKI (Venkataraman and Kellum, 2003). However, it still remains to be elucidated whether these interventions are also beneficial in clinical cases.

Cutrin et al. (2000) have reported that short-term kidney ischemia (25 min) increases GGT activity and also enhances lipid peroxidation, suggesting that the postischemic AKI is related to an increase of GGT activity. In fact, acivicin, a prototype of GGT inhibitor (Capraro and Hughery, 1985), could prevent the ischemia-induced increase in GGT activity, lipid peroxidation enhancement, and morphological alterations (Curtin et al., 2000). However, in addition to inhibiting GGT, acivicin is an antimeabolite and inactivates a number of biosynthetic glutamine amidotransferase enzymes for purine and pyrimidine, amino acids, and amino sugars (Earhart and Neil, 1985). Furthermore, acivicin is known to inhibit other various glutamine amidotransferases, including imidazole glycerol phosphate synthase and guanine monophosphate synthetase (Chittur et al., 2001). Thus, novel and selective GGT inhibitors are required to determine the pathophysiological role of GGT in the postischemic AKI.

GGsTop is a newly developed and selective GGT inhibitor (Han et al., 2007). This compound has no acute toxicities and is chemically stable. Therefore, GGsTop is a very useful compound as a selective GGT inhibitor, especially for in vivo studies. In the present study, we demonstrated that GGsTop produced a dose-dependent preventive effect on the postischemic AKI. The renal dysfunction and histological damage observed in the postischemic kidney were markedly improved by treatment with GGsTop.

To explore possible mechanisms underlying the renoprotective effects of GGT inhibitor against ischemia/reperfusion-induced renal injury, we first evaluated the effect of GGsTop on renal GGT activity. GGT activity rapidly increased after ischemia/reperfusion, and the obtained time course of GGT activity was similar to that of GSH (substrate of GGT) degradation in the postischemic kidney, as reported by Slusser et al. (1990). We also observed decreased GSH content in the kidney exposed to 45-min ischemia. Therefore, we evaluated the effects of GGsTop on the alterations of renal GGT activity and GSH content after ischemia/reperfusion. Preischemic

Fig. 5. Time course of O₂ production in the kidneys of vehicle-treated AKI rats and the effect of preischemic treatment with GGsTop on O₂ production in the kidney after reperfusion. GGsTop was intravenously given at 5 min before ischemia. Each column and bar represents the mean ± S.E.M., * P < 0.05; ** P < 0.01, compared with vehicle-treated AKI rats. RLU, relative light units.

Fig. 6. Time course of MDA levels in the kidneys of vehicle-treated AKI rats and the effect of preischemic treatment with GGsTop on MDA levels in the kidney after reperfusion. GGsTop was intravenously given at 5 min before ischemia. Each column and bar represents the mean ± S.E.M., * P < 0.05; ** P < 0.01, compared with vehicle-treated AKI rats.

Fig. 7. Renal GSH content in the kidneys of vehicle-treated AKI rats and the effect of preischemic treatment with GGsTop. GGsTop was intravenously given at 5 min before ischemia. Each column and bar represents the mean ± S.E.M., ** P < 0.01, compared with vehicle-treated AKI rats.
treatment with GGsTop completely elevated an elevation of GGT activity after reperfusion. On the other hand, the renal GSH content was markedly elevated by the GGsTop treatment. The rationale for the dose setting of GGsTop in our study is based on findings that the effective dosage of acivicin to exert antioxidative effects is 50 to 300 mg/kg (Slusser et al., 1990; Cutrin et al., 2000) and GGsTop possesses more than 100 times higher activity in inhibiting GGT than that of acivicin (Han et al., 2007).

Next, we evaluated how the increase of GGT activity is associated with renal O$_2$ production. Our interest was not only to see the level of O$_2$ production but also to investigate the relationship between GGT activity and O$_2$ production. Results indicated that O$_2^*$ production in the postischemic kidney increased markedly from 6 to 29 h after reperfusion. This is consistent with our previous report (Nakajima et al., 2006). In the present study, when GGsTop was given before ischemia, renal O$_2^*$ production enhanced by the ischemia/reperfusion was suppressed completely. Thus, it seems likely that GGsTop exerted a potent renoprotective effect by suppressing the oxidative stress after the augmentation of GGT activity.

According to Stark et al. (1993), the metabolism of GSH catalyzed by GGT results in the generation of thyl radicals and other oxidant species that are responsible for cytotoxicity and lipid peroxidation. In addition, this GGT-dependent metabolism of GSH causes the reduction of ferric ion (Fe$_{3+}$) into ferrous ion (Fe$_{2+}$) that reduces molecular oxygen to produce O$_2^*$ and subsequently initiates a lipid peroxidation chain reaction (Tien et al., 1982; Stark et al., 1994). Another factor for breakdown of oxidative balance would be the reduction of antioxidative GSH itself. The decrease of intracellular GSH level occurs concomitantly with increases in GGT activity and O$_2^*$ production (Slusser et al., 1990; Dudek et al., 2010). Based on the finding that the decrease in GSH level weakens the antioxidant defense in vascular endothelial cells (Dudek et al., 2010), the antioxidative properties of GSH could be a major factor for AKI tolerance.

Correlating to renal O$_2^*$ production, a MDA level (as an index for lipid peroxidation) was markedly increased ~6 to 29 h after reperfusion, and this increment was abolished by preischemic treatment with GGsTop. Taken together, it is likely that GGsTop exerts its renoprotective effect by inhibiting a GGT activity-dependent ROS production and the following augmentation of lipid peroxidation.

The findings in the present study clearly indicate that GGsTop is a promising novel agent with a potent renoprotective effect and the potential to be evaluated in clinical trials for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI. In the report of a phase II clinical study with acivicin, a reversible neurological toxicity was evidenced for patients with AKI.

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