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Effect of glucose-cysteine adduct as a cysteine prodrug in rats

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Summary. Effect of intraperitoneal administration (5 mmol/kg of body weight) of glucose-cysteine adduct (glc-cys) as a cysteine prodrug in rat tissues was studied. Cysteine levels in liver and kidney increased to 1.08 and 1.98 μmol per g or ml, respectively, at 2 h after the administration. GSH levels did not change substantially. However, when glc-cys was injected to rats treated with diethyl maleate, a GSH-depleting agent, the decreased GSH levels were restored rapidly. The recoveries in liver and kidney were 72% at 1 h and 66% at 2 h, respectively, after glc-cys administration. Metabolism of glc-cys was assessed by urinary excretion of glc-cys, sulfate and taurine. Average excretion of glc-cys was 2.86 mmol/kg/24 h after glc-cys administration. Increased excretions of sulfate and taurine were 0.77 and 0.14 mmol/kg/24 h, respectively. Data show that, although glc-cys excretion was relatively rapid, glc-cys was effectively utilized for GSH synthesis in GSH-depleted tissues.

Keywords: Amino acids – Glucose-cysteine adduct – Cysteine prodrug – Glutathione – Cysteine – Sulfate

Introduction

Glutathione (GSH) is the most abundant sulfhydryl compound present in most mammalian cells in the range from about 0.8 mM to about 12 mM (Kosower and Kosower, 1978) and functions as an important protective agent. In many tissues, GSH is thought to act to maintain protein sulfhydryl groups, to remove peroxides and free radicals and to serve for disulfide exchange reactions and other enzymatic reactions (Meister, 1975). Another function of GSH is detoxification by mercapturic acid formation, of which liver is the major site. Tissue GSH concentrations are maintained by active synthesis and degradation through γ-glutamyl cycle (Meister, 1975) and its half-lives in the rat liver was estimated to be about 2 to 4 h (Waelsch and Rittenberg, 1942; Sekura and Meister, 1974) and those in the mouse liver and kidney were 145 and 29 min, respectively (Sekura and Meister, 1974).

In contrast to high tissue GSH levels, cysteine (and/or cystine) concentrations in the cell are low except for cystinotic cells. Cysteine seems to be maintained at low levels by its utilization for GSH and protein syntheses and its metabolism to sulfur compounds, mainly sulfate and taurine. In fact, cysteine injection to rats resulted in the increase in sulfate and taurine excretion, and there seems to exist a sulfur equilibrium between injected and excreted sulfur (Ubuka et al., 1995). In mammals, cysteine is supplied as a component of dietary proteins and is formed through the transsulfuration pathway from methionine, which was also supplied by dietary proteins. Therefore, it seems to be necessary to provide cysteine when cysteine demand is elevated due to the impairment of the transsulfuration pathway (Gomez et al., 1994) or the elevation of GSH demand (Vina et al., 1980). However, high concentrations of cysteine and cystine are toxic to animals (Meister A, 1988). Therefore, cysteine prodrugs are considered as a means of low or non-toxic cysteine supply. As such cysteine prodrugs, effects of L-2-oxothiazolidine-4-carboxylate (OTC) (Williamson et al., 1982) and N-acetyl-L-cysteine (NAC) (Lauterburg et al., 1983) have been studied. In the previous report we described the effect of glucose-cysteine adduct (glc-cys) [2-(D-gluco-pentahydroxypentyl)-thiazolidine-4-carboxylic acid, the condensation product of D-glucose and L-cysteine] on the GSH level in *in situ* perfused rat liver (Yao et al., 1996). In the present paper, we report *in vivo* effect of glc-cys as a cysteine prodrug in intact rats and those treated with diethyl maleate (DEM), a GSH-depleting agent (Boyland and Chasseaud, 1970).

Materials and methods

Materials

Male Wistar strain rats weighing 250–350 g were used, and maintained on laboratory diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum throughout this study. Glc-cys was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). DEM and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Glutathione reductase (EC 1.6.4.2) was from Oriental Yeast Co., Ltd.

Injection of glc-cys and DEM

Glc-cys and DEM were administered to rats by a single intraperitoneal injection. Glc-cys solution of 0.75 mmol/ml in water (adjusted to pH 7.0 with sodium hydroxide solution) was injected at a dose of 5 mmol per kg of body weight except otherwise indicated. DEM was injected as a suspension in corn oil (1 g/2 ml) at a dose of 1 g per kg of body weight.

