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Report

Inhibitory Activity and Proposed Binding Model of γ-Glutamyl Cysteine, the Precursor of Glutathione, on Angiotensin Converting Enzyme

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Owing to their various physiological activities, thiol compounds, such as l-cysteine with UV-protection properties and captopril that inhibits the catalytic activity of angiotensin converting enzyme (ACE), are currently used as supplements and pharmaceuticals. Glutathione (GSH) plays an important role in intracellular protective effects and is currently used for the treatment of cataract and detoxification from metal poisoning. In contrast to GSH, the GSH precursor γ-glutamyl cysteine (γ-EC) has been reported to exhibit neuroprotective effects, thus making it an attractive key biological protective molecule. However, its characteristics are largely unknown. Here, we evaluated the ACE inhibitory function of γ-EC and its mechanism by comparing it with that of GSH *in vitro*. ACE inhibitory analysis showed that the IC_{50} of GSH and γ-EC against ACE were 8.3 μM and 3.9 × 10^2 μM, respectively. These data suggested that γ-EC exerted ACE inhibitory activity, but it was weaker than that of GSH. Docking simulation showed that the ACE inhibitory activity of both compounds was due to the interaction of their carboxyl groups of Glu with Zn^{2+} in the active center of ACE. Moreover, GSH could fit more compactly in the pocket of ACE, forming more hydrogen bonds with the enzyme than γ-EC. By analyzing its kinetics and *in vivo* efficacy, we hope that γ-EC could be used as a promising compound for lowering blood pressure in applications with moderate activity, such as functional foods.

Key words thiol, γ-glutamyl cysteine, angiotensin converting enzyme

INTRODUCTION

Thiol compounds have high reactivity and exert various physiological activities, such as antioxidant (SH/S-S exchange reactions), metallic chelation, and nucleophilic addition reactions. Thus, they are currently used in a variety of supplements and pharmaceuticals; such examples include l-cysteine, which protects against UV-induced skin damage, captopril, which inhibits the catalytic activity of the angiotensin converting enzyme (ACE) and lowers blood pressure, N-acetyl-l-cysteine, which cleaves disulfide bonds (S-S) in mucus proteins exerting expectorant effects, thiamazole, which inhibits the catalytic activity of thyroid peroxidase and lowers the levels of thyroid hormones, and tiopronin, which chelates hydrargyrum, detoxifying the body.[1–5] Among them, glutathione (GSH), which is the most abundant thiol compound in cells, plays an important role in intracellular protective effects such as the removal of active oxygen and exclusion of foreign compounds.[6–8] Based on these actions, GSH is currently used for the treatment of cataract and the detoxification from metal poisoning. In this context, GSH has been thoroughly studied as a key biological protective molecule, and many findings have already been reported on its protective effects.

The biosynthetic precursor of GSH, γ-glutamyl cysteine (γ-EC), is also a thiol compound (Fig. 1). More specifically, γ-EC is the product of the first step of the GSH biosynthetic process.

(1) Glu + Cys + ATP → γ-Glu-Cys (γ-EC) + ADP + Pi
(2) γ-Glu-Cys (γ-EC) + Gly + ATP → γ-Glu-Cys-Gly (GSH) + ADP + Pi

It has been reported that the antioxidant activity of γ-EC is due to the thiol group of the cysteine residue, as is the case

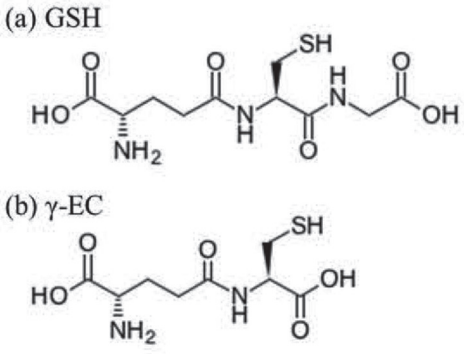

Fig. 1. The Structure of GSH and Its Precursor γ-EC

Structural formulas of (a) GSH and (b) γ-EC. GSH is a tripeptide consisting of Glu, Cys, and Gly. γ-EC is a dipeptide consisting of Glu and Cys. Both have unusual γ-peptide bonds between Glu and Cys.

