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MOLECULAR MECHANISMS OF CHLOROACETALDEHYDE-INDUCED CYTOTOXICITY IN ISOLATED RAT HEPATOCYTES

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Abstract—2-Chloroacetaldehyde (CAA) induced a loss in hepatocyte viability in a concentration- and time-dependent manner. Three phases before cytotoxicity ensued could be distinguished. Glutathione (GSH) was depleted immediately upon addition of CAA but only partial depletion occurred with subtoxic CAA concentrations. GSH-depleted hepatocytes were much more susceptible to CAA toxicity, indicating that CAA was detoxified by GSH. The second phase of changes involved a steady decrease in protein thiol levels, mitochondrial respiration, transmembrane potential and ATP levels. The third phase involved lipid peroxidation which commenced at around 60 min with a CAA concentration that caused 50% cytotoxicity in 120 min. Addition of antioxidants (diphenylphenylenediamine, butylated hydroxyanisole) and iron chelators (desferoxamine) at 40 min prevented lipid peroxidation and delayed CAA-induced cytotoxicity without restoring protein thiols, hepatocyte respiration or preventing further ATP depletion. Addition of dithiothreitol at 40 min, however, restored protein thiols and hepatocyte respiration, and prevented further ATP depletion and cytotoxicity. CAA-induced hepatocyte cytotoxicity therefore involved reversible thiol protein adduct formation, mitochondrial toxicity and lipid peroxidation.

Key words: Aldehyde toxicity, GSH, mitochondrial toxicity, lipid peroxidation, xanthine oxidase

2-Chloroacetaldehyde (CAA)† is an alkylating agent and potent mutagen [1], forms etheno compounds with DNA adenosine and cytosine [2], induces interstrand DNA cross-links [3], and inhibits DNA synthesis [4]. It is also a major reactive metabolite of a large number of industrial chemicals such as vinyl chloride [5], ethylene dichloride [6] and ethylene chlorohydrin. CAA is found in the urine of rats given the alkylating anticancer drug cyclophosphamide [7]. Clinical studies show that the cyclophosphamide analogue ifosfamide used in antitumor chemotherapy undergoes side chain oxidation in humans to form CAA [8], which may be responsible for the neurotoxic side-effects of ifosfamide chemotherapy [9]. The anticancer drug 1-(2 chloroethyl)-3-cyclohexyl-l-nitrosourea is also metabolised to 2-chloroethanol [10].

The metabolism of CAA *in vitro* or *in vivo* has not been investigated thoroughly. However, horse liver alcohol dehydrogenase has been shown to catalyse the NADH-dependent reduction of CAA to form 2-chloroethanol, whereas yeast aldehyde dehydrogenase catalyses the NAD^+-dependent oxidation of CAA to form 2-chloroacetic acid [5]. CAA also depletes glutathione (GSH) in lymphocytes from patients receiving ifosfamide [11], probably as a result of a nonenzymic reaction with GSH. Chloroethanol does not react with GSH but depletes hepatic GSH *in vivo* presumably following oxidation by alcohol dehydrogenase to CAA [12].

In a recent study [13], the liver was the primary target organ when mice were given 0.1 g/L CAA in their drinking water. Hepatocellular necrosis, hepatocellular hyperplasia and chronic active inflammation were induced by CAA. CAA also induced increases in absolute liver weights and caused liver tumors in these animals [13]. Rat liver GSH is depleted markedly after an oral dose of CAA [14]. In the present study, the molecular mechanisms involved in CAA-induced cytotoxicity in isolated rat hepatocytes were investigated. GSH depletion, inhibition of respiration, ATP depletion and lipid peroxidation were found to precede cytotoxicity. Cytotoxicity was delayed by antioxidants or iron chelators, but could be prevented by adding dithiothreitol (DTT) 40 min after CAA.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (280–300 g), fed a standard chow diet and fed water *ad lib.*, were used in all experiments.

Chemicals. 2-Chloroacetaldehyde 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, thiobarbituric acid, fluoro-2,4-dinitrobenzene and iodoacetic acid were obtained from Sigma (St. Louis, MO). Other chemicals were of the highest commercial grade available.

Isolation and incubation of hepatocytes. Hepa-

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† Abbreviations: CAA, 2-chloroacetaldehyde; GSH, glutathione; DTT, dithiothreitol; MDA, malondialdehyde; and DPPD, *N*,*N*′-diphenyl-1,4-phenylenediamine.

tocytes were obtained by collagenase perfusion of the liver as described by Moldeus *et al.* [15]. Approximately 85% of the hepatocytes excluded trypan blue. Cells were suspended at a density of 10^6 cells/mL 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 an atmosphere of 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 chemicals of CAA were freshly prepared prior to use. GSH-depleted hepatocytes were obtained by preincubating hepatocytes with *n*-bromoheptane (100 μM) for 30 min before addition of CAA [16].

