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Short communication

Glycine facilitates γ-glutamylcysteinylethyl ester-mediated increase in liver glutathione level

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Abstract

γ-Glutamylcysteinylethyl ester (γ-GCE) increases reduced glutathione (GSH) levels in GSH-depleted rat hepatocytes. Because glycine, a constituent of GSH, exists at 0.3 to 0.4 mM in rat plasma, we examined the influence of glycine added to the medium on the action of γ-GCE to increase GSH levels in the rat hepatocytes. Glycine (0.2-0.8 mM) dose-dependently enhanced γ-GCE-mediated increase in intracellular GSH levels with an increase in intracellular γ-GCE levels. These results indicate that exogenous glycine facilitates γ-GCE-mediated increase in intracellular GSH levels in rat hepatocytes possibly by enhancing the uptake of γ-GCE into the cells. © 1997 Elsevier Science B.V.

Keywords: γ-glutamylcysteinylethyl ester; Glutathione, reduced; Hepatocyte; Glycine; *Tert*-butyl hydroperoxide

1. Introduction

Glutathione is a tripeptide composed of glutamic acid, cysteine and glycine, i.e., γ-glutamylcysteinylglycine, and exists in many tissues. The liver is one of the tissues with the highest content of glutathione and the main tissue participating in glutathione synthesis (Kaplowitz et al., 1985). Glutathione is synthesized in two steps catalyzed by γ-glutamylcysteine synthetase (first step) and glutathione synthetase (second step), and the first step is known to be rate limiting for cellular glutathione synthesis (Kaplowitz et al., 1985). A reduced form of glutathione (GSH) present in liver cells has been demonstrated to possess a variety of physiological functions, including protection of the cell against oxidative damage and detoxification of xenobiotics via chemical and enzymatic reactions (Reed, 1990).

Depletion of liver GSH often occurs in humans and experimental animals with liver injuries (Reed and Fariss, 1984; Uhling and Wendel, 1992). However, exogenous GSH is unable to cross liver cell membranes as the intact tripeptide under physiological conditions (Hahn et al., 1978; Garcia-Ruiz et al., 1992). Recently, we have demonstrated that in isolated rat hepatocytes, γ-glutamylcysteinylethyl ester (γ-GCE) is hydrolyzed to γ-glutamylcysteine (γ-GC) by esterase, resulting in its conversion to GSH by glutathione synthetase (Nishida et al., 1996). γ-GCE is also known to be transported into GSH-depleted rat hepatocytes much easier than GSH itself (Nishida et al., 1996). Thus, γ-GCE is able to increase the intracellular GSH concentration through its conversion to GSH in GSH-depleted rat hepatocytes. In addition, it has been reported that intravenously administered γ-GCE can prevent ischemia/reperfusion-induced liver injury with a decrease in hepatic GSH levels in rats possibly by maintaining hepatic GSH levels and that this protective effect due to γ-GCE is much greater than that due to GSH (Kobayashi et al., 1992; Ozaki et al., 1994). We also have observed that intraperitoneally administered γ-GCE can prevent the progression of acute liver injury by enhancing decreased hepatic GSH levels in mice intoxicated with carbon tetrachloride (unpublished data). However, in our previous study on the conversion of γ-GCE to GSH in isolated rat hepatocytes experiments were done in the glycine-free incubation medium (Nishida et al., 1996). Glycine is one of three amino acids constituting GSH and a substrate for intracellular GSH synthetase (Kaplowitz et al., 1985). It is known that glycine concentration in rat plasma is 0.3 to 0.4 mM (Fernstrom et al., 1971). Accordingly, it is conceivable that glycine present in rat plasma might affect γ-GCE-mediated increase in hepatocellular GSH levels in vivo.

In this study, we examined the influence of glycine added to the incubation medium on the action of γ-GCE to

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increase GSH levels in isolated rat hepatocytes pretreated with diethylmaleate, a GSH depletor. It has been shown that *N*-acetyl-L-cysteine (NAC) is taken up by hepatocytes, resulting in an increase in the intracellular GSH levels by supplying cysteine, a key precursor of GSH synthesis (Nakano et al., 1995), and that GSH levels in GSH-depleted hepatocytes are increased by exogenously added GSH (Nishida et al., 1996). Therefore, we further compared the influence of glycine added to the incubation medium on the action of GSH or NAC to increase GSH levels in GSH-depleted hepatocytes with that on the action of γ-GCE.