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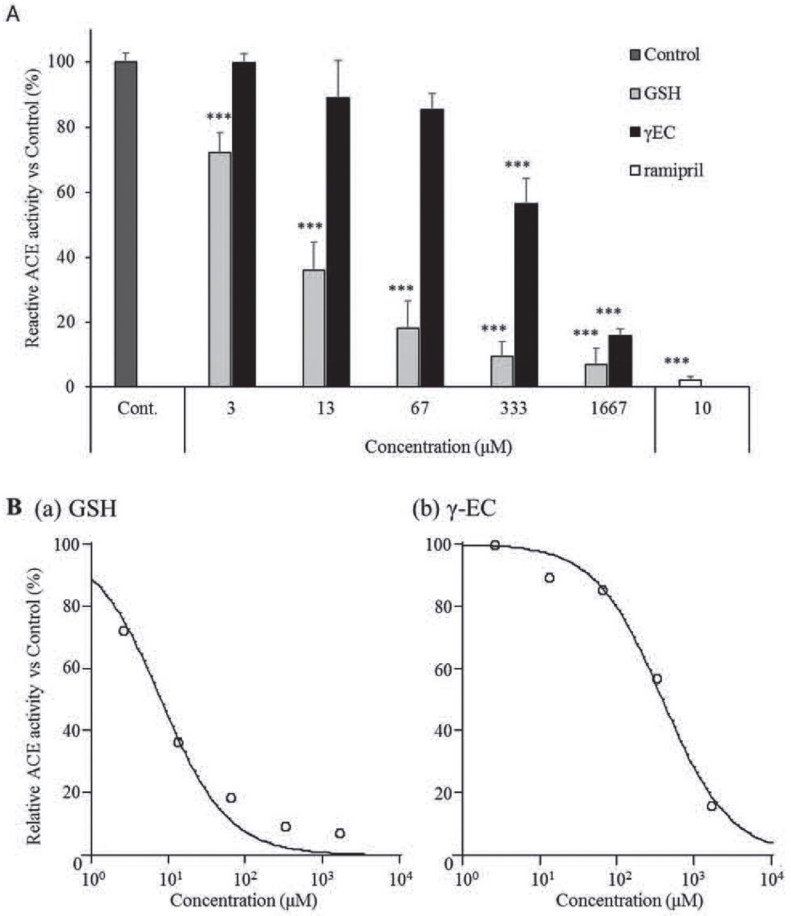

Fig. 2. ACE Inhibitory Activity of GSH and γ-EC

A: γ-EC, GSH, and ramipril as an ACE inhibitor (positive control) were mixed with ACE and the substrate analog, 3-hydroxybutyryl-Gly-Gly-Gly. The amount of 3-hydroxybutyric acid produced from the substrate analog following ACE catalysis was quantified. The ACE inhibitory activity was determined using a fluorescent assay. Data are expressed as the mean ± SD (n = 3, ***$P < 0.001$ versus control by Dunnett's test). B: After plotting the ACE activity at each concentration, the IC_{50} was calculated through fitting.

of GSH. In addition, γ-EC has been reported to have neuroprotective effects, in contrast to GSH.[9–11] Furthermore, as the molecular weight of γ-EC is smaller than that of GSH, its use is expected to reduce production costs and improve intestinal absorption. All these properties make γ-EC an attractive compound as a key biological molecule.

However, as the *in vivo* production of γ-EC is a rate-determining reaction in GSH biosynthesis, the characteristics of γ-EC, such as its bioactivity and kinetics, have remained largely unknown.

Here, we evaluated the characteristics and mechanism of action of γ-EC, focusing on ACE inhibition, a known function of thiol compounds, by comparing it with that of GSH *in vitro*.

MATERIALS AND METHODS

Reagents γ-EC, GSH, and ramipril were purchased from Bachem (Bubendorf, Switzerland), Nacalai Tesque (Kyoto, Japan), and Selleck (Houston, TX, USA), respectively.