Determination of malondialdehyde (MDA) in hepatocytes. Lipid peroxidation in hepatocytes was measured by the thiobarbituric acid assay as described by Ottolenghi [17] and expressed as the amount of MDA formed using an absorption coefficient of 1.56×10^6 mol cm^{-1} at 532 nm after incubating deproteinated aliquots of cellular mixture with thiobarbituric acid in a boiling water bath for 15 min [17].

Glutathione determination. The total GSH and oxidized glutathione (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) [18]. 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.

Protein sulfhydryl group determination. Protein sulfhydryl groups were determined using Ellman's reagent as described by Sedlak and Lindsay [19] and modified by Albano *et al.* [20]. Total protein was assayed using a modified Lowry procedure [21].

Determination of ATP. ATP in hepatocytes was extracted using an alkaline extraction procedure and quantified by HPLC, using a C18 μBondapak reverse phase column (Waters Associates) as described previously by Stocchi *et al.* [22].

Isolation and incubation of mitochondria. Liver mitochondria were prepared as described by Jocelyn and Cronshaw [23]. Mitochondria (2 mg protein/mL) were preincubated in the absence or presence of 1-chloro-2,4-dinitrobenzene (CDNB; 10 nmol/mg) for 2 min before Ca^{2+} loading.

Unless otherwise stated, all incubations were preformed in buffer consisting of 210 mM mannitol, 70 mM sucrose and 3 mM HEPES, pH 7.4 (Buffer A), supplemented with 5 mM succinate plus 1 μM rotenone, at 37°. All spectrophotometric assays were carried out on a DW2000 spectrophotometer.

Calcium release was monitored in freshly isolated mitochondria by measuring the absorbance change of the metal chromatophoric dye Arzenazo III (40 μM) [2,2-(1,8-dihydroxy-3,6-disulfonapthalene-2,7-bisazo)bis(arsenic acid)], using the wavelength pair 654–685 nm. Mitochondria (2.0 mg protein/mL) were loaded with Ca^{2+} before addition of CAA.

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

RESULTS

As shown in Fig. 1A, CAA at a concentration of 0.3 mM induced a 50% loss in hepatocyte viability in 2 hr as measured by trypan blue exclusion. The cytotoxicity of CAA was concentration and time dependent. Removal of any unmetabolized CAA at 10 min by resuspending the hepatocytes in fresh buffer had no effect on subsequent cytotoxicity, indicating that CAA was metabolised rapidly and that the cytotoxic effects of CAA were irreversible.

As shown in Fig. 1B, toxic concentrations of CAA depleted 90% of cellular GSH within the first 3 min of addition. At subtoxic CAA concentrations, however, GSH was only partially depleted. CAA also caused depletion of protein thiols within the first 10 min of addition of a toxic concentration of CAA (Fig. 1C). Addition of the thiol reductant DTT 40 min after CAA (see Table 2) fully restored protein thiols and averted cytotoxicity.

CAA inhibited hepatocyte respiration in a time-dependent manner (Table 1). Hepatocyte respiration was still inhibited at 60 min after the initial CAA addition. DTT reversed CAA-induced inhibition of hepatocyte respiration even if added 40 min after CAA. Hepatocyte mitochondrial membrane potential was also partly dissipated 30 min after CAA addition as measured by rhodamine 123 uptake (results not shown). CAA also induced a marked decrease in hepatocyte ATP levels (Fig. 2), but not if DTT was present. DTT also stopped further ATP depletion when added 40 min after CAA. The antioxidant *N,N'*-diphenyl-1,4-phenylenediamine (DPPD) was unable to prevent ATP depletion.

Low concentrations of CAA inhibited mitochondrial respiration with glutamate or β-hydroxybutyrate as substrates but not with succinate (results not shown). This suggests that the respiratory chain site most sensitive to inhibition is localised prior to the NADH-ubiquinone oxidoreductase. Mitochondrial respiration was restored by the subsequent addition of DTT (results not shown).

GSH-depleted hepatocytes were more sensitive to CAA-induced cytotoxicity (Table 2). The antioxidant DPPD and DTT prevented cytotoxicity. ATP depletion was also more rapid in GSH-depleted hepatocytes following CAA addition (results not shown), as was the rate of mitochondrial membrane collapse and the inhibition of hepatocyte respiration. CAA was also more effective at inhibiting mitochondrial respiration with glutamate or β-hydroxybutyrate as substrates if mitochondrial GSH was depleted beforehand (results not shown).

