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Protective effect of glucose-cysteine adduct on the *in situ* perfused rat liver

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Summary. *In situ* perfusion of rat liver was performed with a medium containing glucose-cysteine adduct [2-(D-gluco-pentahydroxypentyl)-thiazolidine-4-carboxylic acid, glc-cys] and its effect on glutathione (GSH) and ATP levels and bile production was examined. The GSH content in the liver was maintained at the original level during perfusion with 1 mM glc-cys for 2 h, while it decreased significantly in the absence of glc-cys. After 4 h of perfusion without glc-cys, ATP content and bile production decreased significantly besides the decrease in GSH content, but they were maintained at the original levels with glc-cys. When the perfusion was performed with the liver of rats injected with diethyl maleate (DEM), the GSH level, which was decreased to 6.0% of the control by DEM injection, was restored to 22.6% of the original level by perfusion with 2 mM glc-cys for 30 min. Data indicate that glc-cys is a cysteine prodrug with protective action on the liver.

Keywords: Amino acids – Glucose-cysteine adduct – Cysteine prodrug – Liver perfusion – Glutathione – Diethyl maleate

Introduction

Glutathione (GSH) is an important protective agent contained in high concentrations in mammalian tissues (Kosower and Kosower, 1978). It is synthesized and degraded through γ-glutamyl cycle (Meister, 1988), in which L-cysteine is used for the synthesis of γ-glutamylcysteine. In contrast to the high concentrations of GSH in the cell, excess cyst(e)ine is toxic to animals (Meister et al., 1986). Therefore, it is needed to supply L-cysteine with less toxic cysteine prodrugs (Yao et al., 1994) when tissue GSH level is decreased by the administration of GSH-depleting agents (Vinã et al., 1980; Lauterburg et al., 1983; Ruffman and Wendel, 1991; Traber et al., 1992; Yao et al., 1994) or decreased cysteine formation due to impaired trassulfuration pathway (Gomez et al., 1994). Intraperitoneal administration of cysteine prodrugs restores reduced glutathione (GSH) contents in the liver of rats and mice

treated with hepatotoxic substances such as acetaminophen (Vinã et al., 1980; Lauterburg et al., 1983; Ruffman and Wendel, 1991), diethyl maleate (DEM) (Yao et al., 1994) and phorone (Traber et al., 1992). Such cysteine prodrugs include *N*-acetyl-L-cysteine (NAC) (Vinã et al., 1980; Lauterburg et al., 1983; Ruffman and Wendel, 1991; Traber et al., 1992; Yao et al., 1994), L-2-oxothiazolidine-4-carboxylate (OTC) (Williamson et al., 1982; Porta et al., 1991), and condensation products of L-cysteine and reducing monosaccharides (Roberts et al., 1987) such as 2-(D-gluco-pentahydroxypentyl)-thiazolidine-4-carboxylic acid (D-glucose-L-cysteine, glc-cys) (Roberts et al., 1987; Gomez et al., 1994) and 2-(D-ribo-tetrahydroxybutyl)thiazolidine-4-carboxylic acid (D-ribose-L-cysteine, rib-cys) (Roberts et al., 1992). Condensation products of L-cysteine and carbonyl compounds have also been shown to act as cysteine prodrugs (Włodek et al., 1993). However, only a few papers on the glc-cys as a cysteine prodrug (Roberts et al., 1987; Gomez et al., 1994) have been reported.

In the previous paper, we reported that *in situ* perfusion of the rat liver with a NAC-containing medium resulted in the increase in cysteine and GSH concentrations in the perfused liver (Yao et al., 1994). In the present study, we examined the action of glc-cys as a cysteine prodrug in the *in situ* perfused liver of intact and DEM-treated rats and have confirmed that glc-cys has protective effects on the liver as checked by measuring GSH and ATP levels and bile production.

Materials and methods

Materials

Male Wistar strain rats weighing 250–350 g were used in this study, and they were maintained on MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water. Glc-cys was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from yeast, myokinase (EC 2.7.4.3) from rabbit muscle, pyruvate kinase (EC 2.7.1.40) from rabbit muscle and lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle were obtained from Böhringer-Mannheim (Mannheim, Germany). Glutathione reductase (EC 1.6.4.2) from yeast, NADH, NADP and phosphoenol pyruvate were purchased from Oriental Yeast Co. 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) and DEM were products of Wako Pure Chemical Ind. (Osaka, Japan).

