Involvement of γ-Glutamyl Transpeptidase in Ischemia/Reperfusion-Induced Cardiac Dysfunction in Isolated Rat Hearts

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INTRODUCTION

γ-Glutamyl transpeptidase (GGT) is a heterodimeric membrane-bound enzyme that plays a physiological role in the metabolism of extracellular reduced glutathione (GSH) and its S-conjugates via breaking the γ-glutamyl amide bond by hydrolysis and/or transpeptidation. Since this enzyme results in the release of cysteine, which is regarded as one of pivotal rate-limiting steps for the intracellular synthesis of GSH, GGT is considered to be an antioxidant enzyme. However, cysteinyl–glycine generated in the degradation process of GSH by GGT is an exceptionally reactive thiol compound. Previous studies demonstrated that cysteinyl–glycine reduces ferric iron (Fe3+) into the ferrous form (Fe2+) under physiological conditions, leading to the reduction of oxygen to a superoxide anion (O2−) and consequently concluded that GGT produces reactive oxygen species (ROS) through a sequence of reactions. Although the pathophysiological role of GGT itself remains unclear because of this dual nature, the serum level of this enzyme has been utilized as an indicator of hepatic dysfunction and biliary tract disease. Furthermore, clinical usability of serum GGT as a predictor of the development of cardiovascular disease has recently been proposed.

GGsTop is a highly potent and specific, and irreversible γ-glutamyl transpeptidase (GGT) inhibitor without any influence on glutamine amidotransferases. The aim of the present study was to investigate the involvement of GGT in ischemia/reperfusion-induced cardiac dysfunction by assessing the effects of a treatment with GGsTop. Using a Langendorff apparatus, excised rat hearts underwent 40 min of global ischemia without irrigation and then 30 min of reperfusion. GGT activity was markedly increased in cardiac tissues exposed to ischemia, and was inhibited by the treatment with GGsTop. Exacerbation of cardiac functional parameters caused by ischemia and reperfusion, namely the reduction of left ventricular (LV) developed pressure and the maximum and negative minimum values of the first derivative of LV pressure, and the increment in LV end-diastolic pressure was significantly attenuated by GGsTop treatment. The treatment with GGsTop suppressed excessive norepinephrine release in the coronary perfusate, a marker for myocardial dysfunction, after ischemia/reperfusion. In addition, oxidative stress indicators in myocardium, including superoxide and malondialdehyde, after ischemia/reperfusion were significantly low in the presence of GGsTop. These observations demonstrate that enhanced GGT activity contributes to cardiac damage after myocardial ischemia/reperfusion, possibly via increased oxidative stress and subsequent norepinephrine overflow. GGT inhibitors have potential as a therapeutic strategy to prevent myocardial ischemia/reperfusion injury in vivo.

Key words γ-glutamyl transpeptidase; GGsTop; ischemia–reperfusion; cardiac dysfunction; superoxide

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MATERIALS AND METHODS

Animals  Male Sprague-Dawley rats weighing 250–350g provided by Japan SLC, Inc. in Japan were used. Rats were raised under a 12-h light/dark cycle and were permitted to arbitrarily access to food and water. The present study complied with experimental protocols and animal care methods certified by the Experimental Animal Research Committee of Osaka University of Pharmaceutical Sciences.

Procedure for Isolated Rat Heart Model  Rats were anesthetized, and then hearts of rats were excised after no response to stimulus. Isolated rat hearts were retrogradely irrigated with Krebs–Henseleit buffer at a fixed pressure of 80 mmHg. The hemodynamic parameters (left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximum and negative minimum values of the first derivative of left ventricular pressure (dP/dt max and dP/dt min)) were measured via a water-filled latex balloon as previously reported.18) Coronary flow (CF) was observed as well.

