Mechanism of Protection by S-(1,2-Dicarboxyethyl)glutathione Triester against Acetaminophen-Induced Hepatotoxicity in Rat Hepatocytes

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Treatment with the triester of S-(1,2-dicarboxyethyl)glutathione (DCE-GS) prevented the hepatotoxicity induced by acetaminophen via elevation of the glutathione (GSH) level in rat hepatocytes. This elevation of the GSH level in rat hepatocytes by DCE-GS triester was dose- and time-dependent (2.1-fold in 24 h with 0.5 mM). DCE-GS triester increased the GSH level much more effectively than GSH, DCE-GS, and DCE-GS monooester and diester. Furthermore, the activity of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH biosynthesis, was also increased by DCE-GS triester treatment (1.4-fold in 24 h with 1.0 mM). In contrast, with a rat liver homogenate, DCE-GS increased the γ-GCS activity, whereas DCE-GS triester had no effect on this activity. These results suggested that DCE-GS triester, which is transported into hepatocytes much more effectively than DCE-GS and other DCE-GS esters due to its greater lipophilicity, was hydrolyzed to DCE-GS, and then the DCE-GS produced increased the GSH level via activation of γ-GCS in rat hepatocytes.

Key words glutathione; γ-glutamylcysteine synthetase; acetaminophen; S-(1,2-dicarboxyethyl)glutathione

In 1963 S-(1,2-dicarboxyethyl)glutathione (DCE-GS, Fig. 1) was isolated from calf lenses and its chemical structure was determined by comparison with the synthetic compound. By HPLC after reaction with 2,4-dinitrofluorobenzene we previously determined the content of this peptide in the lenses of various vertebrates, in various tissues of rat, and in the subcellular fraction of rat liver. We also reported that DCE-GS was enzymatically synthesized using glutathione (GSH) and l-malate, and that the enzyme catalyzing this reaction was purified from the rat liver cytosolic fraction. Successively, we found that DCE-GS had potent inhibitory effects on blood coagulation and platelet aggregation, and an enhancing effect on epidermal growth factor-stimulated DNA synthesis in primary cultures of rat hepatocytes. We also determined the levels of GSH and DCE-GS in rat liver during the regeneration of rat liver with time after partial heptatectomy. The GSH and DCE-GS levels increased to a great extent in regenerating rat liver. Recently we showed that oral administration of DCE-GS triester, S-(1,2-diethoxy-carbonyl)glutathione isopropyl ester, to rats inhibited the aceticaminophen (APAP)-induced hepatotoxicity via an increased hepatic GSH content.

However, it remains unclear how DCE-GS triester is transported into hepatocytes and how DCE-GS triester transported into hepatocytes increases the hepatic GSH content. Therefore, in order to clarify the mechanism of protection by DCE-GS triester against APAP-induced hepatotoxicity, we examined the mechanism as to the enhancement of the hepatocellular GSH level on DCE-GS triester treatment using isolated rat hepatocytes.

MATERIALS AND METHODS

Animals Male Wistar rats aged 6 weeks (150—180 g) were used in this study. The animals were obtained from Shizuoka Laboratory Animal (Shizuoka, Japan). Water and food (MF; Oriental Yeast) were provided ad libitum for at least 1 week before use.

Chemicals The structures of DCE-GS and its esters are shown in Fig. 1. DCE-GS, S-(1,2-dicarboxyethyl)glutathione isopropyl ester (monoester), S-(1,2-diethoxy-carbonyl)glutathione (diester), and S-(1,2-diethoxy-carbonyl)glutathione isopropyl ester (triester) were supplied by Senju Pharmaceutical Co., Ltd. (Osaka, Japan). Cell culture reagents were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Other compounds were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Preparation and Primary Culture of Isolated Hepatocytes Hepatocytes, exhibiting more than 85% initial viability, as measured as trypan blue exclusion, were isolated from rats by means of the in situ perfusion technique with collagenase. The isolated hepatocytes were suspended in William’s medium E supplemented with 5% fetal bovine serum, 1 µg/ml insulin, 1 µM dexamethasone and 100 µg/ml kanamycin, inoculated at a cell density of 1 × 10^5 cells/0.2 ml/cm² onto 16- or 35-mm diameter Corning plastic dishes, which had been coated with rat tail collagen, and then cultured under a humidified atmosphere of 5% CO₂ : 95% air at 37°C. After an attachment period of 2 h, the medium was replaced by a serum-free medium containing 100 µg/ml kanamycin, followed by culturing for a further 21 h. DCE-GS and its esters were added to the medium, followed by culturing for various periods of time.