Determination of cysteine, glc-cys and GSH in liver, kidney and blood plasma

Cysteine and glc-cys were determined by acidic ninhydrin reaction (Gaitonde, 1967). Because glc-cys reacted with acidic ninhydrin reagent with a color value of 50% of cysteine, cysteine (and cystine) and glc-cys were separated by ion-exchange chromatogra-

phy as follows. Tissue samples were homogenized in 4% perchloric acid: 1.0 g of liver or kidney with 4 volumes of 5% perchloric acid, and 2 ml of blood plasma with 2 volumes of 6% perchloric acid. After elimination of precipitated protein by centrifugation, perchloric acid in the resulting supernatant was removed by titration with potassium hydroxide and centrifugation. The supernatant (2.0 ml) was applied to a column of Dowex 50W (100–200 mesh, 7 × 35 mm, H^+ form). The column was eluted with 60 ml of 0.1M HCl followed by 30 ml of 1M HCl, collecting 3-ml fractions. Glc-cys was eluted by 0.1M HCl and cysteine (and cystine) by 1M HCl. Fractions containing glc-cys and cysteine (and cystine) were pooled separately and subjected to acidic ninhydrin reaction. All these procedures were processed quickly in order to minimize spontaneous hydrolysis of glc-cys.

Total GSH was determined according to the method of Tietze (Tietze, 1969) using DTNB and glutathione reductase.

Determination of glc-cys, cysteine, sulfate and taurine in urine

Rats were housed separately in metabolic cages and weighed every morning. Twenty-four hour urine was collected in a 100-ml Erlenmeyer flask containing 5 ml of 50% acetic acid and 1 ml of toluene. A urine sample (1.0 ml of the rats injected with glc-cys or 3.0 ml of the control rats) was applied to a Dowex 50W column, and glc-cys and cystine were separated and determined as described above. Total sulfate (free plus ester) was determined by ion chromatography after hydrolysis as described (Fujiwara et al., 1995). Taurine, and hypotaurine if any, was determined by reversed phase high-performance liquid chromatography after conversion to 4-dimethylaminoazobenzene-4′-sulfonyl-taurine (dabsyl-taurine), and dabsyl-hypotaurine if any (Futani et al., 1994).

Statistical analysis

Data were expressed as mean ± SD, and statistical significance was assessed by Student's *t* test.

Results

Tissue contents of glc-cys and cysteine after intraperitoneal injection of glc-cys

Although there might be a possibility that glc-cys is formed in the cell, it was not detected in tissue extracts and urines under the analytical conditions used in this study. However, as shown in Fig. 1, substantial amounts of glc-cys were detected in liver, kidney and blood plasma after the intraperitoneal injection of glc-cys. Its contents in these tissues were 4.02 ± 0.38, 3.83 ± 0.31 and 3.21 ± 0.22 μmol/g of fresh tissue or ml, respectively, at 1 h after glc-cys injection. These values decreased gradually and contents at 2 h after the administration were 49 (1.96 ± 0.18 μmol), 41 (1.98 ± 0.29 μmol) and 48% (1.54 ± 0.15 μmol), respectively, of those at 1 h. Still at 8 h, 0.66 ± 0.11 and 0.24 ± 0.07 μmol/g of glc-cys were detected in liver and kidney, respectively.

Figure 1 also shows total cysteine (cysteine plus cystine, expressed as cysteine) contents in liver, kidney and blood plasma after intraper-

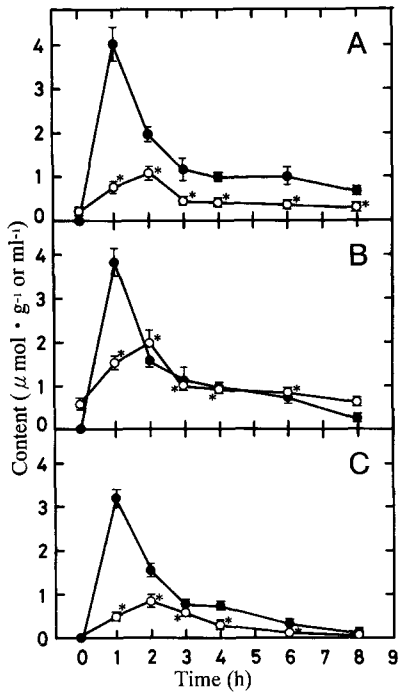

Fig. 1. Contents of glucose-cysteine adduct (glc-cys) and cysteine in rat tissues after intraperitoneal injection of 5 mmol glc-cys per kg of body weight. **A** Liver, **B** kidney and **C** blood plasma. Values are means ± SD of 5 to 7 animals. Statistical difference compared to the value at time 0 was assessed: *$p < 0.01$; • glc-cys; ○ cysteine

