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CHLOROACETALDEHYDE-INDUCED HEPATOCYTE CYTOTOXICITY

MECHANISMS FOR CYTOPROTECTION

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Abstract—2-Chloroacetaldehyde (CAA)-induced cytotoxicity in isolated hepatocytes was enhanced markedly if hepatocyte alcohol or aldehyde dehydrogenase was inhibited prior to CAA addition. Hepatocyte GSH depletion, ATP depletion and lipid peroxidation by CAA were also enhanced markedly. Furthermore, CAA was about 10- and 70-fold more cytotoxic than its oxidative or reductive metabolite chloroacetate or chloroethanol, respectively. Nutrients such as lactate, xylitol, sorbitol or glycerol, which increase cytosolic NADH levels, prevented CAA cytotoxicity in normal hepatocytes but further enhanced cytotoxicity toward alcohol dehydrogenase inactivated hepatocytes, suggesting that increased cytosolic NADH reduces CAA via alcohol dehydrogenase in normal hepatocytes but prevents CAA oxidation in alcohol dehydrogenase inactivated hepatocytes. However, increasing cytosolic NADH levels with ethanol or NADH-generating nutrients after CAA had been metabolized also prevented cytotoxicity and caused a partial ATP recovery, whereas oxidation of cytosolic NADH with pyruvate markedly increased cytotoxicity. This indicates that cytotoxic CAA concentrations cause oxidative stress and that ATP levels can be restored if cellular redox homeostasis is normalized with reductants. Furthermore, except for fructose, nutrients that did not increase NADH did not affect CAA-induced cytotoxicity. Fructose also caused a partial ATP recovery, and its protection was prevented by the glycolytic inhibitor fluoride. Hepatocytes isolated from fasted animals were 4- to 6-fold more susceptible to CAA-induced ATP depletion and cytotoxicity. No lipid peroxidation occurred at these lower CAA concentrations. Furthermore, all nutrients, including alanine, glutamine and glucose, prevented cytotoxicity toward hepatocytes isolated from fasted animals. The susceptibility of hepatocytes to CAA cytotoxicity, therefore, depends on both cellular redox homeostasis and cellular energy supply.

Key words: nutrient; antidote; aldehyde toxicity; cytoprotection

CAA† is a known alkylating agent and potent mutagen [1]. CAA forms etheno compounds with DNA adenosine and cytosine bases [2], induces interstrand DNA cross-links [3], and inhibits DNA synthesis in animals cells [4]. CAA is a major reactive metabolite of a large number of industrial chemicals including vinyl chloride [5], ethylene dichloride [6], and 2-chloroethanol. CAA is also found as a urinary metabolite in rats fed the alkylating anticancer drug cyclophosphamide [7]. Clinical studies show that the alkylating anticancer drug ifosphamide, an analogue of cyclophosphamide, undergoes N-dealkylation in humans to liberate CAA [8], which may be responsible for the neurotoxic side-effects of ifosphamide chemotherapy [9]. 2-Chloroethanol is a metabolite of the anticancer drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) [10].

Recently, we reported that the molecular mechanism of CAA-induced toxicity involves protein thiol depletion, mitochondrial toxicity resulting in ATP depletion, and lipid peroxidation [11]. The cell permeable reductant dithiothreitol was able to prevent cytotoxicity if added some time after the initial addition of CAA. Antioxidants and iron chelators, on the other hand, only delay the onset of cytotoxicity [11]. The current study, an extension of Ref. 11, demonstrates that CAA cytotoxicity was prevented by either ethanol or those nutrients that increase cytosolic NADH levels even when added after CAA had been metabolized, suggesting that CAA causes oxidative stress.

Hepatocytes isolated from fasted animals were also much more susceptible to CAA-induced cytotoxicity; however, this cytotoxicity was also prevented by nutrients that supply ATP without increasing cytosolic NADH. These results suggest that the susceptibility of a cell to CAA cytotoxicity depends on the ability of the cell to maintain both redox homeostasis and ATP levels.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (280–300 g), fed a standard chow diet and water *ad lib.*, were used in all experiments. Where indicated, hepatocytes were isolated from animals fasted for an 18- to 24-hr period.

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† Abbreviations: CAA, 2-chloroacetaldehyde; MDA, malondialdehyde; GSH, glutathione; and GSSG, oxidized glutathione.

