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Polyamines and thiols in the cytoprotective effect of L-cysteine and L-methionine on carbon tetrachloride-induced hepatotoxicity

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Summary. The relationship between cellular glutathione (GSH), protein-SH levels, and lactate dehydrogenase (LDH), with respect to the effect of polyamines on the cytoprotective ability of L-cysteine and L-methionine, the most important components in the sulfur amino acid metabolic pathway, in carbon tetrachloride (CCl_4)-induced toxicity in isolated rat hepatocytes was studied. CCl_4 induced a LDH release and decreased cellular thiols and polyamines levels but treatment with L-cysteine and L-methionine reversed these decreases. Treating with methylglyoxal bis-(guanylhydrazone), MGBG, an irreversible inhibitor of S-adenosylmethionine decarboxylase, which is a key enzyme in spermidine and spermine biosynthesis, and therefore used to deplete cellular polyamines, prevented the protective effect of L-cysteine and L-methionine, but the addition of exogenous polyamines inhibited the influence of MGBG. These results suggest that the cytoprotective effect of L-cysteine and L-methionine in CCl_4-induced toxicity were via maintenance of cellular polyamines, GSH and protein-SH concentrations and prevention of LDH leakage.

Keywords: Amino acids – L-Cysteine – L-Methionine – Polyamines – Glutathione – Protein sulfhydryl – Hepatocytes

Introduction

Cysteine and methionine, two sulfur amino acids, are well known as the essential components for glutathione (GSH) synthesis, influencing synthesis of taurine (Stipanuk et al., 1992), and also an adequate supply of these sulfur amino acids is crucial for maintaining a normal hepatic GSH level (Wang et al., 1997). Cysteine and methionine are also necessary in the synthesis of polyamines, polycationic physiologically important compounds (Acuff and Smith, 1983). Polyamine synthesis involves L-methionine and L-ornithine as precursor amino acids and two key enzymes, S-adenosylmethionine decarboxylase and L-ornithine decarboxylase (ODC), yielding polyamine

(Bacchi, 1981). Dietary cysteine has been shown to stimulate the biosynthesis and increase tissue concentration of polyamines (Acuff and Smith, 1983).

Sulfhydryl groups in both proteins and non-proteins are involved in the maintenance of various cellular functions, including many enzymatic activities (Stamler, 1983). Thus, perturbation of intracellular thiol homeostasis has been found to be critically involved in the development of chemically induced cell damage. GSH, the major non-protein thiol in mammalian cells, is involved in many cellular functions including amino acid transport (Meister, 1973), and decreases in response to oxidative stress caused by radiations, chemical compounds, drugs, hyperoxia, and ischemia/reperfusion (Ishikawa and Sies, 1984; Ceconi et al., 1985). Free sulfhydryl groups in proteins play the role as highly reactive functional groups in biological systems and participate in several different reactions, such as alkylation, arylation, oxidation, thiol-disulfide exchange, etc. Therefore the modification of protein thiol groups can result in severe functional damage, including loss of enzyme activity (Orrenius, 1985). Polyamines (putrescine, spermidine and spermine) are ubiquitous polycationic metabolites in prokaryotic and eukaryotic cells (Tabor and Tabor, 1984), and are essential for a normal growth rate in mammalian cells. Several studies have reported that polyamine synthesis is required for regeneration in several tissues, including the liver (Pösö and Pegg, 1982; Higaki et al., 1994; Luk, 1986). We recently reported that the mechanism of protection provided by taurine against CCl_4 and hydrazine hepatotoxicity involved a prevention of leakage of polyamines to maintain membrane integrity (Wu et al., 1997).

In this study, the protective effects against the toxicity of CCl_4 in isolated rat hepatocytes by cysteine and methionine, the precursor amino acids of taurine, were investigated.

Materials and methods

Materials

L-Cysteine and L-methionine, CCl_4, collagenase, sodium pyruvate, 5-sulfosalicylic acid dihydro, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and diethyl maleate (DEM) were obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Methylglyoxal bis-(guanylhydrazone) (MGBG), putrescine, spermidine, spermine, sodium NADH and GSH (reduced form) were obtained from Sigma-Aldrich Fine Chemicals (Tokyo, Japan).