ACE Inhibition Test The ACE inhibitory activities of ramipril, γ-EC, and GSH were evaluated using the ACE Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, this kit can detect the amount of 3-hydroxybutyric acid produced from the reaction of ACE with its substrate analog, 3-hydroxybutyryl-Gly-Gly-Gly, by measuring fluorescence at 450 nm.

Structural Analysis of the Binding of GSH and γ-EC to ACE Maestro (Schrödinger K.K., New York City, NY, USA) was used for the computational docking simulation. Natesh *et al*. have reported the cocrystal structure of ACE with lisinopril, an ACE inhibitor.[12] Using PDB 1O86 reported by Natesh *et al*. as a template for ACE, we calculated the interaction between the enzyme and the active groups in GSH and γ-EC.

Statistical Analysis Data were expressed as the mean ± SD. Statistical analyses were conducted using KyPlot 6.0 (KyensLab Inc., Tokyo, Japan). Statistical significance was set at $P < 0.05$. IC_{50} values were estimated from the semilogarithmic plots of the γ-EC or GSH concentration versus relative ACE activity (%), using KaleidaGraph 3.6J (Synergy Software, Mount Penn, PA, USA).

RESULTS AND DISCUSSION

The ACE Inhibitory Activity of γ-EC To analyze the ACE inhibitory activities of γ-EC, GSH, and ramipril, we measured 3-hydroxybutyric acid levels following the reaction between ACE and its substrate analog 3-hydroxybutyryl-Gly-Gly-Gly. Figure 2A shows that the amount of 3-hydroxybutyric acid was significantly reduced in the ramipril-treated group, which was used as a positive control, compared with

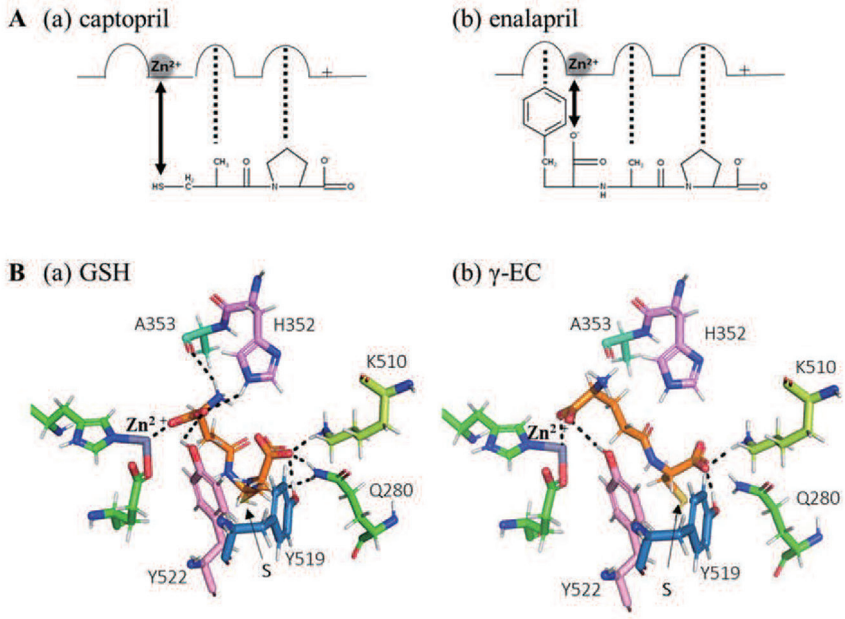

Fig. 3. Docking Simulation of γ-EC and GSH Against ACE for Structural Analysis of Mechanism of Action

A: Interaction of (a) captopril (thiol compound) and (b) enalapril (nonthiol compound) in the active site of ACE. ACE contains Zn^{2+} at its active site. The carboxyl group in enalapril and the thiol group in captopril bind to Zn^{2+}, as indicated by the arrows. Both enalapril and captopril form hydrophobic interactions with ACE, as shown by the broken lines. B: Interaction model of (a) GSH and (b) γ-EC in the active site of ACE using docking simulation. Z^{2+}, sulfur atoms, oxygen atoms, nitrogen atoms, and hydrogen atoms are indicated by gray, yellow, red, blue, and white, respectively.

that in the control group. We also found that the amount of 3-hydroxybutyric acid in the GSH-treated and γ-EC-treated groups was significantly reduced in a concentration dependent manner, compared with that in the control group. These data suggest that GSH and γ-EC inhibited ACE activity *in vitro*. GSH and γ-EC can likely lower blood pressure, although their efficacy is yet to be verified in animal models.