Chemicals. CAA was obtained from the Aldrich Chemical Co. (Milwaukee, WI) as a 50% aqueous solution. Collagenase (from *Clostridium histoliticum*) and HEPES were purchased from Boehringer–Mannheim (Montreal, Canada). Trypan blue, GSH, trichloroacetic acid, thiobarbituric acid, fluoro-2,4-dinitrobenzene and iodoacetic acid were obtained from the Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest commercial grade available.

Isolation and incubation of hepatocytes. Hepatocytes were obtained by collagenase perfusion of the liver as described by Moldéus *et al.* [12]. Approximately 85% of the hepatocytes excluded trypan blue. Cells (10^6 cells/mL) were suspended in round-bottomed flasks rotating in a water bath maintained at 37° in Krebs–Henseleit buffer (pH 7.4), supplemented with 12.5 mM HEPES under carbogen (95% O_2/5% CO_2). Cell viability was determined by measuring the exclusion of trypan blue (final concentration: 0.16%, w/v). Hepatocytes were preincubated for 30 min prior to addition of chemicals. Stock solutions of CAA were freshly prepared prior to use. Hepatocytes under 10% O_2/5% CO_2/85% N_2 showed a similar susceptibility to CAA and antidotal effects of NADH-generating nutrients.

Determination of malondialdehyde in hepatocytes. Lipid peroxidation in hepatocytes was measured by the thiobarbituric acid assay as described by Ottolenghi [13]. After incubating deproteinated aliquots of the hepatocyte mixture with thiobarbituric acid in a boiling water bath for 15 min, the amount of MDA formed was determined using an absorption coefficient of 1.56×10^5 mol cm^{-1} at 532 nm.

Glutathione determination. The total GSH and GSSG content of the hepatocytes was measured on deproteinated samples (5% metaphosphoric acid), after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene, by HPLC, using a C18 μBondapak NH_2 column (Waters Associates, Milford, MA) [14]. GSH and GSSG were used as external standards. A Waters 6000A solvent delivery system equipped with a model 600 solvent programmer, a Wisp 710A automatic injector, and a Data Module were used for analysis.

Determination of ATP. ATP in hepatocytes was extracted using an alkaline extraction procedure and quantified using HPLC, using a C18 μBondapak reverse phase column (Waters Associates) as described previously by Stocchi *et al.* [15].

Lactate/pyruvate assays. Lactate and pyruvate were determined in acid extracts by the enzymatic methods as described previously [16, 17].

Statistics. Statistical significance of differences between treatment group in these studies was determined by Student's *t*-test. The minimal level of significance chosen was $P < 0.05$.

RESULTS

As shown in Table 1, the concentration of CAA required to cause 50% cytotoxicity in 2 hr was 0.3 mM, whereas the required concentration of the reductive metabolite 2-chloroethanol or the oxidative metabolite 2-chloroacetic acid was 20 and 3 mM, respectively. Removal of any unmetabolized CAA at 10 min by resuspending the hepatocyte suspension in fresh buffer had no effect on cytotoxicity, indicating that CAA was metabolized or bound rapidly; however, removal of any unmetabolized CAA at 2 min completely protected hepatocytes against CAA cytotoxicity. Furthermore, less than 10% of the original concentration of CAA could be detected by gas chromatography in acid hepatocyte extracts 30 min after CAA addition, but approximately 90% of the original concentration was recovered as the reductive metabolite (2-chloroethanol) of CAA (results not shown).

As shown in Table 2, inhibition of alcohol dehydrogenase with methyl pyrazole [18] greatly increased the susceptibility of hepatocytes to CAA. Similarly, the aldehyde dehydrogenase inhibitors (cyanamide [19], chloral hydrate [20]) also increased 2-fold the susceptibility of hepatocytes to CAA, including cytotoxicity, GSH depletion (Fig 1A) and lipid peroxidation (Fig. 1B). Furthermore, hepatocytes isolated from animals pretreated with phenobarbital to induce cytosolic aldehyde dehydrogenase [21] were resistant to CAA (results not shown).

