Protective role of fructose 1,6-bisphosphate during CCl₄ hepatotoxicity in rats

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Rats were injected intraperitoneally with CCl₄ (2.5 ml/kg body wt.) and the hepatotoxicity was compared with that of rats receiving the same dose of CCl₄ and an intraperitoneal injection of fructose 1,6-bisphosphate (2 g/kg body wt.). A 50–70 % decrease in plasma aspartate aminotransferase and alanine aminotransferase activities was observed in the latter treatment, indicating a protective role of the sugar bisphosphate in CCl₄ hepatotoxicity. The protection was accompanied by elevated hepatic activities of ornithine decarboxylase at 2, 6 and 24 h, S-adenosylmethionine decarboxylase at 6 h, and spermidine N¹-acetyltransferase at 2 h. The increase in the enzymes involved in polyamine metabolism was shown in our previous work [Rao, Young & Mehendale (1989) *J. Biochem. Toxicol.* 4, 55–63] to correlate with increased polyamine synthesis or interconversion, which was related to the extent of hepatocellular regeneration. The hepatic contents of fructose 1,6-bisphosphate and ATP significantly decreased after CCl₄ treatment, and administration of the sugar bisphosphate increased hepatic ATP. Fructose 1,6-bisphosphate, an intermediary metabolite of the glycolytic pathway, may decrease CCl₄ toxicity by increasing the ATP in the hepatocytes. The ATP generated is useful for hepatocellular regeneration and tissue repair, events which enable the liver to overcome CCl₄ injury.

INTRODUCTION

The polyhalogenated compound CCl₄ is a well-known hepatotoxin (Slater, 1984), and exposure to this chemical is known to result in hepatocellular necrosis in rodents. Depending on the dose of exposure to CCl₄ and prior exposure to other chemicals, extensive liver damage results in total hepatic failure and animal lethality (Curtis *et al.*, 1979; Klingensmith & Mehendale, 1982; Mehendale, 1984). Several investigators have shown the protective role of amino acids (De Toranzo *et al.*, 1983), cystamine (Castro *et al.*, 1973; Macdonald *et al.*, 1986) and various chemicals (DeFerreira *et al.*, 1977; Lindstrom & Anders, 1978) but none of these agents can ameliorate CCl₄ toxicity by interrupting mechanisms thought to be responsible for the progression of liver injury.

Fructose 1,6-bisphosphate has been reported to have a protective effect in ischaemic renal injury in rats (Didlake *et al.*, 1985). Even in humans a protective role has been suggested for fructose 1,6-bisphosphate during acute myocardial infarction (Marchionni *et al.*, 1985). However, no work has been reported on the protective effect, if any, of fructose 1,6-bisphosphate during CCl₄-induced hepatotoxicity. The objective of the present work was to examine if external supplementation with an energy source affords protection against CCl₄ hepatotoxicity. We evaluated the role of fructose 1,6-bisphosphate as a possible protectant against CCl₄ hepatotoxicity. Rats were exposed to a toxic dose of CCl₄ and injected with 2 g of fructose 1,6-bisphosphate/kg body wt., and compared with those receiving CCl₄ alone. The protective action was monitored by measuring serum transaminases. Two other biochemical events important in cellular regeneration and tissue repair were also studied. The

enzymes involved in polyamine metabolism, which are important for hepatocellular regeneration and recovery (Holttä *et al.*, 1973; Oratz *et al.*, 1980; Rao *et al.*, 1989), were determined. Hepatic ATP contents were also monitored. We report here a 50–70 % protection from CCl₄ hepatotoxicity by fructose 1,6-bisphosphate. We suggest that the protective role of fructose 1,6-bisphosphate is due to the ready availability of this sugar bisphosphate, thus conserving energy required for its synthesis and also short-circuiting the glycolytic pathway to generate the much-needed ATP for cell division in the liver exposed to CCl₄.

EXPERIMENTAL

Materials

Chemicals and enzymes. DL-[1-¹⁴C]Ornithine (49.1 mCi/mmol), S-adenosyl-L-[carboxy-¹⁴C]methionine (59.5 mCi/mmol) and [acetyl-1-¹⁴C]acetyl-CoA (57.2 mCi/mmol), 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from New England Nuclear (Boston, MA, U.S.A.). Pyridoxal phosphate, dithiothreitol, L-ornithine, Hyamine hydroxide, hydroxylamine hydrochloride, S-adenosyl-L-methionine (*p*-toluenesulphonate salt), kits for aminotransferase assay, putrescine dihydrochloride, spermidine trihydrochloride, D-fructose 1,6-bisphosphate (sodium salt), ATP (disodium salt), D-fructose 1,6-bisphosphatase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, hexokinase and NADP⁺ were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). CCl₄ was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals used were of reagent grade, and scintillation cocktail was prepared from scintillation-grade toluene.

