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Thiols and Polyamines in the Cytoprotective Effect of Taurine on Carbon Tetrachloride-Induced Hepatotoxicity

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ABSTRACT: The mechanism by which taurine (2-aminoethanesulfonic acid) protects hepatocytes injury induced by carbon tetrachloride (CCl_4) is not fully understood. In a previous study, we reported that cellular polyamines play an important role in this mechanism. The relationship between cellular glutathione (GSH), protein-SH levels, and lactate dehydrogenase (LDH), with respect to the effect of polyamine on the cytoprotective ability of taurine in CCl_4-induced toxicity in isolated rat hepatocytes, was examined. CCl_4 induced a LDH release and decreased cellular thiols and polyamine levels. Treating with taurine reversed these depletions. The effect of CCl_4 was also reversed by the addition of exogenous polyamines. Pretreating with α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, which is a key enzyme in polyamine biosynthesis and therefore used to deplete cellular polyamine, prevented the protective effect of taurine. Adding diethyl maleate, a cellular glutathione-depleting agent, reduced the effect of exogenous polyamines. The role of polyamine in the cytoprotective effect of taurine in CCl_4-induced toxicity may therefore be by preventing, among others, GSH and protein-SH depletions. © 1998 John Wiley & Sons, Inc. J Biochem Toxicol 13: 71–76, 1999

KEY WORDS: **Taurine, Polyamine, Thiol, Lipid Peroxide, Carbon Tetrachloride, Hepatocytes.**

INTRODUCTION

Taurine (2-aminoethanesulfonic acid), a sulfur amino acid, is present in almost all mammalian tissues. It is not incorporated into proteins and is in fact the most abundant free amino acid in many tissues (1). Taurine is taken in via the diet in carnivores and omnivores and is also synthesized from cysteine or methionine present in the diet (2). The depletion of liver taurine in rats increases their susceptibility to the hepatotoxicity of CCl_4 (3). Taurine also protects against the cytotoxicity of hydrazine and 1,4-naphthoquinone in isolated rat hepatocytes (4).

CCl_4 has been widely studied as a hepatotoxic agent. The mechanism of this hepatotoxicity involves bioactivation of CCl_4 to active free radicals, which initiate lipid peroxidation on the one hand and covalently bind to cellular macromolecules on the other (5,6). Lipid peroxidation has been proposed as a major mechanism for CCl_4 hepatotoxicity (7).

Polyamines (putrescine, spermidine, and spermine) are ubiquitous polycationic metabolites in prokaryotic and eukaryotic cells (8) 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 (9,10). We recently reported that the mechanism of protection provided by taurine against CCl_4 and hydrazine hepatotoxicity involved a prevention of leakage of polyamines, which are involved in maintaining membrane integrity (11).

Thiol groups play a complex role in biological systems, and, in particular, glutathione (GSH) exhibits coenzymatic, regulatory, protective, and reparative roles (12). GSH levels of tissues decrease in response to oxidative stress caused by radiation, chemical compounds, drugs, hyperoxia, and ischemia/reperfusion (12–14). 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 (15).

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Both polyamine and GSH have been found to act as protective agents against oxidative stress (16,17) and also possess radioprotective abilities (18). Both have also been found to be endogenous mediators of acute gastroprotection involving gastric mucosal defense (19). Polyamines were found to be highly effective against gastric lesions induced by various ulcerogens, and they acted as primary mediators of EGF-induced gastroprotection (20). Depletion of glutathione, which is one of the endogenous protective chemicals in the gastric mucosa, resulted in aggravating the chemical- and stress-induced gastric erosions and ulcers (21). The present study was therefore carried out to assess what relationship existed between cellular thiol levels and the effect of polyamine on taurine cytoprotection against the toxicity of CCl_4 in isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

Taurine, CCl_4, collagenase, sodium pyruvate, 5-sulfosalicylic acid dihydrate, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and diethyl maleate (DEM) were obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Putrescine, spermidine, spermine, sodium NADH, and GSH (reduced form) were obtained from Sigma-Aldrich Fine Chemicals (Tokyo, Japan). α-Difluoromethylornithine (DFMO) was kindly supplied by Dr. P. McCann (Marion Merrell Dow Research Institute, Cincinnati, OH).