Methods

Hepatocytes preparation and culture

Hepatocytes were isolated from male Sprague-Dawley rats weighing 200 to 250 g by collagenase perfusion (Moldéus et al., 1993). The viability of the isolated hepatocytes was over 90% as determined by 0.2% trypan blue exclusion. The cells were plated in 35-mm plastic dishes at a density of 2.5×10^5 cells/ml in 2 ml Williams' medium E supplemented with 5% FBS, 1 nM insulin and 10 nM dexamethasone. The cells were cultured in humidified atmosphere of 5% CO_2 and 95% air at 37°C overnight. This medium was

changed for Hanks buffer (137.0 mM NaCl, 5.37 mM KCl, 1.25 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.44 mM KH_2PO_4, 0.34 mM $Na_2HPO_4 \cdot 12H_2O$, 4.17 mM $NaHCO_3$, 5.55 mM glucose, 0.017 mM phenol red, pH 7.4) with or without L-cysteine, L-methionine and polyamines. One hr after changing the medium, CCl_4 or DEM was added and the cells were incubated for 2 hrs. L-cysteine, L-methionine and the polyamines remained in the incubation buffer till harvest. The suspension buffer was used for assay of lactate dehydrogenase (LDH) activity, and the cells were used for assay of polyamine contents, GSH and protein-SH concentrations. Experimental agents such as L-cysteine, L-methionine, polyamines and MGBG were added directly to the buffer. CCl_4 was dissolved in dimethyl sulfoxide (DMSO) (3:5 by volume) just before being added to the incubation buffer. DMSO had no interfering effect on the outcome of the experiments.

LDH assay

Measuring the LDH activity in the suspension buffer as previously described (Bergmeyer et al., 1965), monitored the degree of cell injury. Assay conditions included 0.6 mM sodium pyruvate, 0.18 mM NADH, and a suitable volume of enzyme solution at 25°C in a total volume of 3.15 ml. The initial rate of NADH loss, measured as a reduction in absorbance at 340 nm, was used as an indication of LDH activity. Under these assay conditions the loss of NADH was linear with respect to time and enzyme concentration over the range of enzyme activity monitored.

Cellular GSH and protein-SH assay

Cellular GSH and protein-SH concentrations were measured as previously described (Beutler et al., 1963). Cells (5×10^5) were collected and an extract was obtained by treating with 0.5 ml 2% 5-sulfosalicylic acid, and centrifuged at 3,000 rpm for 10 min. The supernatant obtained was used for the assay of intracellular GSH concentration and the cell pellet suspended in 1.2 ml 0.5 M Tris-HCl, pH 7.6 was used for the protein-SH assay. DTNB (at a final concentration of 100 μM) was then added and after 20 min the absorbance was measured at 412 nm. Data were expressed as nmoles SH/5×10^5 cells, calculated on the basis of a GSH calibration curve.

Polyamine assay

Polyamine was measured as previously described (Matsui-Yuasa et al., 1994). Cells (5×10^5) collected by centrifugation were extracted with 0.3 ml of 0.4 N perchloric acid. The supernatant was stored at −20°C until being used. The polyamines were separated on a STR ODS-II column (4.6 × 150 mm, particle size 5 μm, Shimadzu Techno-Research, Kyoto, Japan), with a solvent composed of 10 mM 1-hexanesulfonic acid sodium salt/100 mM sodium perchloric acid as solvent A and solvent A/methanol (1:3) as solvent B. The sample was eluted with 96% of solvent A and 4% of solvent B for 3 min, and then with a programmed solvent gradient using a linear gradient curve. The gradient changed from 20% to 55% of solvent B from 3.1 min to 25 min at a flow rate of 0.7 ml/min. The fractions eluted were mixed with O-phthalaldehyde (0.7 ml/min), and the fluorescence was measured with an excitation wavelength of 345 nm and an emission wavelength of 440 nm for assay of the polyamines with RF 535 Shimadzu fluorescence monitor. DNA content was determined as previously described (Burton, 1968), using calf thymus DNA as the standard.

Statistical analysis

Results were presented as means ± S.D of three experiments followed by Duncan's multiple range tests to determine significant ($p < 0.05$) differences between means.