Aminooxyacetate, an aspartate aminotransaminase inhibitor [22] that prevents the oxidation of cytosolic NADH by the mitochondrial malate

Table 1. Cytotoxicity (EC_{50} 2 hr) of 2-chloroethanol, 2-chloroacetaldehyde and 2-chloroacetic acid

Additions	Concn	% Cytotoxicity			
		Time (min)			
		30	60	120	180
Normal hepatocytes					
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 2
+2-Chloroethanol	20 mM	23 ± 3	31 ± 3	38 ± 4	40 ± 4
+2-Chloroacetaldehyde	0.3 mM	36 ± 3	40 ± 3	49 ± 4	56 ± 5
+2-Chloroacetic acid	3.0 mM	26 ± 3	40 ± 3	49 ± 4	52 ± 4

Hepatocytes (10^6 cells/mL) were preincubated at 37° in Krebs–Heinslett buffer, pH 7.4, with the toxin indicated. Percent cytotoxicity was determined by trypan blue uptake. Results are the means ± SEM of at least three separate experiments.

Table 2. Increasing cytotoxicity of CAA by inhibiting alcohol and aldehyde dehydrogenases

Additions	Concn	% Cytotoxicity Time (min) 30	60	120	180
Normal hepatocytes					
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 3
+CAA	0.5 mM	28 ± 3	39 ± 5	60 ± 6	100
+Methyl pyrazole	0.1 mM	39 ± 4	66 ± 7	100*	
+Chloral hydrate	1.0 mM	33 ± 3	64 ± 6	100*	
+Cyanamide	0.2 mM	28 ± 3	69 ± 7	100*	
+Rotenone	5 μM	39 ± 4	66 ± 7	100*	
+Cyanide	0.3 mM	34 ± 3	41 ± 4	74 ± 8*	
+Methyl pyrazole	0.1 mM	18 ± 2	20 ± 2	22 ± 2	23 ± 3
+Chloral hydrate	1.0 mM	16 ± 2	17 ± 2	20 ± 2	21 ± 2
+Cyanamide	0.2 mM	15 ± 2	17 ± 2	19 ± 2	22 ± 3
+Rotenone	5 μM	18 ± 3	19 ± 2	21 ± 2	24 ± 2
+Cyanide	0.3 mM	17 ± 2	18 ± 2	23 ± 2	25 ± 3

Hepatocytes (10^6 cells/mL) were preincubated at 37° with inhibitor for 10 min prior to addition of CAA. Concentrations of inhibitors used in these experiments did not cause hepatocyte cytotoxicity. Percent cytotoxicity was determined by trypan blue uptake. Results are the means ± SEM of at least three separate experiments.

* $P < 0.05$ vs control and single treatment group (CAA).

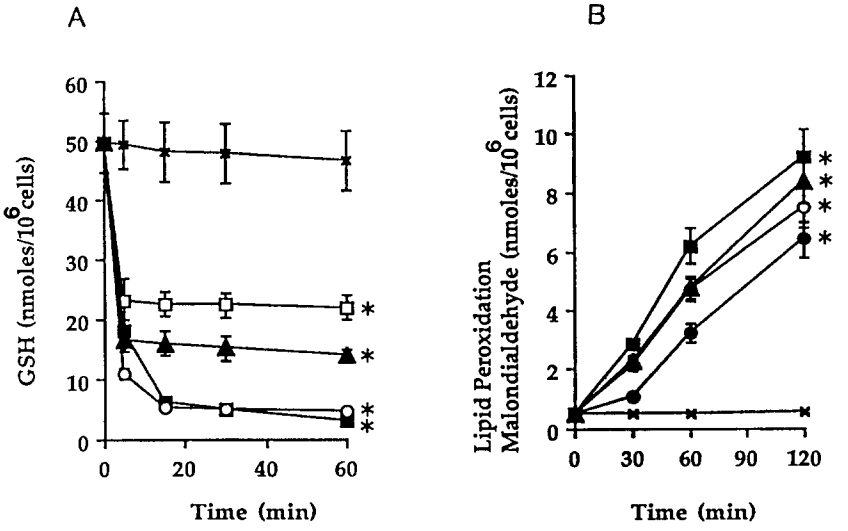

Fig. 1. (A) Effect of alcohol and aldehyde dehydrogenase inhibitors on CAA-induced GSH depletion. Key: (x) control, (□) CAA 0.2 mM, (▲) CAA 0.2 mM + chloral hydrate 1 mM, (○) CAA 0.2 mM + cyanamide 0.2 mM, and (■) CAA 0.2 mM + methylpyrazole 0.1 mM. (B) Effect of alcohol and aldehyde dehydrogenase inhibitors on CAA-induced lipid peroxidation (MDA). Key: (×) control, (●) CAA 0.5 mM, (▲) CAA 0.5 mM + chloral hydrate 1 mM, (○) CAA 0.5 mM + cyanamide 0.2 mM, and (■) CAA 0.5 mM + methylpyrazole 0.1 mM. Values in each panel are the means ± SEM of at least three separate experiments. Key: (*) $P < 0.05$ vs control and single treatment group (CAA).