Experimental Protocol  Hearts were exposed to 40 min of global ischemia by clamping of the aortic cannula, followed by 30 min of reperfusion. The test group was divided into the following three groups: no addition, 0.1 µM GGsTop, and 1 µM GGsTop. GGsTop was irrigated from 15 min prior to ischemia through 5 min after the beginning of reperfusion apart from the ischemia period. Coronary outflow was collected to evaluate the amount of norepinephrine (NE) content for 5 min after the beginning of reperfusion. Hearts were obtained at a variety of time points during procedure and cryopreserved to evaluate GGT activity, O2− production and malondialdehyde (MDA) level. Seventy-seven rats were used in the present study.

GGT Activity  The enzymatic activity of GGT was measured in the no addition and 1 µM GGsTop groups according to the method as reported previously.2) Briefly, left ventricles were homogenized in 10 volumes of 0.1 M Tris–HCl buffer (pH 7.4), and cardiac GGT activity was assessed by a standard spectrophotometric technique with γ-glutamyl-p-nitroanilide as the substrate. GGT activity was expressed as U (µmol p-nitroaniline formed per minute) per milligram of protein.

Quantitative Analysis of O2− Production  O2− production from cardiac slices was determined 30 min after reperfusion using chemiluminescence of lucigenin, as reported previously.18) The value of O2− production from cardiac slices was expressed as relative light units per minute per milligram of dried cardiac tissue weight.

MDA Assay  Left ventricles were homogenized in 10 volumes of 0.1 M Tris–HCl buffer, pH 7.4, and cardiac MDA steady-state levels were assessed. MDA was determined 30 min after reperfusion following the method of Poli et al.19) with slight modifications. MDA levels were expressed as nmol per milligram of protein.

NE Assay  NE in the coronary outflow was determined using HPLC accompanying an amperometric detector (ECD-100 manufactured by Eicom in Japan), as described previously.18) NE levels were expressed as ng per 5 min.

Drugs  GGsTop was synthesized at the Institute for Chemical Research, Kyoto University following the previously reported method.3) GGsTop was dissolved in ultrapure water to 100 and 10 mM for stock solution, and each solution was then diluted to 1 and 0.1 µM with Krebs–Henseleit buffer just prior to the experiment.

Statistical Analysis  Data were presented as mean ± standard error of the mean (S.E.M.), which was statistically processed by statistical software (Graph Pad Prism 7.0 software or Instat developed by Graph-PAD Software in U.S.A.). In statistical analyses, the two-way ANOVA followed by Bonferroni’s multiple comparisons test (Fig. 1) and one-way ANOVA combined with Bonferroni’s multiple comparisons test (Figs. 2, 3) were used. Values of $p < 0.05$ were considered as signifi-
RESULTS

Time-Dependent Change for Cardiac GGT Activity during I/R

We initially investigated time-dependent changes in cardiac GGT activity during ischemia (20 and 40 min after the onset of ischemia) and reperfusion (5 and 30 min after the onset of reperfusion). Enzyme activity in non-ischemic hearts was comparable between without and with treatment with GGsTop. The activity peaked after 20 min of ischemia, and then gradually decreased to the basal level (Fig. 1). On the flip side, cardiac GGT from ischemia through early reperfusion phase was significantly less active in the presence of 1 µM GGsTop.

Effects of GGsTop on I/R-Induced Cardiac Dysfunction

All cardiac functional parameters prior to ischemia, such as LVDP, LVEDP, dP/dt_max, and −dP/dt_min, were not affected by the treatment with GGsTop (Fig. 2). LVDP, dP/dt_max, and −dP/dt_min were markedly reduced and LVEDP was increased from pre-ischemic levels by global ischemia, while these values gradually recovered during reperfusion. Treatments with low or high doses of GGsTop did not change pre-ischemic cardiac function from no addition, and significantly attenuated post-ischemic cardiac systolic dysfunction (the depression of LVDP and dP/dt_max) and diastolic dysfunction (the depression of −dP/dt_min and elevation of LVEDP).