Animals. Male Sprague-Dawley rats (125–150 g) obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) were maintained for 10 days on a 12 h photoperiod in the Central Animal Facility at $25 \pm 1^\circ\text{C}$ and 50–80% relative humidity. They received powdered rat chow (Purina Co., St. Louis, MO, U.S.A.) and water *ad libitum* and were housed over corn-cob bedding.

Treatment. Rats were administered intraperitoneally with CCl_4 dissolved in corn-oil vehicle at a dose of 2.5 ml/kg body wt. (Klingensmith & Mehendale, 1982). Another group of rats received the same dose of CCl_4 , but was also injected intraperitoneally with 2 g of fructose 1,6-bisphosphate/kg body wt. in 0.9% NaCl. Some rats were only injected with 2 g of fructose 1,6-bisphosphate/kg body wt. as sugar bisphosphate controls, whereas others received only the respective amount of corn oil (vehicle for CCl_4) and 0.9% NaCl (vehicle for sugar bisphosphate) as vehicle controls.

Methods

Post-treatment analysis. Animals were killed under diethyl ether anaesthesia at 2, 6 and 24 h after treatment. Blood was drawn from the dorsal aorta in a heparinized syringe, and plasma was separated for measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by the method of Reitman & Frankel (1957). Livers of these rats were perfused with 0.9% NaCl, removed, blotted, and weighed after separation of fat and connective tissue. Weighed portions of the minced liver were quickly placed in three separate ice-cold tubes. All work was performed with tissue samples in ice, and completed within 24 h after death of the animals.

Polyamine-related enzyme assay. Part of the liver was homogenized in 50 mM-phosphate buffer (1:5, w/v), pH 7.2, and the supernatant obtained as described by Russell & Snyder (1969) was used for ornithine decarboxylase assay by the method described by Ono *et al.* (1972). One unit of specific activity was defined as 1 nmol of $^{14}\text{CO}_2$ formed/30 min per mg of protein.

S-Adenosylmethionine decarboxylase was measured by the method described by O'Brien *et al.* (1975), on the same supernatant used to measure ornithine decarboxylase. Enzyme activity was determined by measuring the release of $^{14}\text{CO}_2$ from *S*-adenosyl-L-[carboxy- ^{14}C]methionine in the presence of added putrescine as an acceptor of the propylamino moiety derived from decarboxylated *S*-adenosylmethionine (Heby & Russell, 1973). One unit of specific activity was defined as 1 nmol of $^{14}\text{CO}_2$ formed/60 min per mg of protein.

Spermidine *N*¹-acetyltransferase was measured by the method of Libby (1978) as modified and described in detail, including the method of preparation of enzyme, by Shinki *et al.* (1985, 1986). One unit of specific activity was defined as 1 nmol of *N*¹-acetylspermidine formed/5 min per mg of protein.

Assays of fructose 1,6-bisphosphate and ATP. A portion of the chilled liver was homogenized in 4 ml of 0.75 M-HClO₄, and the supernatant obtained on centrifugation was neutralized to pH 7 with 1 M-NaOH by using a micro-electrode pH-meter. This supernatant was used to measure fructose 1,6-bisphosphate by the method modified from that described by Geller & Byrne (1975). The assay mixture contained 1 ml of 25 mM-Tris/HCl buffer (pH 7.5) containing 2.5 mM-MgSO₄, 75 mM-KCl and 50 μM -EDTA, 0.65 ml of neutralized supernatant,

0.1 ml of 15 mM-NADP⁺ and 0.25 ml of each of the following enzymes: fructose-1,6-bisphosphatase (1 unit), phosphoglucosomerase (2 units) and glucose-6-phosphate dehydrogenase (1 unit). The mixture was incubated at 37°C for 15 min and the A_{340} measured, with appropriate blanks to avoid any error caused by endogenous NADPH. The standard plot with fructose 1,6-bisphosphate was linear up to a concentration of 400 nmol for the respective amounts of enzymes and NADP⁺ used in the assay system.