To evaluate the ACE inhibitory activity of GSH and γ-EC in detail, we plotted the enzymatic activity of ACE at each concentration of GSH and γ-EC, and then used curve fitting to calculate each IC_{50}. Figure 2B (a) and (b) show that the IC_{50} of γ-EC was 3.9×10^2 μM, whereas that of GSH was 8.3 μM. These findings suggested that the ACE inhibitory activity of γ-EC was lower than that of GSH.

Functional foods that could be used for the prevention of diseases and reduction of medical costs have been attracting increased attention lately. Unlike the balance between the main and side-effects, which is important for pharmaceuticals, safety is the most important aspect in functional foods. As GSH has already been used as a pharmaceutical, it cannot be used as a functional food. However, γ-EC is a component of the body and moderately inhibits ACE activity *in vitro*. Therefore, γ-EC would be a more suitable functional food component, although it is necessary to verify its efficacy and safety in prehypertensive mouse models and patients.

Structural Analysis of Mechanism of Conformational Stability between γ-EC and ACE Despite the fact that the structures of γ-EC and GSH differ in only a single glycine, the activity of γ-EC was demonstrated to be approximately 470-folds lower than that of GSH in the ACE inhibitory analysis. Therefore, to evaluate the mechanism of action, we predicted the stable conformations of γ-EC and GSH against ACE using docking simulations.

The ACE enzyme, which catalyzes the conversion of the angiotensin II pressor protein, has Zn^{2+} in its active center. Thus, various compounds that inhibit the interactions of Zn^{2+} in the active center of ACE with its substrates have been developed as antihypertensive drugs. For example, captopril, a thiol compound, inhibits the catalytic activity of ACE through the interaction of its thiol group with Zn^{2+} in ACE (Fig. 3A (a)). Likewise, interaction of the carboxyl group of enalapril, a nonthiol compound, with Zn^{2+} in ACE, inhibits the catalytic activity of ACE (Fig. 3A (b)). 1) Therefore, we speculated that γ-EC and GSH also inhibit the catalytic activity of ACE through the interaction between their active groups and Zn^{2+} in ACE.

Docking simulation of γ-EC and GSH against ACE showed that both γ-EC and GSH could interact with Zn^{2+}; however this interaction was not accomplished through the thiol group of Cys, but through the carboxyl group of Glu. Moreover, apart from Zn^{2+}, the carboxyl group of Glu was also shown to interact and form hydrogen bonds with amino acid residues in the ACE active center. Furthermore, the carboxyl groups of the amino acid residues located at the opposite end of Glu (Cys for γ-EC, Gly for GSH) interacted with other amino acid residues in ACE by hydrogen bonding. We thus inferred that both ends of GSH and γ-EC bound to ACE.

Whereas γ-EC fit linearly in the ligand-binding pocket of ACE, GSH fit compactly in the pocket by bending to allow the approach of the carboxyl groups at both ends. In fact, the distance between the carboxyl carbons at both ends was shorter for GSH (5.3 Å) than that for γ-EC (7.7 Å). In addition, Fig. 3B shows that the number of hydrogen bonds between (a) GSH and ACE (8 bonds) was greater than that between (b) γ-EC and ACE (4 bonds). These data suggested a stronger inhibition of the catalytic activity of ACE by GSH, because GSH can fit more compactly in the pocket of ACE allowing it to form more hydrogen bonds with the active site of the enzyme.