Effects of GGsTop on I/R-Induced NE Overflow

I/R caused a significant increase in NE release in the coronary effluent from the basal level before ischemia. In parallel with the case of cardiac function, NE overflow observed after I/R was significantly suppressed at both low and high doses of GGsTop (Fig. 3A).

Effects of GGsTop on I/R-Induced O₂⁻ Production and MDA Formation

To evaluate the contribution of ROS to the protective effects of GGsTop on I/R-induced cardiac injury, we determined cardiac O₂⁻ production and MDA formation before ischemia as the basal level and after 30 min of reperfusion. O₂⁻ production and MDA formation after I/R were significantly increased compared to those at basal levels. Cardiac O₂⁻ production after I/R was significantly suppressed by the treatment with GGsTop (Fig. 3B). Figure 3C shows that the treatment with GGsTop reduced I/R-induced cardiac MDA formation, and a significant difference was not observed at the low dose.

DISCUSSION

In the present study, cardiac GGT activity was significantly elevated by ischemia, and then gradually returned to the basal level during reperfusion. The increment observed in cardiac GGT activity during ischemia is consistent with the findings reported by Zheng et al., who demonstrated that the activity of this enzyme was increased in the left ventricle of rats with experimental myocardial infarction. Additionally, Ravens and colleagues showed that particle-bound GGT activity in the canine heart was transiently decreased, but thereafter increased by coronary occlusion. On the other hand, previous
studies reported that cardiac GGT activity remained constant even after exposure to ischemia and reperfusion. However, it is important to note that cardiac GGT activity in these studies was only observed at a particular time point. Based on the present results, the change in local GGT activity induced by ischemia and reperfusion may be temporary. In any event, cardiac GGT activity in the present study increased during ischemia, and this was suppressed by the treatment with GGsTop, thereby enabling us to infer the pathophysiological role of GGT in myocardial I/R injury.

Although GGT is associated with the incidence and progression of coronary heart disease, the present study demonstrated for the first time the pathophysiological role of GGT activity in cardiac functional injury. Briefly, the treatment with GGsTop, which suppressed the increment in cardiac GGT activity during ischemia, reduced the severity of myocardial dysfunction. As an aside, this treatment did not affect the basal GGT activity and cardiac function. Furthermore, this drug exerted significant inhibitory effects on NE overflow, which serves as an indicator of myocardial dysfunction after I/R. These results suggest that enhancements in cardiac GGT activity contribute to myocardial dysfunction associated with I/R.

Myocardial I/R causes excessive ROS production and this augmented ROS is implicated in the development of cardiac injury. In this regard, the catabolic process of GSH breaking down into cysteinyl-glycine by GGT also triggers the generation of O$_2^-$ and subsequent increases in ROS. This is because its metabolite produces the reduction of Fe$^{3+}$ into Fe$^{2+}$ and reduces molecular oxygen to O$_2^-$. Moreover, the generation of O$_2^-$ via increased GGT activity promotes the consumption of the antioxidant GSH. This decrease in intracellular GSH activates GGT to maintain GSH homeostasis, further increasing the generation of O$_2^-$. A previous study reported that acivicin, a prototype GGT inhibitor, suppressed elevations in H$_2$O$_2$, a major O$_2^-$ metabolite, in post-myocardial infarction myocytes. Based on these findings, we observed the effects of GGsTop on O$_2^-$ and MDA production in the left ventricle after I/R, and evaluated the contribution of ROS to myocardial I/R injury via local GGT hyperactivity. The results obtained showed that the treatment with GGsTop significantly suppressed I/R-induced significant increments in cardiac O$_2^-$ production. Similarly, while a significant increase in MDA formation was observed in hearts exposed to I/R, this was also inhibited by the treatment with GGsTop. These results indicate that GGsTop exerts its cardioprotective effects, at least in part, by suppressing oxidative damage as a result of GGT activation during ischemia and immediately after reperfusion.