In situ liver perfusion

In situ liver perfusion was performed according to the previously reported method (Yao et al., 1994). In brief, surgical operation was performed under anesthesia with pentobarbital, and the liver was perfused *in situ* through portal vein with a perfusion medium warmed at 32°C and oxygenated with 95% O_2-5% CO_2 at a flow rate of 25 ml per min. The perfusion medium was not circulated and flowed from inferior *Vena cava*. Glc-cys was added to the perfusion medium at concentrations as indicated below. After various time intervals of perfusion, GSH, ATP and ADP in the liver tissue were determined. Bile was collected for 1 h interval during the perfusion through a canula placed in the bile duct (Sugano et al., 1978).

When liver perfusion was performed with rats injected with DEM, 1 g of DEM per 1 kg of body weight was injected intraperitoneally (Yao et al., 1994) and the *in situ* liver perfusion was started as above at 60 min after the DEM injection. In this experiment, the

perfusion with a medium containing 1, 2 or 5 mM glc-cys was performed for 30 min after the initial perfusion for 30 min without glc-cys as that in the previous experiments with NAC and L-cysteine (Yao et al., 1994). After washout with the medium without glc-cys for 10 min, GSH, ATP and ADP in the perfused liver were determined. Bile was collected for 30 min during the perfusion with glc-cys.

Analyses

Total GSH (reduced plus oxidized) was determined by the method of Tietze (Tietze, 1969). ATP (Trautschold et al., 1985) and ADP (Jaworek and Welsch, 1985) were determined enzymatically.

Results

Effect of glc-cys on the GSH, ATP and ADP contents and bile production in the in situ *perfused rat liver*

Figure 1 shows GSH contents in the liver after perfusion with 1 mM glc-cys for 4 h. The GSH level in the liver perfused with glc-cys was maintained at the original level for 2 h, while the GSH level in the control liver perfused without glc-cys decreased significantly at 2 h and later. Thus, the difference between the GSH levels of the 2 groups was evident after 2 h of perfusion.

The ATP content in the liver perfused with glc-cys was well maintained at the original level as shown in Fig. 2A. However, the ATP level in the control liver perfused without glc-cys decreased significantly after 3 h, and the difference between 2 groups was also statistically significant after 3 h of perfusion.

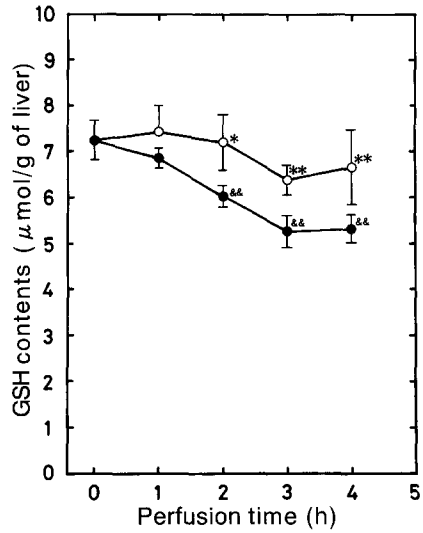

Fig. 1. Glutathione (GSH) contents in the rat liver perfused with glucose-cysteine adduct (glc-cys). *In situ* liver perfusion was performed with a medium containing 1 mM glc-cys and GSH levels were determined. Values are means ± SD of at least 5 animals. ○, 1 mM glc-cys; ●, control. Statistical difference of the values compared to 0-time control (&) or between those of the same time (*) was assessed with Student's *t*-test. &&, **: $p < 0.01$; &, *: $p < 0.05$

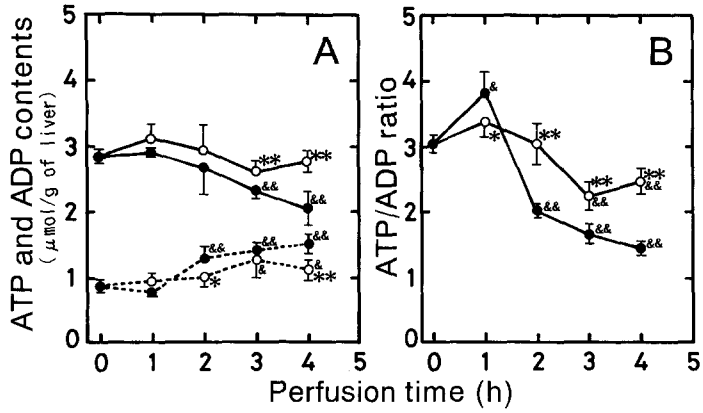

Fig. 2. ATP and ADP contents in the rat liver perfused with glucose-cysteine conjugate (glc-cys). *In situ* liver perfusion was performed with a medium containing 1 mM glc-cys, and ATP and ADP levels were determined. ATP (—) and ADP (---) levels are shown in panel A and ATP/ADP ratio in panel B. Values are means ± SD of at least 5 animals. ○, 1 mM glc-cys; ●, control. Statistical difference was assessed as in Fig. 1