aspartate shuttle, prevented CAA-induced cytotoxicity (see Table 4). However, noncytotoxic concentrations of the mitochondrial respiratory inhibitors cyanide or rotenone (see Table 2) markedly increased CAA-induced cytotoxicity. These inhibitors prevent oxidation of mitochondrial NADH and have been shown to inhibit acetaldehyde metabolism in isolated rat liver mitochondria [23].

As shown in Table 3, nutrients that generate cytosolic NADH (as determined by the lactate/pyruvate ratios), such as lactate, sorbitol, xylitol or glycerol, prevented CAA-mediated hepatocyte killing. Increasing cytosolic NADH levels with ethanol also prevented CAA cytotoxicity. These nutrients or ethanol also prevented CAA-induced GSH depletion and lipid peroxidation (Fig. 2).

Table 3. Nutrient modulation of CAA-induced cytotoxicity in alcohol dehydrogenase inactivated hepatocytes

Additions	Concn	% Cytotoxicity			
		Time (min)			
		30	60	120	180
Normal hepatocytes					
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 3
+CAA	0.5 mM	28 ± 3	39 ± 5	60 ± 6	100
+Lactate	10 mM	22 ± 4	25 ± 4	29 ± 3	32 ± 3*
+Ethanol	20 mM	27 ± 3	28 ± 2	29 ± 3	30 ± 3*
+Xylitol	10 mM	24 ± 1	25 ± 2	30 ± 3	37 ± 3*
+Sorbitol	10 mM	22 ± 2	24 ± 3	29 ± 3	35 ± 3*
+Glycerol	10 mM	25 ± 3	26 ± 2	30 ± 3	35 ± 3*
+Pyruvate	10 mM	44 ± 4	55 ± 5	80 ± 6*	100
+Acetaldehyde	10 mM	49 ± 4	70 ± 6	100*	
+Oxaloacetate	10 mM	18 ± 2	20 ± 2	31 ± 3	46 ± 5*
Hepatocytes with inactive alcohol dehydrogenase					
Control		15 ± 2	16 ± 2	21 ± 2	23 ± 3
+CAA	0.3 mM	32 ± 3	42 ± 5	60 ± 6	80 ± 8
+Lactate	10 mM	37 ± 4	60 ± 7	85 ± 5	100*
+Xylitol	10 mM	36 ± 3	50 ± 4	75 ± 5	100*
+Sorbitol	10 mM	30 ± 3	50 ± 5	78 ± 6	100*
+Glycerol	10 mM	30 ± 3	48 ± 4	88 ± 8	100*
+Pyruvate	10 mM	33 ± 3	35 ± 3	36 ± 4	50 ± 5*
+Acetaldehyde	10 mM	66 ± 6	75 ± 7	100*	
+Oxaloacetate	10 mM	35 ± 3	40 ± 4	43 ± 4	100*

Hepatocytes (10^6 cells/mL) were preincubated at 37° for 10 min in Krebs–Henseleit buffer, pH 7.4, with nutrients. Where indicated, hepatocyte alcohol dehydrogenase was inactivated by incubating with 0.1 mM methyl pyrazole for 5 min prior to nutrient addition. Where indicated, CAA was added to the incubation mixture. Cell viability was determined by trypan blue uptake. Results are the means ± SEM of at least three separate experiments.

* $P < 0.05$ vs single treatment group (CAA).