ATP was measured by the method of Lamprecht & Trautschold (1974) in the same neutralized supernatant used to measure fructose 1,6-bisphosphate. The same procedures were used to measure plasma sugar bisphosphate and ATP after deproteinization with 0.75 M-HClO₄ and adjustment of the supernatant to pH 7 with 1 M-NaOH.

Proteins were measured by the method of Lowry *et al.* (1951). The data were analysed statistically (Gad & Weil, 1986) and the statistical criterion for a significant difference was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

The plasma transaminases (ALT, AST) are known to be increased significantly in rats after exposure to a toxic dose of CCl_4 in a dose-dependent manner, and this is related to the extent of liver damage seen in these animals (Agarwal & Mehendale, 1983; Klingensmith & Mehendale, 1982). By using the same index of liver toxicity, it was observed that there were 50–70% decreases in ALT and AST at 6 and 24 h after exposure of rats to a combination of CCl_4 and fructose 1,6-bisphosphate compared with CCl_4 treatment alone (Table 1).

Increased hepatic contents of polyamines with time after exposure to CCl_4 have been reported by many investigators (Holtta *et al.*, 1973; Matsui & Pegg, 1980; Poso & Pegg, 1982; Rao *et al.*, 1989). Further, the increase in polyamines is known to occur in regenerating rat liver (Holtta *et al.*, 1973; Russell *et al.*, 1970). Recovery of rats from CCl_4 toxicity depends on the extent to which the rat liver can regenerate (Lockard *et al.*, 1983a,b; Mehendale, 1984; Bell *et al.*, 1988). The immediate response of the liver exposed to CCl_4 is to increase putrescine (Matsui & Pegg, 1980; Rao *et al.*, 1989). This is accomplished by two independent mechanisms: direct synthesis of putrescine, and enhancement of the pathways leading to interconversion of higher polyamines into putrescine. Accordingly, ornithine decarboxylase, the key enzyme for putrescine synthesis (Seiler *et al.*, 1985), was significantly increased in rat liver at 6 and 24 h (Fig. 1) after CCl_4 + fructose 1,6-bisphosphate treatment compared with CCl_4 alone. *S*-Adenosylmethionine decarboxylase, responsible for the conversion of putrescine into spermidine and spermine, was also increased at 6 h by 50% in the liver of rats treated with CCl_4 + fructose 1,6-bisphosphate compared with CCl_4 alone (Fig. 1). From 6 to 24 h the value remained stable after the former treatment, but increased significantly after the latter (Fig. 1). Increased spermidine *N*¹-acetyltransferase activity (Matsui *et al.*, 1981), responsible for the conversion of spermine into spermidine into putrescine, provides an immediate additional mechanism for maintaining the putrescine content (Seiler *et al.*, 1985). Spermidine *N*¹-acetyltransferase activity is

Table 1. Plasma transaminase levels at various time points after treatment

One Sigma–Frankel unit of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) will form 0.482 nmol of glutamate/min at pH 7.5 and 25 °C. Data for rats receiving only corn oil or saline as respective controls have not been indicated, since the values in these cases at all time points were within the normal range. Each point represents mean $\pm$ S.E.M. of four separate experiments. Asterisk indicates statistically significant difference in values between rats injected with CCl₄ and fructose 1,6-bisphosphate compared with those receiving CCl₄ alone ($P \leq 0.05$).

Time ... Treatment	Activity (units/ml)							
	0 h		2 h		6 h		24 h	
	ALT	AST	ALT	AST	ALT	AST	ALT	AST
Fructose 1,6-bisphosphate (2 g/kg body wt.)	42 $\pm$ 3	99 $\pm$ 4	40 $\pm$ 3	95 $\pm$ 5	39 $\pm$ 2	99 $\pm$ 4	34 $\pm$ 2	94 $\pm$ 3
CCl ₄ (2.5 ml/kg body wt.)	47 $\pm$ 4	101 $\pm$ 4	120 $\pm$ 25	380 $\pm$ 110	1065 $\pm$ 300	3000 $\pm$ 645	2050 $\pm$ 240	3700 $\pm$ 325
CCl ₄ + fructose 1,6-bisphosphate	41 $\pm$ 5	95 $\pm$ 3	55 $\pm$ 15*	130 $\pm$ 35*	400 $\pm$ 56*	750 $\pm$ 155*	1055 $\pm$ 125*	1600 $\pm$ 300*