Ruthenium red, an inhibitor of mitochondrial calcium uptake, delayed CAA-induced cytotoxicity. CAA also induced mitochondrial calcium release (Fig. 3) in isolated mitochondria, which was prevented by DTT. CAA was much more effective at inducing mitochondrial Ca^{2+} release from GSH-depleted mitochondria.

Lipid peroxidation (Fig. 1D), as measured by the

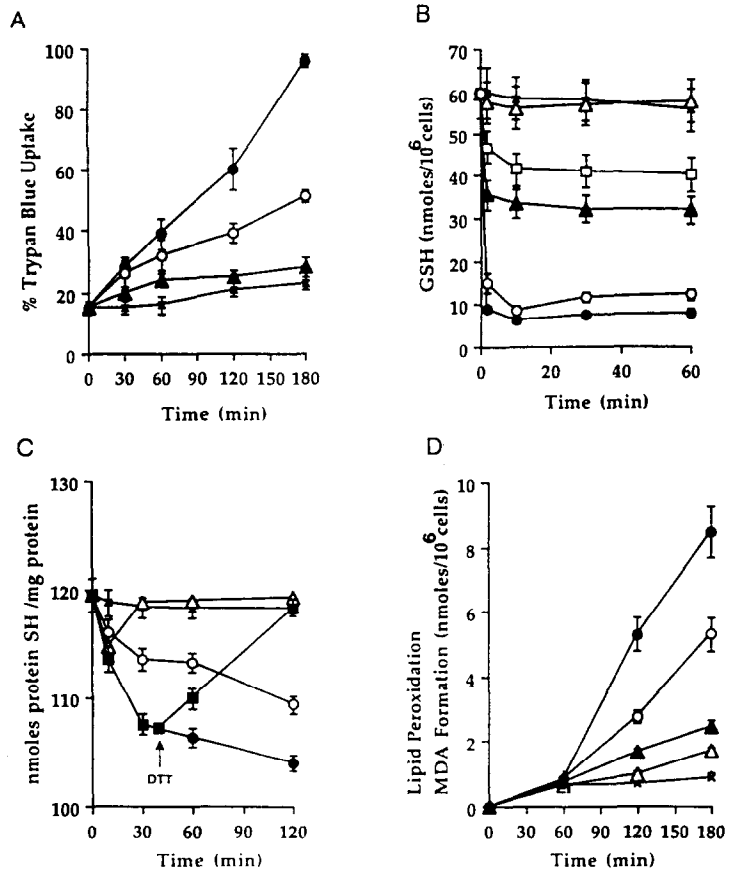

Fig. 1. (A) CAA-induced cytotoxicity towards isolated rat hepatocytes (10^6 cells/mL) under aerobic conditions. Cell viability was determined by trypan blue uptake at 30-min time intervals. Key: (×) control, (▲) CAA (0.2 mM), (○) CAA (0.3 mM), and (●) CAA (0.5 mM). (B) CAA-induced GSH depletion. Key: (×) control, (△) CAA (0.075 mM), (□) CAA (0.150 mM), (▲) CAA (0.2 mM), (○) CAA (0.3 mM), and (●) CAA (0.5 mM). (C) CAA-induced changes in protein thiols. Key: (×) control, (●) CAA (0.5 mM), (△) CAA (0.5 mM) + DTT (0 min), (■) CAA (0.5 mM) + DTT (40 min), and (○) CAA (0.3 mM). (D) CAA-induced lipid peroxidation (MDA formation). Key: (×) control, (△) CAA (0.075 mM), (▲) CAA (0.2 mM), (○) CAA (0.3 mM), and (●) CAA (0.5 mM). Values in each panel are the means ± SEM of at least three separate experiments.

Table 1. CAA-induced inhibition of hepatocyte respiration

Additions	Rate of O_2 uptake (nmol O_2)/min/10^6 cells) Time (min)		
	30	60	120
Normal hepatocytes			
Control	15.3	14.7	13.0
+CAA (0.5 mM)	9.6	7.8	6.7
+CAA (0.5 mM) + DTT (5 mM) (40 min)	9.6	14.5	13.1
GSH-depleted hepatocytes			
Control	14.0	12.5	11.1
+CAA (0.5 mM)	6.1	4.8	0.1
+CAA (0.5 mM) + DTT (5 mM) (40 min)	6.1	10.0	8.5

O_2 uptake was measured using a Clark electrode in 2 mL Krebs-Henseleit buffer, containing HEPES (12.5 mM) at a cell density of 10^6 cells/mL and at 37°. GSH-depleted hepatocytes were prepared by preincubation with 0.2 mM *n*-bromoheptane for 30 min prior to addition of CAA. Results are the means of at least three determinations with two different batches of cells.