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\begin{align*}
\text{HOOC-CH₂-CH₃CH₂CONH₂} & \quad \text{NH₃} \\
\text{CH₃CONHCH₂CH₂COOR} & \quad \text{S-R₂}
\end{align*}
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Fig. 1. Structures of DCE-GS and Its Esters

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Determination of the Levels of GSH, and DCE-GS and Its Esters in Hepatocytes For determination of the levels of these peptides in hepatocytes, cells that had been cultured in 16-mm dishes were washed three times with ice-cold phosphate buffered saline (PBS), and then 250 µl of 2% perchloric acid was added to the cell pellet, followed by keeping at 4 °C for 30 min. Two hundred microliters of the perchloric acid solution was transferred to an Eppendorf tube and then neutralized with 40 µl of 4 M KH₂PO₄, followed by centrifugation at 5000 × g for 5 min. The supernatant was used for determination of GSH, and DCE-GS and its esters. GSH was determined by the enzymatic recycling method using glutathione reductase and 5,5'-dithiobis-(2-nitrobenzoic acid). Previous results have shown that the treatment with DCE-GS triester caused the elevation of the GSH content in hepatocytes. In order to clarify the mechanism of the elevation of GSH content by DCE-GS triester treatment, the effect of DCE-GS triester on the activity of γ-GCS, the rate-limiting enzyme in the GSH synthesis, in hepatocytes was examined. As shown in Table 2, with 0.5 and 1 mM DCE-GS triester, the increases in the γ-GCS activity reached 139 and 150%, respectively.

Protection against APAP-Induced Hepatotoxicity by DCE-GS Triester The protective effect of DCE-GS triester against APAP-induced hepatotoxicity was examined. The degree of hepatotoxicity was judged as AST activity in the culture medium. As shown in Fig. 2 a marked increase of AST activity in the culture medium (about 12.5-fold over the control) was observed on APAP treatment of hepatocytes. The addition of 0.5 mM DCE-GS triester reduced the AST activity leakage induced by 2 mM APAP to the control level. This protection by DCE-GS triester was dose-dependent (data not shown).

Effect of DCE-GS Triester on the GSH Concentration in Hepatocytes Previously we reported that DCE-GS triester pretreatment significantly prevented the hepatic GSH depletion produced by APAP. Therefore, the effect of DCE-GS triester on the GSH content of rat hepatocytes was examined. After rat hepatocytes had been treated with DCE-GS triester for 5, 10, 20, 22 or 24 h, the cellular GSH content was determined (Fig. 3(a)). The treatment with DCE-GS triester (0.25, 0.5 or 1 mM) caused dose- and time-dependent increases in the intracellular GSH content. In the same manner, the elevating effect of DCE-GS triester on the GSH content of APAP-treated hepatocytes was examined. As shown in Fig. 3(b) treatment with APAP significantly decreased the GSH content of hepatocytes. The addition of DCE-GS triester to the culture medium prevented the GSH depletion caused by APAP in dose- and time-dependent manners. Table 1 summarizes the effects of GSH, and DCE-GS and its esters on the GSH level in rat hepatocytes. DCE-GS monoester, diester and triester at the concentration of 0.5 mM increased the GSH level in rat hepatocytes up to 122, 144 and 210% over the control level after 24 h, respectively, while GSH and DCE-GS had no elevating effect on the GSH level. DCE-GS triester increased the GSH level much more effectively than GSH, DCE-GS, and DCE-GS monoester and diester.

Effects of DCE-GS Triester on the γ-Glutamylcysteine Synthetase (γ-GCS) Activity in Hepatocytes It was shown that the treatment with DCE-GS triester caused the elevation of the GSH content in hepatocytes. In order to clarify the mechanism of the elevation of GSH content by DCE-GS triester treatment, the effect of DCE-GS triester on the activity of γ-GCS, the rate-limiting enzyme in the GSH synthesis, in hepatocytes was examined. As shown in Table 2, with 0.5 and 1 mM DCE-GS triester, the increases in the γ-GCS activity reached 139 and 150%, respectively.