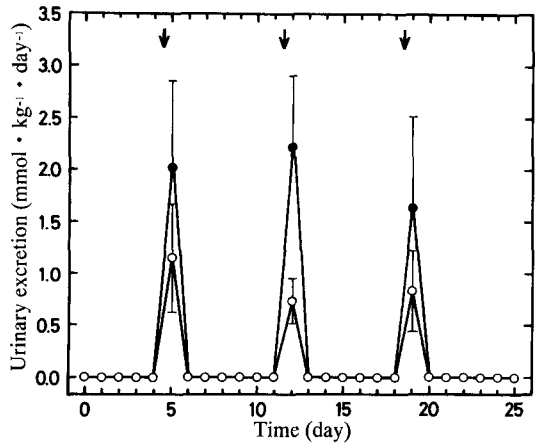

Fig. 2. Excretion of glucose-cysteine adduct (glc-cys) in the 24-h urine of rats after intraperitoneal administration of 5 mmol glc-cys per kg of body weight. Cysteine (○) detected in the urine sample was concluded to be formed from glc-cys (•) during urine collection. See the text. Arrows indicate the time of glc-cys injection. Values are means ± SD of 5 to 6 animals

itoneal injection of glc-cys. Cysteine contents before the administration were 0.21 ± 0.04, 0.56 ± 0.15 and 0.05 ± 0.04 μmol/g of fresh tissue or ml of liver, kidney and blood plasma, respectively, and these contents increased to 1.08 ± 0.16, 1.98 ± 0.29 and 0.83 ± 0.13 μmol/g or ml, respectively, at 2h after the glc-cys injection. Then, cysteine contents decreased gradually and returned to the original levels at 8h after the glc-cys administration.

Urinary excretion of glc-cys after glc-cys administration

Figure 2 shows substantial amount of glc-cys was excreted in the urine. Glc-cys excreted in 24-h urines after three glc-cys administrations were 2.02 ± 0.84, 2.21 ± 0.68 and 1.63 ± 0.89 mmol, respectively. No glc-cys was detected thereafter.

Figure 2 also shows total cysteine (cysteine plus cystine as mentioned above) detected in the urine samples. Average excretion of total cysteine before glc-cys administration was 0.54–0.58 μmol/kg of body weight per day and it was excreted as cystine. In the 24-h urine after three glc-cys administrations, the amount of cysteine found in the 24-h urine was 1.15 ± 0.54, 0.73 ± 0.21 and 0.84 ± 0.40 mmol/kg, respectively. Control experiments were conducted to examine the origin of the cysteine in the urine samples and it was concluded that the cysteine was formed from glc-cys by spontaneous hydrolysis during collection of urine. Therefore, glc-cys excretion after three glc-cys administration is presumed to be 3.17, 2.94 and 2.47 mmol/kg per day, respectively.

Tissue GSH contents after intraperitoneal injection of glc-cys

Figure 3 shows GSH contents after glc-cys administration in liver (Fig. 3A), kidney (Fig. 3B) and blood (Fig. 3C) in intact and DEM-treated rats. In the liver, the average GSH content before glc-cys administration was 6.64 ± 0.39 μmol/g of fresh liver. The GSH content did not change considerably in contrast with that of cysteine. Only significant change was 1.3-fold increase (8.59 ± 1.06 μmol/g) at 2h after the glc-cys administration. As shown in Fig. 3A, intraperitoneal injection of DEM (1 g/kg of body weight) at time 0 resulted in a drastic decrease in hepatic GSH contents, which was 0.25 ± 0.05 μmol/g at 1h after the DEM administration. When 5 mmol glc-cys per kg of body weight was injected at time 1h, the decreased liver GSH level was recovered to 4.79 ± 1.38 μmol/g (72% of the original) after 1h. When 2 mmol glc-cys was injected, the recovery of GSH in the same period was 1.47 ± 0.26 μmol/g (22% of the original).