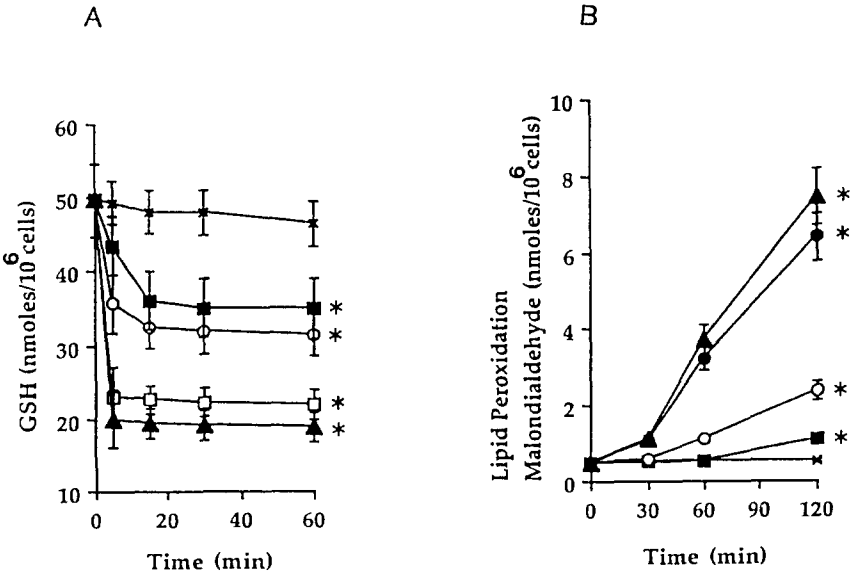

Fig. 2. (A) Effect of lactate and ethanol on CAA-induced GSH depletion. Key: (×) control, (□) CAA 0.2 mM, (■) CAA 0.2 mM + ethanol 20 mM, (○) CAA 0.2 mM + lactate 10 mM, and (▲) CAA 0.2 mM + pyruvate 10 mM. (B) Effect of lactate and ethanol on CAA-induced lipid peroxidation (MDA). Key: (×) control, (●) CAA 0.5 mM, (■) CAA 0.5 mM + ethanol 20 mM, (○) CAA 0.5 mM + lactate 10 mM, and (▲) CAA 0.5 mM + pyruvate 10 mM. Values in each panel are the means ± SEM of at least three separate experiments. Key: (*) $P < 0.05$ vs control and single treatment group (CAA).

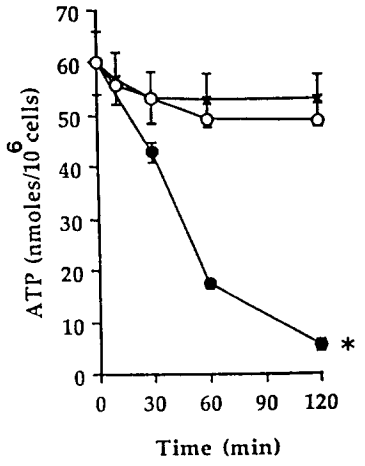

Fig. 3. Effect of lactate on CAA-induced ATP depletion. Key: (×) control, (●) CAA 0.5 mM and (○) CAA 0.5 mM + lactate 10 mM. Values are the means ± SEM of at least three separate experiments. Key: (*) $P < 0.05$ vs control and other treatment group.

However, pyruvate, which oxidizes cytosolic NADH, increased CAA-induced cytotoxicity, GSH depletion and lipid peroxidation. ATP depletion induced by CAA could also be prevented with lactate (Fig. 3). Prevention of CAA-induced cytotoxicity with NADH-generating nutrients was not affected by aminooxyacetate, indicating that their protective effect could be attributed to increased cytosolic NADH levels. In contrast, CAA cytotoxicity toward alcohol dehydrogenase inactivated hepatocytes was increased markedly by increasing cytosolic NADH levels with ethanol, xylitol, sorbitol, glycerol and lactate (Table 3) and decreased by decreasing cytosolic NADH levels with pyruvate. As shown in Table 4, the increase in CAA cytotoxicity in alcohol dehydrogenase inactivated hepatocytes with NADH-generating nutrients was prevented by aminooxyacetate, which correlated with the preservation of cytosolic NADH levels as measured by the lactate/pyruvate ratios.

As shown in Table 5, the following nutrients, which do not increase cytosolic NADH, did not protect hepatocytes against CAA-induced cytotoxicity: glucose, alanine, glutamine, glyceraldehyde and dihydroxyacetone. β-Hydroxybutyrate, which increases mitochondrial NADH levels, increased CAA cytotoxicity; however, acetoacetate, which oxidizes mitochondrial NADH, delayed CAA-induced toxicity. But, if desferoxamine, an iron chelator, was added to prevent the lipid peroxidation component of the cytotoxic mechanism, all the nutrients protected even against twice the normal toxic CAA concentrations (results not shown), suggesting that lipid peroxidation inactivated enzymes of intermediary metabolism. However, fructose protected hepatocytes against CAA-induced toxicity but not if glycolysis was inhibited with fluoride (results not shown), an enolase inhibitor [24], indicating that the antidotal effect of fructose may be attributed to supplying glycolytic ATP. Glucose also protected if a small amount of fructose was present.