2. Materials and methods

2.1. Estimation of GSH, NAC or γ-GCE uptake into hepatocytes

GSH depletion in isolated rat hepatocytes was achieved by incubating the cells (2×10^6 cells/ml) in Hanks' balanced salt solution (HBSS) (pH 7.2) with 1.0 mM diethylmaleate at 37°C for 10 min. These GSH-depleted hepatocytes (2×10^6 cells/ml) were used throughout this study. GSH was determined by the enzymatic recycling method using glutathione reductase and 5,5′-dithiobis(2-nitrobenzoic acid) as described by Tietze (1969). After 10 min-diethylmaleate treatment, GSH concentration in the hepatocytes was less than 25% of the original concentration as previously described (Nishida et al., 1996). GSH-depleted hepatocytes loaded with GSH (GSH-hepatocytes), NAC (NAC-hepatocytes) or γ-GCE (γ-GCE-hepatocytes) were prepared as follows: hepatocytes were incubated in HBSS containing either GSH (5 mM), NAC (5 mM) or γ-GCE (5 mM) at 37°C for 30 min in the presence or absence of various concentrations of glycine. The amount of GSH transported into hepatocytes or GSH derived from NAC or γ-GCE in hepatocytes was estimated from the increase of intracellular GSH content by incubation. GSH was measured by the above-described enzymatic recycling method. After 30 min incubation of GSH-depleted hepatocytes without any treatment, the GSH concentration in the hepatocytes was decreased by about half of that in the cells before the incubation as well as previously described (Nishida et al., 1996). Both γ-GCE transport into hepatocytes and its conversion to GSH within the cells were further estimated by checking the contents of γ-GCE and its related compounds such as GSH, γ-glutamylcysteine (γ-GC), L-cysteine ethyl ester (CysOEt) and cysteine (Cys) in hepatocytes. γ-GCE-hepatocytes with and without glycine treatment were homogenized in 10 vol of ice-cold 2.5% sulfosalicylic acid. Although GSH was determined by the above-described enzymatic recycling method, γ-GCE and its related compounds such as γ-GC, CysOEt and Cys had no significant effect on the determination. For the simultaneous measurements of γ-GCE, GSH, γ-GC, CysOEt and Cys in γ-GCE-hepatocytes, free thiols in the supernatant were converted to their fluorescent derivatives by reaction with monobromobimane. The preparation of the derivatives and their determinations were carried out according to the method of Newton et al. (1981) with some modifications (Nishida et al., 1996).

2.2. tert-Butyl hydroperoxide-induced injury in hepatocytes

γ-GCE-hepatocytes incubated with and without 0.8 mM glycine at 37°C for 30 min were washed with fresh HBSS twice and then treated with 1.0 mM *tert*-butyl hydroperoxide (TBHP) for 30 min according to our previous report (Nishida et al., 1997). Alanine aminotransferase (ALT), a maker enzyme of hepatic cell damage, in the incubation medium was assayed as described in our report (Nishida et al., 1997).

2.3. Statistical analysis

Results obtained are expressed as means ± S.D. Results are analyzed by computerized statistical packages (Super Analysis of Variance, Statview II) if necessary. Each mean value is compared by one-way analysis of variance and Fisher's protected least significant difference for multiple comparisons as the post hoc test. The level of significance was taken as $P < 0.05$.

3. Results

When rat hepatocytes with GSH depletion were incubated with and without either GSH (5.0 mM), NAC (5.0 mM) or γ-GCE (5.0 mM) for 30 min, intracellular GSH concentration in GSH-, NAC- or γ-GCE-hepatocytes was significantly higher than that in untreated hepatocytes and

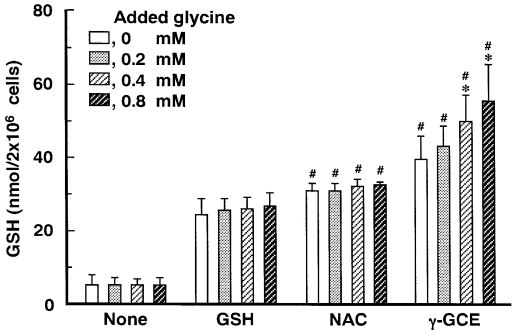

Fig. 1. Effect of glycine co-incubated with GSH, NAC or γ-GCE on intracellular GSH levels in GSH-, NAC- and γ-GCE-hepatocytes. GSH-depleted hepatocytes (2×10^6 cells/ml) were incubated in HBSS containing GSH (5 mM), NAC (5 mM) or γ-GCE (5 mM) with and without glycine at concentrations of 0.2 to 0.8 mM in HBSS at 37°C for 30 min. GSH in the hepatocytes was determined using the enzymatic recycling method as described in Section 2. Each value represents the mean ± S.D. from five independent experiments. * $P < 0.05$ compared with hepatocytes without glycine treatment. # $P < 0.05$ compared with GSH-hepatocytes co-treated with the corresponding concentration of glycine.