In the kidney, the GSH content before glc-cys administration was 2.03 ± 0.21 μmol/g of fresh kidney and it did not change by the administration of 5 mmol glc-cys. Injection of DEM at time 0 resulted in the decrease in GSH content to 0.43 ± 0.02 μmol/g at 1h after the DEM administration. A single intraperitoneal injection of 5 mmol glc-cys per kg of body weight at time 1h

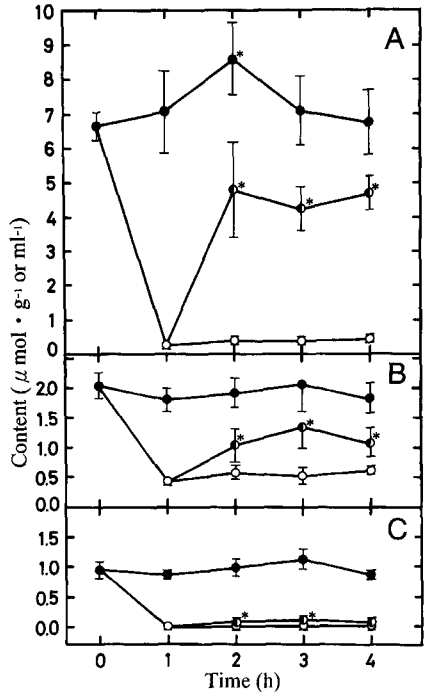

Fig. 3. Tissue glutathione (GSH) contents after intraperitoneal administration of 5 mmol glucose-cysteine adduct (glc-cys) per kg of body weight to rats, which were injected with diethyl maleate (1 g/kg of body weight) (○, ◐) or with the vehicle (●) at time 0. **A** Liver; **B** kidney; **C** blood. Glc-cys was administered at time 0 (●) or at 1 h (◐). Values are means ± SD of 5 to 6 animals. Statistical difference was assessed compared to time 0 (●) or time 1 h (◐): * $p < 0.01$

resulted in GSH levels of 1.02 ± 0.29 (50% of the original) and 1.34 ± 0.38 μmol/g (66% of the original) of fresh kidney at 1 and 2 h, respectively, after the glc-cys administration. When 2 mmol glc-cys was injected, the recovery of GSH in the same periods were 1.19 ± 0.10 (59%) and 1.03 ± 0.09 (51%) μmol/g, respectively.

In the blood, GSH content before glc-cys administration was 0.93 ± 0.18 μmol/ml and it did not change appreciably by 5 mmol glc-cys administration as in the liver and kidney. Injection of DEM resulted in a low GSH level of 0.06 ± 0.03 μmol/ml. Administration of glc-cys resulted in only a small recovery of GSH level.

Urinary excretion of sulfate and taurine after intraperitoneal injection of glc-cys

Figure 4 illustrates excretion of total sulfate. Average daily excretion of total sulfate before glc-cys administration ranged from 1,223 ± 287 to 1,468 ± 169 μmol/kg of body weight per day in 5 control rats. When 5 mmol/kg of glc-cys was intraperitoneally injected to 6 rats at 1 week intervals, a significant increase in the excretion of total sulfate occurred in the 24-h urine after

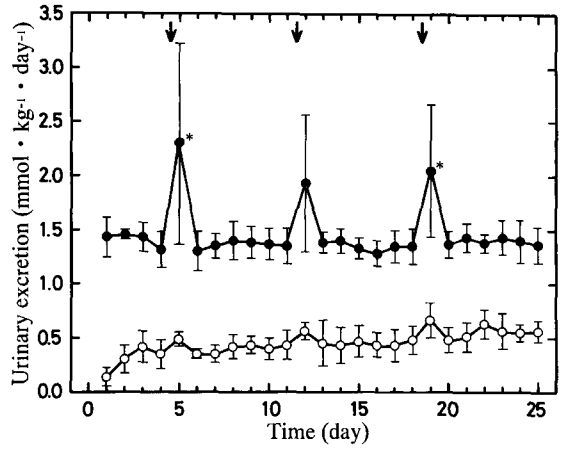

Fig. 4. Excretion of sulfate and taurine after intraperitoneal administration of 5 mmol glucose-cysteine adduct (glc-cys) per kg of body weight to rats. Total sulfate (free and ester) (•) and taurine (○) in 24-h urine of the same rats as those of Fig. 2 were determined. Arrows indicate the time of glc-cys injection. Values are means ± SD and statistical difference compared to that of the previous day was assessed: *$p < 0.01$

the injection. The average excretion after 3 glc-cys injections was 2,098 ± 717 μmol/kg of body weight per day, showing that the average increase in sulfate excretion was 0.77 ± 0.21 mmol/kg per day, which corresponds to 12.6% of the injected glc-cys.