Table 4. Preventing nutrient-increased CAA cytotoxicity in hepatocytes with inactive alcohol dehydrogenase by preventing the oxidation of cytosolic NADH with aminooxyacetate

Additions	Concn	% Cytotoxicity Time (min) 30	60	120	180	[Lactate/Pyruvate] 120 min
Normal hepatocytes						
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 2	11.4 ± 1.2
+CAA	0.3 mM	28 ± 3	36 ± 3	49 ± 4	56 ± 5	1.4 ± 0.2
+Sorbitol	10 mM	22 ± 4	24 ± 4	26 ± 3	29 ± 3*	16.1 ± 1.9*
+Aminooxyacetate	0.075 mM	24 ± 2	26 ± 3	30 ± 3	32 ± 4*	6.5 ± 0.5*
+Aminooxyacetate + sorbitol		26 ± 3	30 ± 4	34 ± 4	38 ± 4*	41.6 ± 3.6*
+Sorbitol	10 mM	16 ± 2	17 ± 2	19 ± 2	21 ± 2	33.4 ± 1.5*
Hepatocytes with inactive alcohol dehydrogenase						
Control		18 ± 2	20 ± 2	22 ± 2	23 ± 3	7.1 ± 0.5
+CAA	0.3 mM	32 ± 3	42 ± 5	60 ± 2	80 ± 5	1.6 ± 0.2
+Sorbitol	10 mM	30 ± 6	50 ± 5	78 ± 4	100*	10.8 ± 2.7*
+Aminooxyacetate	0.1 mM	35 ± 4	46 ± 4	62 ± 3	83 ± 4	38.1 ± 2.0*
+Aminooxyacetate + sorbitol		36 ± 5	44 ± 5	58 ± 3	77 ± 3	56.9 ± 4.3*
+Sorbitol	10 mM	19 ± 2	22 ± 2	23 ± 2	25 ± 4	25.4 ± 2.2*

Hepatocytes (10^6 cells/mL) were preincubated at 37° in Krebs–Henseleit buffer, pH 7.4, with nutrients and/or aminooxyacetate for 10 min. Hepatocyte alcohol dehydrogenase was inactivated by incubating with 0.1 mM methyl pyrazole for 5 min prior to the addition of nutrients and/or aminooxyacetate. Where indicated, CAA was added to the incubation mixture. [Lactate/Pyruvate] samples were taken at 2 hr after CAA addition and analysed as described in Materials and Methods. Percent cytotoxicity was determined by trypan blue uptake. Results are the means ± SEM of at least three separate experiments.

* $P < 0.05$ vs single treatment group (CAA).

Table 5. Nutrient modulation of CAA-induced cytotoxicity in hepatocytes from fed versus fasted animals

Additions	Concn	% Cytotoxicity			
		Time (min)			
		30	60	120	180
Normal hepatocytes: Isolated from fed animals					
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 3
+CAA	0.5 mM	28 ± 3	39 ± 5	60 ± 6	100
+Glyceraldehyde	10 mM	28 ± 3	45 ± 7	74 ± 8	100
+Dihydroxyacetone	10 mM	24 ± 4	32 ± 2	77 ± 2	100
+β-Hydroxybutyrate	10 mM	40 ± 4	48 ± 5	75 ± 6	100
+Acetoacetate	10 mM	32 ± 2	39 ± 2	49 ± 5	85 ± 8
+Glutamine	2 mM	20 ± 2	33 ± 3	70 ± 5	100
+Alanine	10 mM	37 ± 3	45 ± 4	100	
+Glucose	10 mM	29 ± 3	33 ± 3	65 ± 6	100
+Glucose + fructose	0.2 mM	25 ± 3	28 ± 3	35 ± 3	38 ± 3*
+Fructose	10 mM	22 ± 3	28 ± 3	34 ± 4	37 ± 4*
Normal hepatocytes: Isolated from fasted animals					
Control		15 ± 2	16 ± 2	21 ± 2	24 ± 4
+CAA	0.05 mM	35 ± 3	41 ± 5	58 ± 6	65 ± 7
+Glyceraldehyde	10 mM	24 ± 2	28 ± 3	30 ± 3	35 ± 3*
+Dihydroxyacetone	10 mM	24 ± 2	26 ± 2	28 ± 2	32 ± 3*
+β-Hydroxybutyrate	10 mM	26 ± 2	28 ± 3	38 ± 3	40 ± 4*
+Acetoacetate	10 mM	29 ± 3	30 ± 3	38 ± 4	42 ± 4*
+Glutamine	2 mM	28 ± 2	34 ± 3	38 ± 4	41 ± 4*
+Alanine	10 mM	30 ± 3	32 ± 3	36 ± 4	38 ± 4*
+Glucose	10 mM	20 ± 2	25 ± 3	31 ± 3	35 ± 4*
+Glucose + fructose	0.2 mM	24 ± 2	33 ± 3	34 ± 3	35 ± 5*
+Fructose	10 mM	20 ± 2	24 ± 2	28 ± 3	30 ± 3*