Table 1. Bile production of rat liver perfused with glucose-cysteine adduct (glc-cys)

Time (h) of perfusion	Bile production (μl/60 min per g of liver)	
	With 1 mM glc-cys	Control
1 (6)	41.2 ± 3.7*	37.3 ± 1.2
2 (5)	38.5 ± 1.3&	36.9 ± 1.2
3 (3)	35.8 ± 3.2&	32.2 ± 2.3&&
4 (5)	36.9 ± 0.9&**	32.8 ± 2.1&&

In situ liver perfusion was performed as described under Materials and methods using a medium containing 1 mM glc-cys. Bile was collected for each 60 min interval. Number of animals is shown in parentheses and results are expressed as means ± SD. Statistical difference of the value with glc-cys to the control value (*) and of the value to that at 1 h (&) was assessed with Student's *t*-test. **, &&: $p < 0.01$; *, &: $p < 0.05$.

As shown in Fig. 2A, the ADP content in the liver perfused with glc-cys was well maintained at the original level for 2 h. Then, the level increased gradually. The ADP content in the liver perfused without glc-cys increased more eminently than with glc-cys, and the difference between 2 groups was significant at 2 and 4 h of perfusion.

Figure 2B shows the ATP/ADP ratio in the perfused liver of these 2 groups. The ratio in the liver perfused with glc-cys was well maintained at least for 2 h and those at 3 and 4 h were significantly higher than that in the control liver without glc-cys.

Table 1 shows the production of bile in the perfused liver. The bile production decreased slightly with time of perfusion even with glc-cys, but the

Table 2. GSH, ATP and ADP contents and bile production of rat liver after in situ perfusion for 4h with various concentrations of glucose-cysteine adduct (glc-cys)

Glc-cys (mM)	n	Contents (μmol/g)				Bile (μl/g/h)
		GSH	ATP	ADP	ATP/ADP ratio	
0.0	6	5.33 ± 0.29	2.30 ± 0.43	1.50 ± 0.20	1.44 ± 0.12	33.8 ± 2.8
1.0	6	6.63 ± 0.85**	2.89 ± 0.11**	1.13 ± 0.18**	2.45 ± 0.20**	39.8 ± 4.5*
2.0	5	6.51 ± 0.60**	3.08 ± 0.18**	1.18 ± 0.12**	2.61 ± 0.25**	35.3 ± 3.6
5.0	3	4.89 ± 0.33	2.98 ± 0.10**	1.41 ± 0.15	2.03 ± 0.11**	35.0 ± 3.6
10.0	3	5.35 ± 1.05	2.25 ± 0.23	1.51 ± 0.10	1.49 ± 0.06	22.0 ± 1.0**

Rat liver was perfused as described under Materials and methods with a medium containing various concentrations of glc-cys for 4h and contents of GSH, ATP and ADP were determined, which were expressed as means ± SD. Bile was collected for 4h and expressed as μl per g of fresh liver per h. Statistical difference of the value with glc-cys to that without glc-cys was assessed with Student's *t*-test. **, $p < 0.01$; *, $p < 0.05$. Column n shows the number of animals.

decrease was more evident without glc-cys. The difference of bile production between these 2 groups was significant after 1 and 4h of perfusion and the production in the liver perfused with glc-cys was always higher than that in the liver perfused without glc-cys.

Table 2 shows the effect of glc-cys concentration on GSH, ATP and ADP levels and bile production in the liver perfused for 4h. As shown in Figs. 1 and 2 and in Table 1, GSH and ATP levels and bile production in the liver perfused for 4h with 1mM glc-cys were significantly higher than those in the liver perfused without glc-cys. These effects of glc-cys were also observed in the liver perfused with 2mM glc-cys. However, at 5mM or more, glc-cys was less effective than at 1 or 2mM, and bile production decreased significantly when the liver was perfused with 10mM glc-cys.

Effect of glc-cys on the GSH level in the in situ perfused liver of rats injected with DEM

Table 3 shows the effect of glc-cys in the perfusion medium on the *in situ* liver perfusion of DEM-treated rats. The GSH content was decreased to 6% of the original level by a single intraperitioneal injection of 1g of DEM per kg of body weight. After the *in situ* liver perfusion with 1 and 2mM glc-cys for 30min, the GSH content increased to 11.8 and 22.6%, respectively, of the original value. The increase of glc-cys concentration in the perfusion medium to 5mM did not affect the increase in GSH content compared to 2mM. Bile production decreased significantly by DEM injection, and it recovered to the normal level after perfusion with glc-cys. In contrast to the drastic change in the GSH content, DEM injection did not induce substantial change on ATP and ADP contents as shown in Table 3, indicating that DEM affected only the GSH level, and ATP production was not affected under the present experimental conditions.