Results

The protection of L-cysteine and L-methionine against CCl_4 -induced injury in isolated hepatocytes as indicated by LDH leakage was investigated. Figure 1 shows that CCl_4 (3μl/2ml medium) induced a release of LDH, but addition of L-cysteine and L-methionine reduced this effect in a dose-dependent manner (250μM – 1mM). Identical data were obtained with both 500μM and 1mM treatments, hence in subsequent experiments 500μM L-cysteine and L-methionine were used.

In Fig. 2, addition of L-cysteine and L-methionine decreased CCl_4-induced LDH leakage. However, adding DEM (1mM), a cellular thiols depleting agent, inhibited the protection afforded by L-cysteine and L-methionine. Table 1 shows that CCl_4 also decreased cellular GSH and protein-SH. Addition of L-cysteine and L-methionine reduced these depletions, suggesting that maintenance of cellular thiols was necessary in the mechanism of

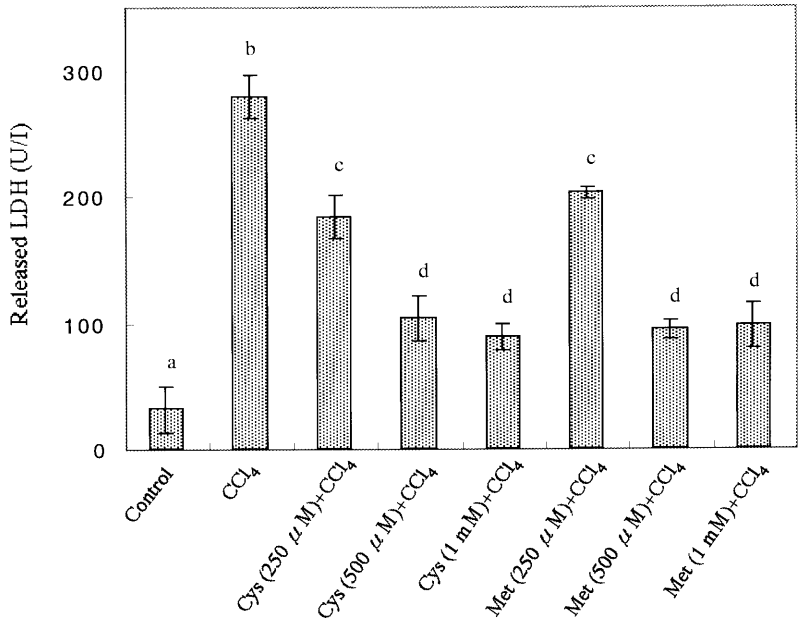

Fig. 1. Dose-dependent effect of L-cysteine and L-methionine on LDH release in CCl_4-treated isolated rat hepatocytes. Hepatocytes were exposed for 2hrs to 3μl/2ml medium CCl_4 (CCl_4 dissolved in DMSO, 3:5) after 1hr pre-incubation with L-cysteine and L-methionine. L-cysteine and L-methionine remained in the medium until harvest. Control, (DMSO 5μl/2ml medium); *Cys*, L-cysteine; *Met*, L-methionine. Results are means ± S.D of three experiments. Data not sharing a common alphabet are significantly different ($p < 0.05$)

Table 1. The effects of L-cysteine and L-methionine on cellular thiols concentrations in CCl_4-treated isolated rat hepatocytes

	Protein-SH (nmol/5 × 10^5 cells)	GSH (nmol/5 × 10^5 cells)
Control	30.0 ± 1.0[a]	14.6 ± 1.2[a]
CCl_4	11.9 ± 1.3[b]	1.0 ± 0.4[b]
L-cysteine + CCl_4	19.0 ± 1.7[c]	6.9 ± 0.7[c]
L-methionine + CCl_4	19.8 ± 2.6[c]	6.2 ± 1.1[c]

Hepatocytes were exposed for 2 hrs to 3 μl/2 ml medium CCl_4 (CCl_4 dissolved in DMSO 3:5) after 1 hr pre-incubation with L-cysteine and L-methionine. L-cysteine and L-methionine remained in the medium until harvest. Control, (DMSO 5 μl/2 ml medium); Cys, L-cysteine (500 μM); Met, L-methionine (500 μM). Results are means ± S.D of three experiments. Data not sharing a common alphabet are significantly different ($p < 0.05$). Test of significance was done using Duncan's test.