Hepatocytes (10^6 cells/mL) were preincubated at 37° for 10 min in Krebs–Henseleit buffer, pH 7.4, with nutrients. Where indicated, CAA was added to the incubation mixture. In the experiments with hepatocytes isolated from starved animals, food was withheld for 18–24 hr. Percent cytotoxicity was determined by trypan blue uptake. Results are the means ± SEM of at least three separate experiments.

* $P < 0.05$ vs single treatment group (CAA).

Glycogen-depleted hepatocytes isolated from fasted animals were 6-fold more susceptible to CAA-induced injury than hepatocytes isolated from fed animals. The concentration of CAA required to kill 50% of "fasted" hepatocytes was now only 50 μM. At this CAA concentration, no lipid peroxidation occurred and all nutrients afforded complete protection against CAA toxicity (Table 5). ATP levels of fasted hepatocytes were 21 ± 4 nmol/10^6 cells, whereas ATP levels of hepatocytes from fed animals were 60 ± 5 nmol/10^6 cells.

DISCUSSION

We recently reported that the molecular mechanism of CAA-induced cytotoxicity toward isolated rat hepatocytes is characterized by rapid protein thiol depletion, ATP depletion and lipid peroxidation [11]. The cell permeable reductant dithiothreitol, even if added 40 min after CAA, is able to restore protein thiols and hepatocyte respiration and prevents further ATP depletion. Preventing lipid peroxidation with iron chelators or antioxidants delays the onset of cytotoxicity without restoring protein thiols and hepatocyte respiration or preventing further ATP depletion [11].

The present studies with isolated rat hepatocytes demonstrate that inactivation of alcohol or aldehyde dehydrogenase increased CAA cytotoxicity. CAA was also more toxic by at least two orders of magnitude than either its oxidative metabolite (2-chloroacetic acid) or reductive metabolite (2-chloroethanol). *In vivo* CAA (LD_{50} 0.006 mL/kg) is also more toxic than 2-chloroethanol (LD_{50} 0.05 mL/kg) following intraperitoneal injections [25]. Inhibition of hepatocyte alcohol or aldehyde dehydrogenase also caused a marked increase in CAA-induced GSH depletion and lipid peroxidation. Hepatocytes isolated from animals pretreated with phenobarbital to induce cytosolic aldehyde dehydrogenase were also more resistant to CAA-induced cytotoxicity.

The decrease in CAA cytotoxicity by ethanol, xylitol, sorbitol, glycerol and lactate but not dihydroxyacetone, glyceraldehyde, glutamine or alanine can be attributed to the ability of the former to increase cytosolic NADH levels [26–29]. Normally, NADH levels are much lower than NAD^+ levels in the cell cytosol [30]. Protection by these compounds was prevented by inactivating alcohol dehydrogenase with methyl pyrazole, suggesting that the increased NADH levels enhanced the reductive detoxification

of CAA by alcohol dehydrogenase. In support of this, pyruvate, which oxidizes cytosolic NADH [31], increased CAA cytotoxicity. Furthermore, hepatocyte GSH depletion by CAA was more rapid and extensive if pyruvate was present but slower if lactate or ethanol was present, indicating that CAA was reductively metabolized by cytosolic NADH and alcohol dehydrogenase.