Fig. 1. Rat liver ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMD) activities at various time points after treatment

The treatment protocol and other details are explained under 'Methods'. The symbols represent the following treatment groups: ●, fructose 1,6-bisphosphate alone; ▲, CCl₄ alone; ■, CCl₄ + fructose 1,6-bisphosphate combination. The final reaction mixture incubated at 37 °C for 30 min for ornithine decarboxylase assay contained 25 μ mol of phosphate buffer, pH 7, 0.1 μ mol of pyridoxal phosphate, 5 μ mol of dithiothreitol, 2.086 μ mol of L-ornithine containing 1 μ Ci of radiolabelled compound, and 3–4 mg of protein in 1 ml final volume. Reaction was stopped by 0.3 ml of 4 M-citric acid, and ¹⁴CO₂ trapped in

enhanced by 100 % at the 2 h time point in rats injected with CCl₄ + fructose 1,6-bisphosphate compared with those receiving CCl₄ alone (Table 2). At the end of 24 h, activity of this enzyme decreased to its 0 h control value in the combination treatment compared with CCl₄ alone (Table 2). All of these changes indicate an early response of liver of rats treated with CCl₄ and fructose 1,6-bisphosphate to augment the contents of polyamines, an event critical for cellular proliferation (Walker *et al.*, 1978; Inoue *et al.*, 1981; Luk, 1986), leading to liver regeneration, hepatolobular restoration and recovery from toxicity.

Polyamines have been suggested to decrease lipid peroxide formation in rodent liver (Kitada *et al.*, 1979). Lipid peroxidation leading to cell membrane damage is known to occur during CCl₄-induced hepatotoxicity (Koster *et al.*, 1977; Wolf *et al.*, 1980; Curtis *et al.*, 1981). Increased content of polyamines at earlier time points after CCl₄ exposure in rat liver also exposed to fructose 1,6-bisphosphate could also be critical in the protection from the haloalkane-induced lipid peroxidation and membrane damage associated with CCl₄ hepatotoxicity.

It is clear that administration of fructose 1,6-bisphosphate resulted in the generation of higher contents of ATP in the liver tissue (Fig. 2). With time, hepatic content of fructose 1,6-bisphosphate and ATP contents decreased after exposure to CCl₄ (Fig. 2). This decrease was greater in rats treated with CCl₄ alone compared

0.2 ml of 1 M-Hyaminate hydroxide was measured in a liquid-scintillation spectrometer by using a blank with inactivated enzyme. S-Adenosylmethionine decarboxylase assay mixture was incubated at 37 °C for 60 min and contained 10 μ mol of phosphate buffer, pH 7.2, 0.01 μ mol of pyridoxal phosphate, 0.5 μ mol of putrescine, 0.02 μ mol of S-adenosylmethionine containing 0.2 μ Ci of radiolabelled compound, and 3–4 mg of protein in 1 ml final volume. All other conditions were the same as those described for ornithine decarboxylase. Values are means $\pm$ S.E.M. for four separate experiments. Asterisk indicates statistically significant difference in values for rats injected with CCl₄ + fructose 1,6-bisphosphate compared with CCl₄ treatment alone ($P \leq 0.05$).

Table 2. Rat liver spermidine *N*¹-acetyltransferase activity at various time points after treatment

The treatment protocol is described under 'Materials'. The final assay mixture incubated at 25 °C for 5 min contained 10 μ mol of Tris/HCl buffer, pH 7.8, 0.3 μ mol of spermidine, 0.8 nmol of [*acetyl*-1-¹⁴C]acetyl-CoA (0.05 μ Ci) and 0.3–0.4 mg of protein in a final volume of 0.1 ml. The reaction was terminated with 20 μ l of 1 M-hydroxylamine hydrochloride, tubes were placed in a boiling-water bath for 3 min, cooled, and 40 μ l of the reaction mixture was placed on a Whatman P-81 phosphocellulose filter disc, and washed with water and ethanol. The radioactivity on the discs was determined by liquid-scintillation spectrometry. Values are means ($\pm$ S.E.M., where included) of four separate experiments. Asterisk indicates a statistically significant difference in values for rats injected with CCl₄ + fructose 1,6-bisphosphate compared with those receiving CCl₄ treatment alone ($P \leq 0.05$).