Effects of DCE-GS and Its Esters on the γ-GCS Activity in a Rat Liver Homogenate The effects of DCE-GS...
and its esters at concentrations of 0.1 mM on the γ-GCS activity were examined. A rat liver homogenate 15000× g supernatant was used as the γ-GCS enzyme source. As shown in Table 3, DCE-GS increased the γ-GCS activity up to 300%, while DCE-GS triester had no effect on this activity.

**DISCUSSION**

The present study showed the mechanism of the enhance-
ment of the hepatocellular GSH level by DCE-GS triester treatment.

In a previous paper we reported that pretreatment with DCE-GS triester markedly prevented APAP-induced liver injury in rats. Furthermore, in this study the protective effect of DCE-GS triester against APAP-induced hepatotoxicity was demonstrated using rat hepatocytes (Fig. 2). The hepatotoxicity of APAP is known to involve the formation of a chemically reactive metabolite, N-acetyl-p-benzoquinonimine (NAPQI), which is produced during cytochrome P-450-mediated oxidation of the drug. NAPQI has been reported to be highly reactive both as an electrophile and as an oxidant species, and it irreversibly arylates protein thiol residues.

Several studies have shown that intracellular GSH is of major importance in protecting cells against damage due to toxic and reactive oxygen compounds. The hepatotoxicity of APAP is prevented by compounds such as N-acetylcysteine or 2-oxothiazolidine-4-carboxylate, which act to maintain the GSH level in the liver and to promote the redox cycle of GSH. Hence, we determined the GSH level in rat hepatocytes after treatment with DCE-GS triester. As shown in Figs. 3(a) and (b) treatment with DCE-GS triester significantly prevented the GSH depletion caused by APAP and also increased the hepatic GSH level.

With respect to the elevating effect of DCE-GS triester on the GSH level, it was examined whether or not other peptides caused elevation of the GSH level. DCE-GS triester elevated the GSH level in rat hepatocytes much more effectively than GSH, and DCE-GS and its esters (Table 1). This elevation of the GSH level in rat hepatocytes caused by DCE-GS esters was lipophilicity-dependent. That is to say, this strong elevating effect of DCE-GS triester on the GSH level is probably due to its easy transport into hepatocytes because of its greater lipophilicity.

Next, we examined the effects of DCE-GS esters on GSH synthesis. The synthesis of GSH is catalyzed by two enzymes, \( \gamma \)-GCS and GSH synthetase. The former is the rate-limiting enzyme and its feedback is inhibited by GSH. This feedback inhibition by GSH, which is not allosteric in nature, appears to involve the binding of GSH to the glutamate site of the enzyme as well as to another enzyme site: this site may require a sulfhydryl group since ophthalmic acid \((\gamma\text{-glutamyl}-\alpha\text{-aminobutyryl}-\text{glycine})\) is a weak inhibitor.

Kondo et al. previously showed the significance of glutathione S-conjugate in the regulation of GSH synthesis using human erythrocyte \( \gamma \)-GCS. They reported that feedback inhibition of \( \gamma \)-GCS by GSH was abolished by the addition of a glutathione S-conjugate such as S-2,4-dinitrophenyl glutathione. As shown in Table 2, treatment with DCE-GS triester increased the \( \gamma \)-GCS activity in rat hepatocytes, whereas in experiments involving a rat liver homogenate 15000× g supernatant DCE-GS esters showed no effect on the \( \gamma \)-GCS activity (Table 3). Furthermore, treatment with DCE-GS triester significantly increased the DCE-GS and DCE-GS monoester levels in rat hepatocytes (Table 4). However, the elevations of the \( \gamma \)-GCS activity, and DCE-GS and DCE-GS monoester induced by the treatment with DCE-GS triester did not occur on treatment with bis-\((\rho\text{-nitrophenyl})\) phosphate, a non-specific esterase inhibitor (data not shown).

Nishida et al. elucidated the mechanism of conversion of \( \gamma \)-glutamylecysteinylethyl ester to GSH using isolated rat hepa-
REFERENCES AND NOTES

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