Figure 4 also shows taurine excretion in the same urine as that of sulfate excretion. Taurine excretion increased in the 24-h urine after glc-cys injection. The average increase compared to the excretion in the 24-h urine of the previous day was 0.14 ± 0.03 mmol/kg of body weight per day, which corresponded to 2.8% of the glc-cys administered. However, there was no statistical significance in the increase in taurine excretion compared to the previous day excretion and between the experimental and control rats.

Growth curve of rats injected with glc-cys

There was no difference between the growth curves of the 2 groups of rats, of which urinary excretions of cystine, glc-cys, sulfate and taurine were determined.

Discussion

Roberts et al. synthesized 8 cysteine prodrugs including glc-cys and studied their capability for GSH synthesis and protective effects against acetaminophen-induced hepatotoxicity (Roberts et al., 1987). It was shown that glc-cys is capable of releasing L-cysteine and glucose by nonenzymatic ring opening and hydrolysis, and that glc-cys was most effective in increasing GSH level in rat hepatocyte preparation *in vitro*. However, it was not signifi-

cantly effective on the survival rate of acetaminophen-treated mice when glc-cys was intraperitoneally administered at 30 min after the acetaminophen injection. Gomez et al. (Gomez et al., 1994) studied the effect of glc-cys and found that it attenuated effectively acetaminophen toxicity in mice when glc-cys was injected at 4h before the intraperitoneal acetaminophen injection or acetaminophen was administered orally. These findings suggested that glc-cys was effective by a slow release of cysteine.

Present experiments have shown that liver contained 4.02 ± 0.38 μmol/g of glc-cys at 1h after its injection and 49% of this glc-cys remained even after 2h. On the other hand, cysteine content reached a peak at 2h after the glc-cys administration, and this is in sharp contrast to the rapid increase in the liver cysteine level after the administration of *N*-acetyl-L-cysteine (Yao et al., 1994). The relationships between glc-cys and cysteine contents in the kidney and blood were similar to that in the liver. As shown in Fig. 2, substantial amount of glc-cys was excreted in the 24h-urine after its administration. The average amount after three administration was 1.95mmol/kg of body weight per day. Moreover, 0.91 ± 0.42mmol/kg of cysteine was detected in the 24-h urine after glc-cys administration. In our previous experiments (Yoshida et al., 1989), most part of injected cysteine was metabolized to sulfate and taurine, and cysteine excretion was negligible. Control experiments have shown that a substantial amount of cysteine, which corresponded to the amount detected in urine samples, was formed during the collection and storage of urine samples for the time of present experiments. Therefore, it was concluded that the cysteine found in the urine was formed by spontaneous hydrolysis during the collection and storage of urine samples. Thus, these results show that glc-cys corresponding to 57.2% (2.86mmol/kg) of the injected (5.00mmol/kg) was excreted as glc-cys without further metabolic change, indicating that *in vivo* metabolism of injected glc-cys is rather slow as suggested (Gomez et al., 1994) and excretion of glc-cys is relatively rapid.

Glc-cys administration to intact rats did not affect the GSH levels in the liver, kidney and blood except for a 1.3-fold increase in the liver at 2h after its administration. In the GSH-depleted liver, 72% of the original GSH level was recovered in 1h after the glc-cys administration. In the GSH-depleted kidney, the recovery was 50% and 66% at 1 and 2h, respectively, after glc-cys administration. The effect of glc-cys for the increase in the GSH levels in GSH-depleted tissues is comparable to that of NAC in rats (Yao et al., 1994) and that of OTC in mice (Williamson et al., 1982).

Administration of glc-cys resulted in the increase in the excretion of total sulfate as shown in Fig. 4. The average increased excretion after three glc-cys administration in the 24-h urine compared to the previous day was 0.77mmol per kg of body weight per day, which corresponded to 15.4% of the glc-cys administered. The increased excretion of sulfate occurred only in the 24-h urine after glc-cys administration as observed after the administration of L-cysteine (Yoshida et al., 1989) and OTC (Yamada et al., 1995). Taurine excretion also increased after the glc-cys administration and the average increase was 0.14mmol per kg per day corresponding to 2.8% of the glc-cys administered, although the increase was statistically insignificant. These re-

sults suggest that glc-cys corresponding to at least 18% of the injected glc-cys was metabolized to sulfate and taurine through L-cysteine formation during 24 h following 5 mmol glc-cys administration.

Results obtained in the present study indicate that 60% of 5 mmol glc-cys injected to rats was excreted as glc-cys and at least about 20% was metabolized to sulfate and taurine during 24-h after the administration and that glc-cys was effective in the elevation of decreased GSH levels in the liver and kidney.

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