Methods

Hepatocytes Preparation and Culture

The protocol of the experiment was approved by the Animal Research Committee of Osaka City University, and care of the animals was in accordance with the standards of this institution (Guide for Animal Experimentation, Osaka City University). Hepatocytes were isolated from male Sprague–Dawley rats weighing 200–250 g by collagenase perfusion (22). 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 taurine (initially dissolved in Hank's solution before being used for a final concentration of 15 mM) and the polyamines (100 μM). As GSH was under investigation, we had to limit the influence of exogenous amino acids such as cysteine and methionine. To lower any interference, we replaced Williams' Medium E, which abounds in amino acids, with Hank's buffer. One hour after changing the medium, CCl_4 or diethyl maleate (DEM, 1.0 mM) was added, and the cells were incubated for 2 hours. DEM was added as stock solution in DMSO for a final concentration of 1.0 mM in the incubation buffer. The DEM concentration used was on the basis of literature (23) and pilot experiments. Taurine and the polyamines remained in the incubation buffer until harvest. The suspension buffer was used for assay of lactate dehydrogenase (LDH) activity, and the cells were used for assay of GSH (nonprotein thiols) and protein-SH concentrations. Polyamines (100 μM) and DFMO (3 mM) were added directly to the buffer after having been dissolved in the same. CCl_4 (at final concentration of 3 μL/2 mL medium) was dissolved in dimethyl sulfoxide (DMSO) (3:5 by volume). All "Control" treatments therefore included DMSO at 5 μL/2 mL medium, and this had no interfering effect on the outcome of the experiments.

LDH Assay

Measuring the LDH activity in the suspension buffer as described in Reference 24 monitored the degree of cell injury. Assay conditions included 0.6 mM sodium pyruvate, 0.18 mM NADH, and 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.

Determination of Polyamine

The measurement of polyamine was described in Reference 25. 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 minutes and then with a programmed solvent gradient using a linear gradient curve. The gradient

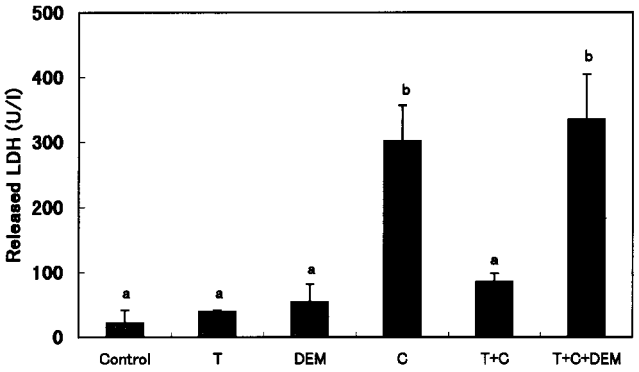

FIGURE 1. The effects of taurine and dimethyl maleate (DEM) on lactate dehydrogenase (LDH) release in CCl_4-treated isolated rat hepatocytes. Hepatocytes were exposed for 2 hours to 3 μL/2 mL medium CCl_4 (CCl_4 dissolved in DMSO 3:5) after 1 hour preincubation with taurine (15 mM). Taurine remained in the medium until harvest. Control (DMSO 5 μL/2 mL medium); C, CCl_4; T, taurine; D, diethyl maleate (DEM) (1 mM). 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.

changed from 20% to 55% of solvent B from 3.1 to 25 minutes 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. The DNA content of the perchloric acid precipitable materials was determined by the methods of Schneider (26) using calf thymus DNA as the standard.

Cellular GSH and Protein-SH Assay

Cellular GSH (nonprotein thiols) and protein-SH concentrations were measured as described in Reference 27. Cells (5×10^5) were collected, and an extract was obtained by treating with 0.5 mL 2% 5-sulfosalicylic acid and centrifuged at 3000 rpm for 10 minutes. The supernatant obtained was used for the assay of intracellular GSH (nonprotein thiols) 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 minutes, 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.

Statistical Analysis

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

RESULTS

CCl_4 (3 μL/2 mL medium) induced a release of LDH, but addition of taurine prevented this (Figure 1). However, adding diethyl maleate (DEM) (1 mM), a cellular thiols depleting agent, inhibited the protection afforded by taurine. Table 1 showed that CCl_4 also decreased cellular protein-SH, GSH (nonprotein thiols), and polyamines. Addition of taurine (15 mM) prevented these depletions.

To examine the relationship between polyamine and cellular thiols, we added polyamine to CCl_4-treated hepatocytes and measured hepatocytes cellular thiol concentrations. As shown in Table 2, addition of exogenous polyamines, putrescine, spermidine, and spermine prevented the cellular thiol reduction caused by CCl_4. Addition of these exogenous polyamines also prevented hepatocytes LDH release induced by CCl_4 (Figure 2). The effect of the exogenous polyamines was, however, inhibited by treatment with DEM. When hepatocytes were treated with DFMO, an irreversible inhibitor of ornithine decarboxylase, which is a key enzyme in polyamine biosynthesis and therefore used to deplete cellular polyamine, the cytoprotection afforded by taurine was inhibited, but addition of polyamine reversed the effect. Pretreating with DFMO inhibited the ability of taurine to prevent the cellular thiol reduction caused by CCl_4 (Table 3). Addition of exogenous polyamines, however, recovered this ability.