Finally, we thermodynamically compared the stabilities of

the 2 conformations. We found that the Glide score, which is used as an index of thermodynamic stability, was lower for GSH (-8.893) than for γ-EC (-6.574). As a smaller Glide score value is known to indicate a higher stability, these findings suggested that the interaction between GSH and ACE is thermodynamically more stable than that between γ-EC and ACE.

Although it has been previously reported that GSH inhibits ACE activity, the underlying molecular mechanism has not been discussed.[13] Therefore, we first showed that γ-EC inhibited ACE activity and proposed a new model in which the carboxyl group of Glu interacts with the Zn^{2+} of ACE via docking simulation.

Conclusions In this study, we revealed that γ-EC, as a GSH precursor, inhibited the catalytic activity of ACE in a concentration-dependent manner, although weaker than GSH. Both γ-EC and GSH could inhibit the activity of ACE through the interaction of the carboxyl group of Glu and not the thiol group of Cys with the Zn^{2+} in the active center of ACE. Furthermore, we demonstrated that GSH exerted a stronger inhibition against the catalytic activity of ACE because GSH can fit more compactly in the pocket of ACE, forming more hydrogen bonds with the enzyme.

By further analyzing its kinetics and *in vivo* efficacy, we hope that in the future γ-EC will be used as a promising component of functional food for lowering blood pressure.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Natesh R, Schwager SL, Evans HR, Sturrock ED, Acharya KR. Structural details on the binding of antihypertensive drugs captopril and enalaprilat to human testicular angiotensin I-converting enzyme. *Biochemistry*, **43**, 8718–8724 (2004).
2) Sheffner AL. The reduction *in vitro* in viscosity of mucoprotein solutions by a new mucolytic agent, N-acetyl-L-cysteine. *Ann. N. Y. Acad. Sci.*, **106**, 298–310 (1963).
3) Davidson B, Soodak M, Neary JT, Strout HV, Kieffer JD, Mover H, Maloof F. The irreversible inactivation of thyroid peroxidase by methylmercaptoimidazole, thiouracil, and propylthiouracil *in vitro* and its relationship to *in vivo* findings. *Endocrinology*, **103**, 871–882 (1978).
4) Funase Y, Toshioka N, Mita I, Sugihara T, Ogura T, Nakamura Y, Kawaguchi S. Some Metal Complexes of 2-Mercaptopropionylglycine. *Chem. Pharm. Bull. (Tokyo)*, **19**, 1618–1626 (1971).
5) Kitoh H, Toshioka N. Effects of tiopronin on excretion and distribution of 203Hg-labeled mercury compounds in mice. *Jpn. J. Pharmacol.*, **28**, 423–431 (1978).
6) Meister A, Anderson ME. Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760 (1983).
7) Homolya L, Váradi A, Sarkadi B. Multidrug resistance-associated proteins: export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors*, **17**, 103–114 (2003).
8) Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed. Pharmacother.*, **57**, 145–155 (2003).
9) Grant CM, MacIver FH, Dawes IW. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast Saccharomyces cerevisiae due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol. Biol. Cell*, **8**, 1699–1707 (1997).
10) Ristoff E, Hebert C, Njålsson R, Norgren S, Rooyackers O, Larsson A. Glutathione synthetase deficiency: is gamma-glutamylcysteine accumulation a way to cope with oxidative stress in cells with insufficient levels of glutathione? *J. Inherit. Metab.*, **25**, 577–584 (2002).
11) Quintana-Cabrera R, Fernandez-Fernandez S, Bobo-Jimenez V, Escobar J, Sastre J, Almeida A, Bolaños JP. γ-Glutamylcysteine detoxifies reactive oxygen species by acting as glutathione peroxidase-1 cofactor. *Nat. Commun.*, **3**, 718 (2012).
12) Natesh R, Schwager SL, Sturrock ED, Acharya KR. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature*, **421**, 551–554 (2003).
13) Basia Z, Turkoglub V. In vitro effect of oxidized and reduced glutathione peptides on angiotensin converting enzyme purified from human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **1104**, 190–195 (2019).