Ethanol ingestion in humans has been shown to shift the normal oxidative metabolism of serotonin [32], norepinephrine [33], and dopamine [34] by NAD^+ and aldehyde dehydrogenase to reductive metabolism. Work with rat liver slices suggests that an alcohol dehydrogenase–NADH complex rather than increasing cytosolic NADH levels is responsible for the reductive metabolism of dopamine [35], as lactate, which also increases cytosolic NADH levels, is not effective [36]. The ethanol-induced increase in chloral hydrate or cyclohexanone reductive metabolism [37, 38] was also attributed to an alcohol dehydrogenase–NADH complex, and direct transfer of deuterated ethanol was demonstrated [37, 38].

The increase in CAA toxicity by xylitol, sorbitol, glycerol and lactate in alcohol dehydrogenase inactivated hepatocytes could be explained if the increased cytosolic NADH levels increased mitochondrial NADH levels via the mitochondrial malate-aspartate shuttle, thereby preventing mitochondrial aldehyde dehydrogenase from oxidatively detoxifying CAA. In support of this, GSH depletion induced by CAA was more rapid and more extensive in alcohol dehydrogenase inactivated hepatocytes if a nutrient was present (results not shown). Furthermore, aminooxyacetate prevented the increase in CAA toxicity by these NADH-generating nutrients in alcohol dehydrogenase inactivated hepatocytes, which correlated with the preservation of cytosolic NADH levels as measured by the lactate/pyruvate ratio. Aminooxyacetate is a specific inhibitor of aspartate aminotransaminase [22], which prevents the oxidation of cytosolic NADH by the mitochondrial malate-aspartate shuttle. Aminooxyacetate, however, did not affect nutrient protection of CAA toxicity in normal hepatocytes. Furthermore, pyruvate, which oxidizes cytosolic NADH, prevented CAA toxicity in alcohol dehydrogenase inactivated hepatocytes.

Nutrients such as dihydroxyacetone, glyceraldehyde, glutamine, alanine, and acetoacetate, which do not increase cytosolic NADH [39], did not prevent CAA-induced hepatocyte cytotoxicity. However, fructose markedly protected hepatocytes from CAA, suggesting that another cytoprotective mechanism exists. Fructose protection was prevented by fluoride, an enolase inhibitor, suggesting that fructose acts by supplying glycolytic ATP, which protects the plasma membrane ionic pumps and prevents cytotoxicity. Further evidence that the susceptibility of hepatocytes to CAA depends on their ability to maintain ATP levels was obtained using glycogen-depleted hepatocytes isolated from fasted rats. The latter hepatocytes were 5-fold more susceptible to CAA-induced cytotoxicity, suggesting that their ability to supply glycolytic ATP was required to maintain hepatocyte viability in the presence of CAA. Furthermore, dihydroxyacetone, glyceraldehyde, glutamine, alanine and acetoacetate, which did not prevent CAA cytotoxicity in hepatocytes from fed animals, were highly effective in protecting hepatocytes isolated from fasted animals from these lower CAA concentrations.

The observation that certain nutrients did not protect against CAA-induced cytotoxicity suggests that their metabolizing enzymes are potential targets for CAA-induced inactivation. Glutamine has been shown to prevent hydrogen peroxide toxicity in injured epithelial cells by supplying ATP [40, 41]; however, glutamine and alanine both failed to protect against CAA-induced toxicity. This suggests that the metabolism or the active transport systems of glutamine and alanine have been disabled by CAA. Antioxidants or the iron chelator desferoxamine prevented CAA cytotoxicity, and higher CAA concentrations were required to cause cytotoxicity. Interestingly, glutamine and alanine now protected hepatocytes from these higher CAA concentrations, suggesting that lipid peroxidation inactivated the alanine or glutamine metabolizing enzymes or membrane transport systems. This could also explain the cytoprotective effectiveness of glutamine and alanine towards "fasted" hepatocytes as lipid peroxidation did not occur at the much lower CAA concentrations required for cytotoxicity, and desferoxamine or antioxidants were not cytoprotective.

The ability to restore hepatocyte redox homeostasis and enhance metabolic detoxification of CAA with readily available nutrients may provide the basis for adjunct nutritional therapy to ameliorate or prevent secondary tissue toxicities caused by CAA during chemotherapy with ifosfamide. Further studies to determine the efficacy of nutrient therapy in ameliorating CAA toxicity *in vivo* are currently under investigation.

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