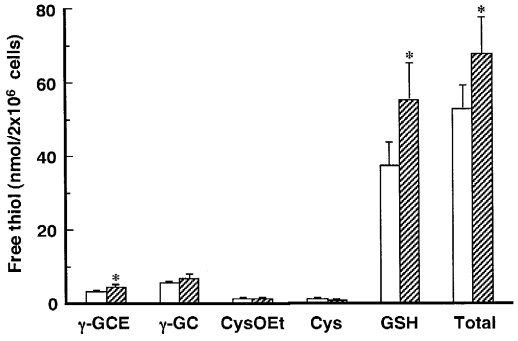

Fig. 2. Concentrations of free thiols in γ-GCE-hepatocytes co-incubated with and without glycine. GSH-depleted hepatocytes (2×10^6 cells/ml) were incubated in HBSS containing γ-GCE (5 mM) with (shaded bar) and without (open bar) 0.8 mM glycine at 37°C for 30 min. γ-GCE, γ-GC, GSH, CysOEt, and Cys in the hepatocytes were determined using high-performance liquid chromatography as described in Section 2. Each point is a mean ± S.D. from five independent experiments. * $P < 0.05$ compared with hepatocytes without glycine.

also the increased GSH concentration in NAC- or γ-GCE-hepatocytes was significantly higher than that in GSH-hepatocytes (Fig. 1). In GSH-, NAC- or γ-GCE-hepatocytes co-incubated with glycine (0.2 to 0.8 mM) for 30 min, any concentration of glycine had no effect on intracellular GSH concentration in GSH- or NAC-hepatocytes, while 0.4 or 0.8 mM glycine further increased intracellular GSH concentrations in γ-GCE-hepatocytes (Fig. 1). This enhancing effect of glycine was significant and occurred in a dose-dependent manner (Fig. 1).

When GSH-depleted hepatocytes were incubated with 5 mM γ-GCE in the presence or absence of 0.8 mM glycine for 30 min, the concentrations of intracellular γ-GCE, GSH and total free thiols in γ-GCE-hepatocytes co-incubated with glycine were significantly higher than those in γ-GCE-hepatocytes without co-incubation with glycine, although there were no differences in intracellular CysOEt and Cys concentrations between γ-GCE-hepatocytes co-incubated with and without glycine (Fig. 2).

Activities of ALT released from GSH-depleted hepatocytes incubated with and without 1.0 mM TBHP were 502.8 ± 84.5 ($n = 5$) and 120.0 ± 24.3 mU/ml ($n = 5$), respectively. The activity of ALT released from γ-GCE-hepatocytes subjected to 1.0 mM TBHP (219.6 ± 40.3 mU/ml, $n = 5$) was significantly lower than that from GSH-depleted hepatocytes subjected to 1.0 mM TBHP ($P < 0.05$). Furthermore, the activity of ALT released from γ-GCE-hepatocytes co-incubated with 0.8 mM glycine in the presence of 1.0 mM TBHP was 164.1 ± 16.8 mU/ml ($n = 5$) and this activity was significantly lower than that from γ-GCE-hepatocytes incubated with 1.0 mM TBHP ($P < 0.05$).

4. Discussion

The present study has clearly shown that exogenously added glycine can facilitate γ-GCE-mediated increase in intracellular GSH levels in isolated rat hepatocytes with GSH depletion. When rat hepatocytes with GSH depletion were incubated with either γ-GCE (5 mM), GSH (5 mM) or NAC (5 mM) in the absence of glycine, an increase in intracellular GSH concentration occurred in γ-GCE-, GSH or NAC-hepatocytes, although the increase in GSH concentration was much higher in γ-GCE-hepatocytes than in GSH- or NAC-hepatocytes. The γ-GCE-mediated increase in intracellular GSH concentration was enhanced by the addition of 0.4 or 0.8 mM glycine to the incubation medium, but not by the addition of 0.2 mM glycine, and this enhancement due to exogenously added glycine occurred in a dose-dependent manner (Fig. 1). In contrast, the GSH-mediated increase in intracellular GSH concentrations was not enhanced by any concentration of glycine added to the incubation medium (Fig. 1). In addition, NAC has been shown to be taken up by hepatocytes and to increase GSH concentration in the cells (Nakano et al., 1995). But, an increase in GSH level in NAC-hepatocytes was not enhanced by any concentration of glycine added (Fig. 1). Therefore, exogenously added glycine may specifically facilitate γ-GCE-mediated increase in intracellular GSH levels in GSH-depleted hepatocytes.