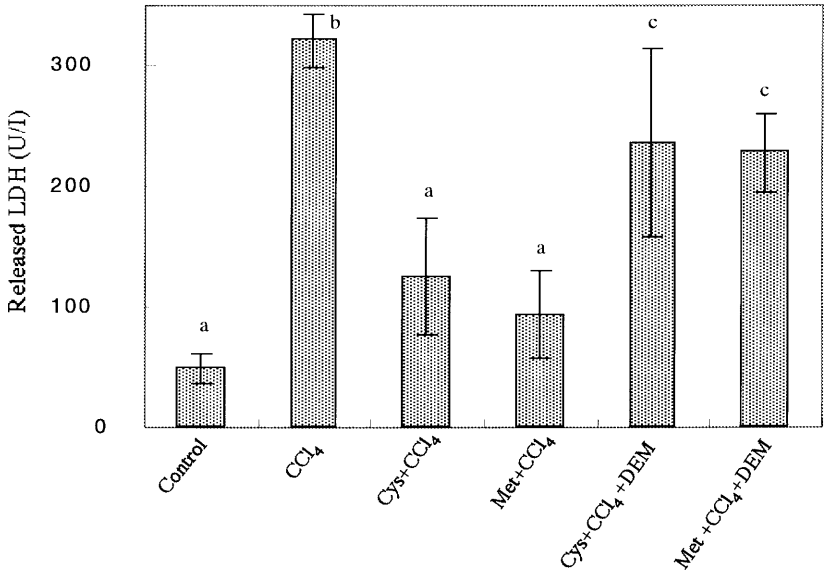

Fig. 2. The effects of L-cysteine, L-methionine and DEM on LDH release in CCl_4-treated isolated rat hepatocytes. Hepatocytes were treated as in legend of Fig. 1. Cys (500 μM); Met (500 μM); DEM (1 mM). Results are means ± S.D of three experiments. Data not sharing a common alphabet are significantly different ($p < 0.05$)

L-cysteine and L-methionine protection against CCl_4-induced hepatocytes injury.

When hepatocytes were treated with MGBG, an irreversible inhibitor of S-adenosylmethionine decarboxylase, which is a key enzyme in spermidine and spermine biosynthesis (Corti et al., 1974), and therefore used to deplete

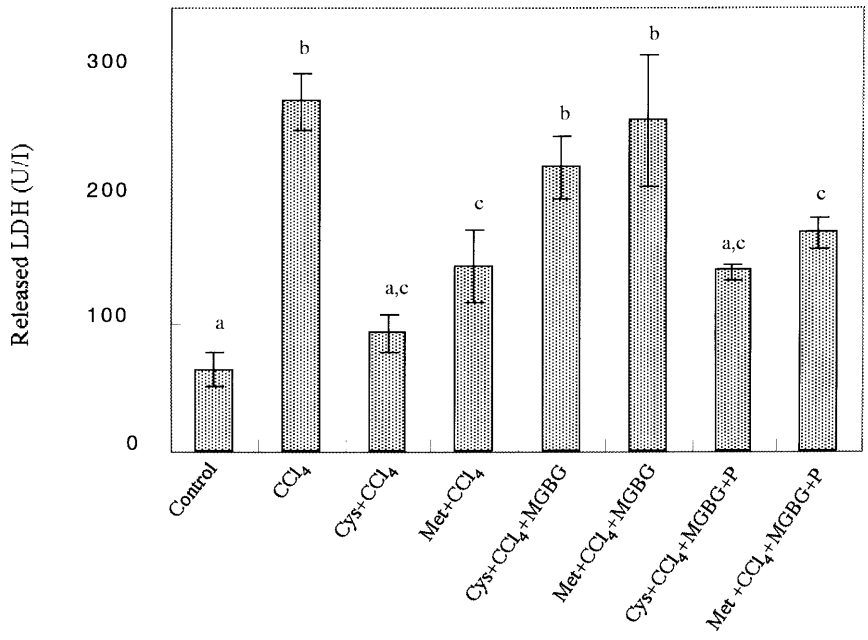

Fig. 3. The effects of L-cysteine, L-methionine, MGBG and polyamines on LDH release in CCl_4-treated isolated rat hepatocytes. Hepatocytes were treated as in legend of Fig. 1, including a 1 hr pre-incubation with polyamines. *MGBG*, methylglyoxal bis (guanylhydrazone) (25μM); *P*, polyamines (putrescine, spermidine, spermine; 100μM each). Results are means ± S.D of three experiments. Data not sharing a common alphabet are significantly different ($p < 0.05$)