Table 3. GSH, ATP and ADP contents and bile production of the liver of diethyl maleate (DEM)-injected rats after *in situ* perfusion with glucose-cysteine adduct (glc-cys)

Group (n)	DEM	Glc-Cys (mM)	Contents (μmol/g)				Bile (μl/g/h)
			GSH	ATP	ADP	ATP/ADP ratio	
A (5)	−	−	6.64 ± 0.39	3.18 ± 0.15	0.95 ± 0.25	3.32 ± 0.38	36.7 ± 0.7
B (3)	+	−	0.40 ± 0.18&&	2.92 ± 0.26	0.92 ± 0.12	3.17 ± 0.14	29.9 ± 1.1&&
C (3)	+	1.0	0.78 ± 0.05**	3.01 ± 0.20	0.94 ± 0.14	3.11 ± 0.23	32.9 ± 1.3*
D (5)	+	2.0	1.50 ± 0.4**	3.15 ± 0.23	1.05 ± 0.06	2.95 ± 0.11	34.7 ± 1.8**
E (5)	+	5.0	1.53 ± 0.17**	3.28 ± 0.23	1.06 ± 0.12	3.06 ± 0.25	36.5 ± 1.9**

DEM (1 g/kg of body weight) was intraperitoneally injected and *in situ* liver perfusion was started at 60 min after the injection and continued for 30 min as described under Materials and methods. Values are expressed as means ± SD, and statistical difference of the value of group B to that of group A (&) and the values of groups C, D or E to that of the group B (*) was assessed with Student's *t*-test. &&, **: $p < 0.01$; *, $p < 0.05$. Number of animals is shown in parentheses.

Discussion

GSH and ATP are important cell components which function as, for example, the intracellular reducing agent and energy carrier, respectively. Therefore, the maintenance of the levels of these components in the cell is important for cell functions. In our previous report, it was shown that *in situ* liver perfusion with a medium containing 10 mM NAC or 10 mM L-cysteine resulted in the increase in cysteine and GSH levels in the perfused liver of DEM-treated rats (Yao et al., 1994). The present study examined the protective effect of glc-cys on the *in situ* perfused rat liver.

As shown in Fig. 1, the decrease in the GSH level in the perfused liver was significantly protected when perfusion was performed with a medium containing glc-cys. Glc-cys also protected significantly the perfused liver from the changes in ATP and ADP contents as shown in Fig. 2. As for the bile production, one of the important liver functions, the protective effect of glc-cys was not so evident as that on GSH and ATP levels, but it was effective when perfusion was prolonged as shown in Table 1.

In the liver of DEM-treated rats, a 17% recovery of the GSH level was attained by 30 min-perfusion with 2 mM glc-cys. This effect is comparable with that with 10 mM NAC (Yao et al., 1994). Thus, the present data show that glc-cys has protective effects on the *in situ* perfused rat liver. Results in Tables 1 and 2 seem to indicate that the preferable concentration of glc-cys is 1–2 mM when it is applied in liver perfusion. This concentration may be applicable to the intravenous administration because glc-cys is considered to be converted non-enzymatically to cysteine.

Roberts et al. (Roberts et al., 1987) synthesized 8 cysteine prodrugs including glc-cys and compared their capability for GSH synthesis and protective effects against acetaminophen-induced hepatotoxicity. They found that glc-cys was most effective in increasing GSH level in rat hepatocyte preparation. However, glc-cys was not significantly effective on the survival rate of

acetaminophen-treated mice when glc-cys was intraperitoneally administered at 30 min after intraperitoneal injection of acetaminophen. Gomez et al. studied the effect of glc-cys on the prevention of the increase in the plasma alanine aminotransferase level induced by acetaminophen (Gomez et al., 1994). They found that glc-cys effectively attenuated acetaminophen toxicity when glc-cys was injected at 4 h before the intraperitoneal acetaminophen injection or acetaminophen was administered orally, and they have concluded that glc-cys may function as a slow release formulation of cysteine. Our data, especially those shown in Table 3, seem to agree with this conclusion. Thus, glc-cys might be effective for the preventive use under hepatotoxic conditions such as liver transplantation. Further study on the metabolism of intraperitoneally injected glc-cys in rats is under investigation.

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