Enhancements in cardiac sympathetic nerve activity and its effects on excessive NE secretion from nerve endings are also regarded as one of factors consequently leading to ischemia-induced myocardial cytotoxicity. A previous study showed that the negative modulation of NE secretion significantly suppressed post-ischemic cardiac functional disorder. Furthermore, our study previously indicated that the superoxide scavengers, Tempol and Tiron, decreased NE overflow and improved cardiac dysfunction caused by ischemia and reperfusion, which indicates that excessive O$_2^-$ production is related to NE overflow and subsequent myocardial I/R injury. These findings also suggest that the cardioprotective effects of GGsTop are closely associated with the decrease in NE overflow from the post-ischemic heart via the reduction of O$_2^-$ production.

Over the last few decades, many studies have examined the mechanisms responsible for the excessive production of ROS, including O$_2^-$, triggering cardiac dysfunction. For example, excessive ROS causes the indiscriminate oxidation and peroxidation of proteins, lipids, and DNA, which leads to irrevers-
able cellular disorders. Accumulating evidence suggests that the impaired post-ischemic left ventricular function results from oxidative damage to cardiac cell components. In addition, ROS have been shown to disrupt calcium homeostasis, which is closely associated with functional performance, in the post-ischemic heart. Many other possibilities remain because ROS regulate a number of different intracellular signaling cascades, such as protein kinases B and C and mitogen-activated protein kinases.

From the perspective of underlying mechanisms other than ROS, it is assumed that leukotriene (LT) can also be a target of GGsTop. Herman et al. observed that GGT contributed to catalyzing the bioconversion from LTC4 to LTD4. LTD4 has been known as an exacerbation factor to worse I/R injury in rat hearts. Hence, there is a possibility that GGsTop prevented the release of LTD4, which contributed in part to the attenuation of the cardiac I/R injury. Deep-dive studies are required to confirm this hypothesis.

As described above, GGT is a heterodimeric membrane-bound enzyme that utilizes extracellular GSH and its S-conjugates as a substrate. However, the perfusate used in the present study originally contained neither glutathione nor its related compounds. Although we cannot specify the source of a substrate for GGT, intracellular GSH may be considered as a candidate. Several years ago, multidrug resistance-associated proteins (MRP) were shown to mediate GSH export from cells under physiological conditions. Furthermore, Park et al. found that the abundance of MRP increased in response to an oxidant insult during regional ischemia. Many researchers have reported the phenomenon of inducing the release of GSH from the isolated perfused heart by ischemia or reperfusion, it, however, currently remains uncertain whether MRP is related to this process. Nevertheless, this is just a hypothesis, and further studies are needed to identify where the substrate comes from.

In conclusion, the treatment with GGsTop exerted protective effects against the exacerbation of I/R-induced cardiac dysfunction by suppressing GGT activity and subsequent ROS production, suggesting that membrane-bound cardiac GGT is a detrimental factor or mediator underlying myocardial I/R injury. In the present study, we used an excised perfused heart model. Further investigations are needed to apply these results to an in vivo pathological model in order to elucidate the precise role of GGT in myocardial I/R injury and its mechanisms.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**