Table 2. The effects of L-cysteine and L-methionine on cellular polyamines concentrations in CCl_4-treated isolated rat hepatocytes

	Polyamines (nmol/mg DNA)		
	Putrescine	Spermidine	Spermine
Control	64.0 ± 2.4[a]	276.2 ± 31.2[a]	237.7 ± 13.9[a]
CCl_4	9.8 ± 4.2[b]	75.8 ± 35.4[b]	66.8 ± 22.8[b]
L-cysteine + CCl_4	30.6 ± 0.4[c]	276.5 ± 24.1[a]	220.5 ± 17.9[a,c]
L-methionine + CCl_4	27.0 ± 1.4[c]	242.4 ± 13.9[a]	187.5 ± 16.1[c]

Hepatocytes were treated as in legend of Fig. 1 and Materials and methods.

cellular polyamines, the cytoprotection afforded by L-cysteine and L-methionine was inhibited, but addition of polyamines reversed the effect (Fig. 3). In Table 2, L-cysteine and L-methionine reduced the decrease in cellular polyamine concentrations. Levels of polyamine in cells treated with DMSO only (as vehicle for CCl_4 and as Control) or L-cysteine and L-methionine only, were identical (data not shown). These results show that cellular polyamines play an important role in the protective mechanism of L-cysteine and L-methionine against CCl_4 induced hepatocyte injury.

Discussion

We investigated the protection by L-cysteine and L-methionine of CCl_4-induced hepatocyte injury, and found that CCl_4 decreased cellular polyamines and thiol concentrations, and the treatment with L-cysteine and L-methionine reduced these decreases. Treating with MGBG reduced the protective effect of L-cysteine and L-methionine, but the addition of exogenous polyamines inhibited the influence of MGBG. These results suggest that the cytoprotective effect of L-cysteine and L-methionine in CCl_4-induced toxicity were via maintenance of cellular polyamines, GSH and protein-SH concentrations.

It has been reported that when the capacity of cells and tissues to maintain GSH homeostasis is lost, injury often follows (Deleve and Kaplowitz, 1990), and GSH depletion and loss of protein thiols precede cell death (Orrenius, 1985). Weis et al. (1996) have shown that the incubation of hepatocytes with 3,5-dimethyl-acetaminophen in the presence of glucose/glucose oxidase and horseradish peroxidase caused a concentration-dependent loss of cell viability which was associated with decreased protein thiol levels. Restoration of the protein thiol levels arrested the cell killing. In this study, adding DEM, a cellular thiols depleting agent, inhibited the protection afforded by L-cysteine and L-methionine, and L-cysteine and L-methionine maintained cellular thiols concentrations decreased by exposure to CCl_4. These show that maintenance of cellular thiols was necessary in the protection afforded by L-cysteine and L-methionine.

On the role of polyamines in the maintenance of cellular GSH, Rigobello et al. (1993) reported that spermine was able to completely inhibit the release of GSH from liver mitochondria induced by Ca^{2+} and phosphate, and proposed that polyamines acted to reduce membrane permeability, thereby preventing GSH release. We have previously established that polyamines are involved in the cytoprotective activity of taurine in CCl_4-induced toxicity and that in the absence of polyamines taurine loses its protective effect (Wu et al., 1997).

The protective effect of L-cysteine and L-methionine was lost on treating with MGBG, but was recovered by the addition of exogenous polyamines. This shows that maintenance of cellular polyamines is necessary in the protective effect. L-Cysteine and L-methionine were found to maintain polyamines concentrations decreased by CCl_4. It has been reported that dietary cysteine and methionine stimulate the biosynthesis and increase tissue concentration of polyamine (Acuff and Smith, 1983), and the importance of polyamines in cell function is reflected in the strict regulatory control of their intracellular concentrations (Pegg, 1988; Heby and Persson, 1990).

It is considered that cysteine and methionine increased GSH synthesis and inhibited GSH efflux to maintain cellular GSH and protein-SH concentrations decreased by exposure to CCl_4. On the other hand, increase in polyamine synthesis to possibly maintain cellular GSH and protein-SH concentrations decreased by treatment with CCl_4, also might have resulted in preventing hepatocytes injury.

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