DISCUSSION

The relationship existing between cellular GSH, protein-SH levels, and the effect of polyamine on the cytoprotective ability of taurine in CCl_4-induced toxicity in isolated rat hepatocytes was examined. Our results showed that CCl_4 decreased cellular thiol and polyamine concentrations, and the treatment with taurine prevented these reductions. This protection of taurine against hepatocyte injury was prevented by DEM and DFMO, agents inhibiting cellular thiol and polyamine, respectively. Addition of exogenous polyamines reversed the increase in LDH release and inhibition by DFMO as well as restoring the protective role of taurine. These findings suggest that the protection of taurine against cytotoxicity of CCl_4 may be due to maintenance of cellular thiol and involve cellular polyamine.

TABLE 1. The Effect of Taurine on Cellular Protein-SH, GSH, and Polyamine Contents in CCl_4-Treated Isolated Rat Hepatocytes

Treatment	*Protein-SH* (*nmoles/5 × 10⁵ cells*)	*GSH*	*Polyamines* *Putrescine*	*Spermidine (nmoles/mg DNA)*	*Spermine*
Control	61.0 ± 2.0[a]	21.7 ± 1.5[a]	81.2 ± 13.4[a]	220.8 ± 35.7[a]	204.1 ± 18.8[a]
CCl_4	26.1 ± 0.8[b]	3.3 ± 0.1[b]	14.9 ± 10.1[b]	39.1 ± 10.5[b]	12.8 ± 13.6[b]
Tuarine + CCl_4	52.6 ± 1.5[c]	17.0 ± 0.5[c]	92.6 ± 21.0[a]	255.7 ± 40.0[a]	205.8 ± 56.2[a]
Taurine	65.3 ± 7.3[a]	20.5 ± 0.2[a]	76.6 ± 13.3[a]	246.6 ± 33.4[a]	187.4 ± 20.4[a]

Hepatocytes were exposed 2 hours to carbon tetrachloride (CCl_4) (3 μL/2 mL medium) after 1 hour preincubation with taurine (15 mM). Taurine remained in the medium till harvest. Control: DMSO (5 μL/2 mL medium). Results are the means ±S.D. of three experiments. Values in the same column not sharing a common superscript letter are significantly different ($p < 0.05$). Test of significance was done using Duncan's test.

TABLE 2. The Effect of Polyamine on Cellular Protein-SH and GSH in CCl_4-Treated Isolated Rat Hepatocytes

Treatment	*Protein-SH* (*nmoles/5 × 10⁵ cells*)	*GSH*
Control	61.0 ± 2.1[a]	21.7 ± 1.5[a]
CCl_4	26.1 ± 0.8[b]	3.3 ± 0.1[b]
PUT + CCl_4	46.6 ± 5.6[c]	11.3 ± 1.9[c]
SPD + CCl_4	59.0 ± 0.8[a]	12.2 ± 2.8[c]
SPM + CCl_4	41.5 ± 1.2[d]	10.1 ± 0.1[c]
PUT	57.5 ± 1.0[a]	19.3 ± 5.2[a]
SPD	58.8 ± 2.8[a]	17.6 ± 3.4[a]
SPM	57.4 ± 3.9[a]	17.7 ± 2.6[a]

Hepatocytes were exposed 2 hours to carbon tetrachloride (CCl_4) (3 μL/2 mL medium) after 1 hour preincubation with polyamines (100 μM each): PUT, putrescine; SPD, spermidine; SPM, spermine. Polyamines remained in the medium until harvest. Control: DMSO (5 μL/2 mL medium). Results are the means ±S.D. of three experiments. Values in the same column not sharing a common superscript letter are significant different ($p < 0.05$). Test of significance was done using Duncan's test.