1. Donald Allison R. gamma-Glutamyl transpeptidase: kinetics and mechanism. Methods Enzymol., 113, 419–437 (1985).
2. Tate SS, Meister A. gamma-Glutamyl transpeptidase from kidney. Methods Enzymol., 113, 400–419 (1985).
3. Han L, Hiratake J, Kaniyama A, Sakata K. Design, synthesis, and evaluation of gamma-phosphono diester analogues of glutaamate as highly potent inhibitors and active site probes of gamma-glutamyl transpeptidase. Biochemistry, 46, 1432–1447 (2007).
4. Stark AA, Zeiger E, Pagano DA. Glutathione metabolism by gamma-glutamyltranspeptidase leads to lipid peroxidation: characterization of the system and relevance to hepatocarcinogenesis. Carcinogenesis, 14, 183–189 (1993).
5. Dominici S, Paolicchi A, Corti A, Maclero E, Pompella A. Prooxidant reactions promoted by soluble and cell-bound gamma-glutamyl transpeptidase activity. Methods Enzymol., 401, 484–501 (2005).
6. Mason JE, Starke RD, Van Kirk JE. Gamma-glutamyltransferase: a novel cardiovascular risk biomarker. Prev. Cardiol., 13, 36–41 (2010).
7. Jiang S, Jiang D, Tao Y. Role of gamma-glutamyltransferase in cardiovascular diseases. Exp. Clin. Cardiol., 18, 53–56 (2013).
8. Kamiyama A, Nakajima M, Han L, Wada K, Mizutani M, Tabuchi Y, Kojima-Yuasa A, Matsu-Yuasa I, Suzuki H, Fukuyama K, Watanabe B, Hiratake J. Phosphonate-based irreversible inhibitors of human γ-glutamyl transpeptidase (GGT). GGsTop is a non-toxic and highly selective inhibitor with critical electrostatic interaction with an active-site residue Lys562 for enhanced inhibitory activity. Bioorg. Med. Chem., 24, 5340–5352 (2016).
9. Watanabe B, Morikitaka T, Tabuchi Y, Kobayashi R, Li C, Yamamoto M, Koeduka I, Hiratake J. An improved synthesis of the potent and selective γ-glutamyl transpeptidase inhibitor GGsTop together with an inhibitory activity evaluation of its potential hydrolysis products. Tetrahedron Lett., 58, 3700–3703 (2017).
10. Yamamoto S, Watanabe B, Hiratake J, Tanaka R, Ohkita M, Matsumura Y. Preventive effect of GGsTop, a novel and selective γ-glutamyl transpeptidase inhibitor, on ischemia/reperfusion-induced renal injury in rats. J. Pharmacol. Exp. Ther., 339, 945–951 (2011).
11. Tuzova M, Jean JC, Hughey RP, Brown LA, Cruikshank WW, Hiratake J, Joyce-Bradly M. Inhibiting lung lining fluid glutathione metabolism with GGsTop as a novel treatment for asthma. Front. Pharmacol., 5, 179 (2014).
12. Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol. Rev., 88, 581–609 (2008).
13. Opie LH. Reperfusion injury and its pharmacologic modification. Circulation, 80, 1049–1062 (1989).
14. Zheng MQ, Tang K, Zimmerman MC, Liu L, Xie B, Rozanski GJ. Role of gamma-glutamyl transpeptidase in redox regulation of K⁺ channel remodeling in postmyocardial infarction rat hearts. Am. J. Physiol. Cell Physiol., 297, C253–C262 (2009).
15. Ravens KG, Gudjiarnason S, Cowan CM, Bing RJ. Gamma-glutamyltranspeptidase in myocardial infarction. Clinical and experimental studies. Circulation, 39, 693–700 (1969).
16. Ondrejicova O, Ziegelhofer A, Gabauer I, Sotnikova R, Styk J, Gibala P, Sedlak J, Horakova L. Evaluation of ischemia-reperfusion injury by malondialdehyde, glutathione and gamma-glutamyl transpeptidase: lack of specific local effects in diverse parts of the dog heart following acute coronary occlusion. Cardiovasc. Res., 4, 225–230 (1993).
17. Paolicchi A, Emdin M, Ghiozzi E, Ciancia E, Passino C, Popoff G, Pompella A. Human atherosclerotic plaques contain gamma-glutamyl transpeptidase enzyme activity. Circulation, 109, 1440 (2004).
18. Koyama T, Tawa M, Yamagishi N, Tsubota T, Sawano T, Ohkita M, Koeduka T, Hiratake J. An improved synthesis of the potent and selective γ-glutamyl transpeptidase inhibitor GGsTop together with an inhibitory activity evaluation of its potential hydrolysis products. Tetrahedron Lett., 58, 3700–3703 (2017).
19. Poli G, Dianzani MU, Cheeseman KH, Slater TF, Lang J, Esteban-H. Separation and characterization of the aldehyde products of lipid peroxidation stimulated by carbon tetrachloride or ADP-iron in isolated rat hepatocytes and rat liver microsomal suspensions. Biochem. J., 227, 629–638 (1985).
20. Lapenna D, de Gioia S, Ciofani G, Mezzetti A, Pierdomenico SD, Di Ilio C, Cucurullo F. Impaired glutathione biosynthesis in the ischemic-reperfused rabbit myocardium. FEBS Lett., 391, 76–78 (1996).
21. Ramirez PR, Ji LL. Glutathione supplementation and training increases myocardial resistance to ischemia–reperfusion in vivo. Am. Biol. Pharm. Bull., 5195
22) Valjevac A, Dzubur A, Nakas-Icindic E, Hadzovic-Dzuvo A, Legara O, Kiseliakovic E, Jadic R. Is γ-glutamyl transferase activity a potential marker of left ventricular function during early postmyocardial infarction period? Future Cardiol., 7, 705–713 (2011).