Treatment	Time...	Activity (nmol of <i>N</i> ¹ -acetylspermidine/ 5 min per mg of protein)			
		0 h	2 h	6 h	24 h
Fructose 1,6-bisphosphate (2 g/kg body wt.)	0.02	0.02		0.01	0.02
CCl ₄ (2.5 ml/kg body wt.)	0.02		0.14 $\pm$ 0.03	0.50 $\pm$ 0.06	0.06
CCl ₄ + fructose bisphosphate	0.02		0.31 $\pm$ 0.05*	0.58 $\pm$ 0.1	0.03*

with those treated with the combination of CCl₄ and sugar bisphosphate (Fig. 2). Plasma fructose 1,6-bisphosphate and ATP contents in all animals were low. Further, no significant changes were observed in the plasma contents of these compounds between rats receiving CCl₄ alone or in combination with fructose 1,6-bisphosphate at 2, 6 and 24 h, and hence these results have not been discussed separately.

It has been reported by Nyfeler *et al.* (1984) that the halogenated alkane halothane decreases the hepatic content of fructose 2,6-bisphosphate. This sugar bisphosphate is known to be an important modulator of the glycolytic pathway, and a decrease in its concentration results in a decrease in 6-phosphofructo-1-kinase activity and a simultaneous increase in fructose-1,6-bisphosphatase activity (Pilkis *et al.*, 1981; Claus *et al.*, 1984). This would result in diminished conversion of fructose 6-phosphate into fructose 1,6-bisphosphate, the intermediary step in glycolysis resulting in diminished generation of ATP. CCl₄ poisoning is known to cause a significant depletion of glycogen in rat liver (Lockard *et al.*, 1983b; Bell & Mehendale, 1987), which would indicate increased glucose 1-phosphate or glucose formation targeted for the glycolytic pathway for ATP synthesis. However, any inhibition of glycolysis will result in energy deficiency in the hepatocytes, owing to decreased ATP synthesis. ATP synthesis is an important and critical requirement of all regenerating cells. It is likely that the exogenous supply of fructose 1,6-bisphosphate to rats injected with CCl₄ short-circuits the glycolytic pathway, thus avoiding the steps involved in the conversion of glucose into fructose 1,6-bisphosphate

Fig. 2. Rat liver ATP and fructose 1,6-bisphosphate (FBP) contents at various time points after treatment

The treatment protocol and other details are explained under 'Methods'. The symbols represent the following treatment groups: ●, fructose 1,6-bisphosphate only; ▲, CCl₄ alone; ■, CCl₄ + fructose 1,6-bisphosphate. The procedure for determination of fructose 1,6-bisphosphate is explained under 'Methods'. ATP was measured in the same supernatant used to measure fructose 1,6-bisphosphate. The assay mixture contained 1 ml of Tris/HCl buffer, pH 7.5, containing 50 mM-KCl, 0.2 mM-EDTA and 6.7 mM-MgCl₂, 1 ml of neutralized sample, 0.1 ml of 1 M-glucose, 0.1 ml of hexokinase (5 units), 0.1 ml of glucose-6-phosphate dehydrogenase (1.9 units) and 0.1 ml of 0.1 M-NADP⁺. After incubation at 37 °C for 15 min the A₃₄₀ readings were taken against a blank. The assay was linear up to 500 nmol of ATP. Values are means $\pm$ S.E.M. for four separate experiments. Asterisk indicates statistically significant difference in values for rats injected with CCl₄ + fructose 1,6-bisphosphate compared with those receiving CCl₄ alone ($P \leq 0.05$).

to augment the generation of ATP. It has been suggested (Galzigna *et al.*, 1976; Markov *et al.*, 1985) that exogenous fructose 1,6-bisphosphate can cross the cellular membrane barrier and undergo further metabolism in the cell. Exogenous supply of an intermediary metabolite of the glycolytic pathway, which is otherwise synthesized from glucose at the expense of 2 mol of ATP, also favours a greater cellular energy conservation. The generation of ATP would greatly help to supply the much-needed energy for the liver cell to increase protein synthesis, a consequence of which will be increased polyamines and subsequently enhanced hepatocellular regeneration. Although several aspects of these mechanisms need further experimental validation, the present

findings, indicating a protective role of fructose 1,6-bisphosphate against CCl₄-induced hepatotoxicity, are supportive of the hypothesis detailed above. Further investigations are warranted to determine the optimal treatment with fructose 1,6-bisphosphate and to unravel the mechanisms underlying the protective role of this bisphosphate sugar.

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