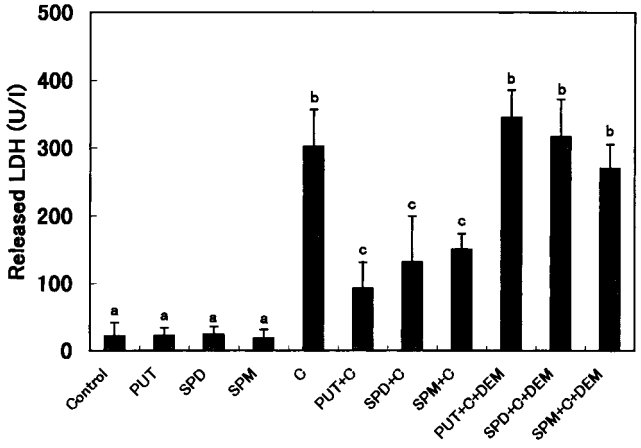

FIGURE 2. The effects of polyamines and dimethyl maleate (DEM) on lactate dehydrogenase (LDH) release in CCl_4-treated isolated rat hepatocytes. Hepatocytes were exposed for 2 hours to 3 μL/2 mL medium CCl_4 (CCl_4 dissolved in DMSO 3:5) after 1 hour preincubation with polyamines (100 μM each). Polyamines remained in the medium until harvest. Control (DMSO 5 μL/2 mL medium); C, CCl_4; PUT, putrescine; SPD, spermidine; SPM, spermine; D, diethyl maleate (DEM). 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.

TABLE 3. The Effect of Taurine, DFMO, and Polyamine on Cellular Protein-SH and GSH Contents, and LDH Release in CCl_4-Treated Isolated Rat Hepatocytes

Treatment	*Protein-SH* (*nmoles/5 × 10⁵ cells*)	*GSH*	*LDH* (*U/L*)
Control	57.6 ± 1.0[a]	16.8 ± 0.4[a]	15.1 ± 17.4[a]
C	28.7 ± 1.9[b]	3.8 ± 1.8[b]	464.1 ± 51.6[b]
T + C	59.7 ± 6.6[a]	15.0 ± 5.4[a]	138.2 ± 11.5[c]
T + C + D	31.7 ± 3.8[b]	4.8 ± 1.1[b]	351.5 ± 12.7[d]
T + C + D + PUT	51.0 ± 5.1[c]	14.9 ± 4.0[a]	123.7 ± 1.9[c]
T + C + D + SPD	48.1 ± 2.2[c]	11.4 ± 2.5[a,c]	181.8 ± 38.2[e]
T + C + D + SPM	49.1 ± 0.7[c]	9.1 ± 2.6[c]	218.9 ± 4.1[e]
DFMO	56.8 ± 1.7[a]	17.5 ± 2.5[a]	11.7 ± 10.3[a]

Hepatocytes were exposed 2 hours to CCl_4 (3 μL/2 mL medium) (CCl_4 dissolved in DMSO 3:5) after 16 hours treatment with DFMO (3 mM) and 1 hour preincubation with taurine (15 mM) and polyamines (100 μM each). Taurine and the polyamines remained in the incubation buffer until harvest: C, CCl_4; D, DFMO; α-difluoromethylornithine; T, taurine; PUT, putrescine; SPD, spermidine; SPM, spermine. Control: DMSO (5 μL/2 mL medium). Results are the means ±S.D. of three experiments. Values in the same column not sharing a common superscript letter are significantly different ($p < 0.05$). Test of significance was done using Duncan's test.

As the major nonprotein thiol in mammalian cells, GSH is involved in many cellular functions. It has been reported that when the capacity of cells and tissues to maintain GSH homeostasis is lost, injury often follows (28), and GSH depletion and loss of protein thiol precede cell death (15). Weis *et al.* (29) 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 that was associated with decreased protein thiol levels. Restoration of the protein thiol levels arrested the cell killing. Taurine, given to monocrotaline (MONO, a hepatotoxic pyrrolizidine alkaloid)-injected animals, normalized many of the biochemical changes caused by the alkaloid. It showed a sparing effect on serum methionine, and corrected the depression in hepatic GSH by stimulating γ-Glu-Cys synthetase and antagonized the MONO-induced changes in GSH transferase (30). In this study, taurine also prevented GSH and protein-SH depletions

and reversed the membrane damage assessed by LDH leakage. Treating with DFMO significantly decreased this effect of taurine. This was, however, recovered after the addition of exogenous polyamines.

Polyamines bind to components of membranes, such as acidic phospholipids or negatively charged residues of membrane-bound proteins, and thus affect some properties of biological membranes (10). On the role of polyamine in the maintenance of cellular GSH, Rigobello *et al.* (16) have 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. Our results show that polyamines prevented the decrease in cellular GSH and protein-SH, as well as a reduction in LDH leakage. Methionine, which is directly involved in the synthesis of polyamine (8), is also involved in the synthesis of GSH via cysteine. It was therefore possible that the inclusion of polyamine in the culture medium enabled more methionine to rather cycle to cysteine and consequently to GSH.

We previously established the involvement of polyamine in the cytoprotective ability of taurine in CCl_4-induced toxicity and that in the absence of polyamine taurine lost its protective effect. From the current study, this role of polyamine in the cytoprotective effect of taurine in CCl_4-induced hepatotoxicity may be by preventing, among other effects, GSH and protein-SH depletions.

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