23) Schömig A, Haass M, Richardt G. Catecholamine release and arrhythmias in acute myocardial ischemia. Eur. Heart J., 12 (Suppl. F), 38–47 (1991).

24) Nagy A, Valen G, Ek B, Sellei P, Sjöquist PO, Vaage J. Effects of a novel, low-molecular weight inhibitor of lipid peroxidation on ischemia–reperfusion injury in isolated rat hearts and in cultured cardiomyocytes. Free Radic. Biol. Med., 24, 1462–1469 (1998).

25) Dhalla NS, Golfman L, Takeda S, Takeda N, Nagano M. Evidence for the role of oxidative stress in acute ischemic heart disease: a brief review. Can. J. Cardiol., 15, 587–593 (1999).

26) Temsah RM, Netticadan T, Chapman D, Takeda S, Mochizuki S, Dhalla NS. Alterations in sarcoplasmic reticulum function and gene expression in ischemia-reperfused rat heart. Am. J. Physiol., 277, H584–H594 (1999).

27) Herman RP, Heller RS, Canavan CM, Herman CA. Bioconversion of leukotriene C4 by the bullfrog heart. Biomed. Biochim. Acta, 47, S174–177 (1988).

28) Mayatepek E, Okun JG, Meissner T, Assmann B, Hammond J, Zschocke J, Lehmann WD. Synthesis and metabolism of leukotrienes in gamma-glutamyl transpeptidase deficiency. J. Lipid Res., 45, 900–904 (2004).

29) Lee CC, Appleyard RF, Byrne JG, Cohn LH. Leukotrienes D4 and E4 produced in myocardium impair coronary flow and ventricular function after two hours of global ischaemia in rat heart. Cardiovasc. Res., 27, 770–773 (1993).

30) Ballatori N, Krance SM, Marchan R, Hammond CL. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. Mol. Aspects Med., 30, 13–28 (2009).

31) Park HA, Kubicki N, Gnyawali S, Chan YC, Roy S, Khanna S, Sen CK. Natural vitamin E α-tocotrienol protects against ischemic stroke by induction of multidrug resistance-associated protein 1. Stroke, 42, 2308–2314 (2011).

32) Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Vissoli O. Oxygen-mediated myocardial damage during ischemia and reperfusion: role of the cellular defences against oxygen toxicity. J. Mol. Cell. Cardiol., 17, 937–945 (1985).

33) Janssen M, Koster JF, Bos E, de Jong JW. Malondialdehyde and glutathione production in isolated perfused human and rat hearts. Circ. Res., 73, 681–688 (1993).