We further compared the intracellular concentrations of total free thiols and γ-GCE and its related compounds such as GSH, γ-GC, CysOEt and cysteine in GSH-depleted hepatocytes co-incubated with 5 mM γ-GCE and 0.8 mM glycine with those in GSH-depleted hepatocytes incubated with 5 mM γ-GCE alone. The intracellular concentrations of total thiols, γ-GCE and GSH in the cells co-incubated with γ-GCE and glycine were significantly higher than those in the cells incubated with γ-GCE alone (Fig. 2). These results suggest that glycine added to the incubation medium might facilitate γ-GCE uptake into GSH-depleted rat hepatocytes and that glycine present in rat plasma might exert such a facilitating action when γ-GCE is systemically administered to rats. Our previous study (Nishida et al., 1996) has shown using the Lineweaver–Burk plot that γ-GCE is taken up into GSH-depleted rat hepatocytes in a diffusion manner. Accordingly, it can be presumed that this diffusion of γ-GCE into GSH-depleted rat hepatocytes is enhanced by exogenously added glycine, although the precise mechanism for this enhancement due to exogenously added glycine is still unclear.

We have observed that glycine is present at concentrations of 0.1 to 0.2 mM in isolated rat hepatocytes with GSH depletion (2×10^6 cells) (unpublished data). We have also confirmed that GSH synthetic activity in isolated rat hepatocytes with diethylmaleate-induced GSH depletion is maintained almost at the same level of freshly isolated rat hepatocytes without GSH depletion (Nishida et al., 1996). It has been reported that GSH synthetase isolated from rat kidney has a K_m value of 0.76 mM for glycine (Oppenheimer et al., 1979), although there is no information on the catalytic properties of GSH synthetase isolated from rat liver. It has also been shown that cultured

rat hepatocytes have a K_m value of approximately 0.17 mM for glycine uptake (Christensen and Handlogten, 1981). From these findings, it can be presumed that glycine added to the incubation medium can be taken up into isolated rat hepatocytes with GSH depletion, resulting in its utilization with γ-GC derived from γ-GCE taken up into the cells for GSH synthesis in the cells. Accordingly, the possibility cannot be ruled out that exogenously added glycine facilitates γ-GCE-mediated increase in intracellular GSH levels in GSH-depleted rat hepatocytes by stimulating intracellular GSH synthesis using γ-GC derived from γ-GCE taken up into the cells. However, this matter should be further investigated.

We have reported that γ-GCE pretreatment inhibits the potentiation of TBHP-induced cell injury in GSH-depleted rat hepatocytes by replenishing intracellular GSH (Nishida et al., 1997). Although hepatocellular injury induced by TBHP was significantly inhibited in γ-GCE-hepatocytes, further inhibition of TBHP-induced hepatocellular injury was observed in γ-GCE-hepatocytes co-incubated with 0.8 mM glycine. These results indicate that exogenously added glycine can increase the protective action of γ-GCE on TBHP-induced cell injury in GSH-depleted hepatocytes possibly by facilitating γ-GCE-mediated increase in intracellular GSH level.

In conclusion, the present results indicate that exogenously added glycine facilitates γ-GCE-mediated increase in intracellular GSH levels in GSH-depleted rat hepatocytes possibly by enhancing the uptake of γ-GCE into the cells, and suggest that such a facilitating effect of glycine might occur when γ-GCE is systemically administered to rats. The concentration of glycine present in human plasma is approximately 0.25 mM (Record et al., 1976; Harvey et al., 1977) and this human plasma glycine concentration is similar to the rat plasma glycine concentration. It is also known that plasma glycine concentrations increase up to 0.5 mM in patients with acute liver diseases (Harvey et al., 1977). Accordingly, when γ-GCE is systemically administered to humans, especially in patients with acute liver diseases, plasma glycine may facilitate γ-GCE-mediated increase in intracellular GSH levels in the hepatic cells by enhancing the uptake of γ-GCE into the cells, although the uptake of γ-GCE into human hepatocytes with GSH depletion has not been examined.

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