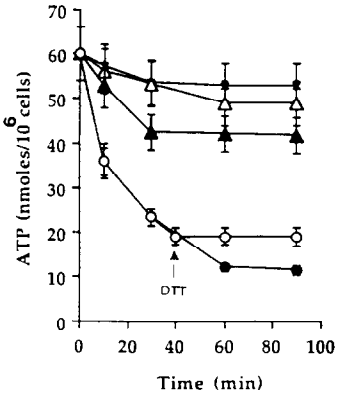

Fig. 2. CAA-induced ATP depletion. Key: (×) control, (△) CAA (0.5 mM) + DTT (0 min), (▲) CAA (0.2 mM), (○) CAA (0.5 mM) + DTT (40 min), and (●) CAA (0.5 mM). Values are the means ± SEM of at least three separate experiments.

formation of MDA, commenced at 60–90 min following the addition of a cytotoxic concentration of CAA and steadily increased until cytotoxicity occurred. The extent and rate of MDA formation increased as CAA concentration was increased. When lipid peroxidation was prevented by low concentrations of the antioxidants DPPD or butylated hydroxyanisole (BHA), cytotoxicity was delayed. DPPD was more effective at delaying CAA-induced cytotoxicity than BHA. The iron chelator desferoxamine also completely prevented lipid peroxidation and delayed cytotoxicity. Under hypoxic conditions (95%N_2:5%CO_2), lipid peroxidation did not occur and hepatocytes were much more resistant to CAA even though GSH depletion occurred. Oxypurinol, a xanthine oxidase inhibitor, prevented lipid peroxidation and hepatocyte susceptibility to CAA (Table 2).

DISCUSSION

A rapid depletion of GSH and a loss of protein thiols occurred when CAA was added to hepatocytes.

Table 2. Modulating CAA-induced cytotoxicity with antioxidants, oxygen removal or GSH depletion

Additions	Concentration	Cytotoxicity (% trypan blue uptake) Time (min) 30	60	120	180
Normal hepatocytes					
Aerobic (95% O_2:5% CO_2)					
Control		15 ± 2	16 ± 2	21 ± 2	22 ± 2
CAA	0.5 mm	28 ± 3	39 ± 5	60 ± 6	100
+ DPPD (0 min)	20 μM	25 ± 3	28 ± 3	36 ± 4	70 ± 7
+ DPPD (40 min)	20 μM	31 ± 3	32 ± 4	39 ± 4	100
+ DPPD (60 min)	20 μM	31 ± 3	36 ± 4	82 ± 3	100
+ BHA (0 min)	25 μM	28 ± 2	36 ± 4	49 ± 6	85 ± 8
+ BHA (40 min)	25 μM	32 ± 2	38 ± 4	62 ± 6	100
+ BHA (60 min)	25 μM	42 ± 2	47 ± 5	80 ± 8	100
+ Des (0 min)	0.2 mM	22 ± 2	25 ± 3	33 ± 3	78 ± 6
+ Des (40 min)	0.2 mM	32 ± 2	40 ± 4	42 ± 4	100
+ DTT (0 min)	5 mM	26 ± 2	27 ± 3	32 ± 3	35 ± 3
+ DTT (40 min)	5 mM	26 ± 2	28 ± 4	38 ± 4	43 ± 4
+ DTT (60 min)	5 mM	30 ± 2	32 ± 4	68 ± 4	79 ± 8
+ Allopurinol	1 mM	26 ± 3	32 ± 3	58 ± 5	100
+ Oxypurinol	50 μM	23 ± 3	28 ± 3	32 ± 3	45 ± 5
+ Ruthenium red	5 μM	22 ± 3	26 ± 3	31 ± 3	85 ± 8
Hypoxic (95% N_2:5% CO_2)					
Control			29 ± 3	32 ± 3	35 ± 4
CAA	0.5 mM		24 ± 2	34 ± 3	65 ± 6
GSH-depleted hepatocytes					
Aerobic (95% O_2:5% CO_2)					
Control		12 ± 1	15 ± 3	26 ± 3	28 ± 3
CAA	0.3 mM	34 ± 3	68 ± 7	80 ± 8	100

Hepatocytes (10^6 cells/mL) were preincubated at 37° for 5 min in Krebs-Henseleit buffer, pH 7.4, with antioxidants (DPPD, BHA); iron chelators (desferoxamine); DTT, allopurinol, oxypurinol or ruthenium red. Where indicated CAA was added to the incubation mixture at the time indicated. GSH-depleted hepatocytes were prepared as described in Materials and Methods. Cell viability was determined by trypan blue uptake. Hypoxic conditions were generated by maintaining hepatocyte incubation mixtures under an atmosphere of 95% N_2:5% O_2. Three separate experiments were carried out and results reported are means ± SEM. Abbreviations: CAA, chloroacetaldehyde; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; BHA, butylated hydroxyanisole; Des, desferoxamine; and DTT, dithiothreitol.

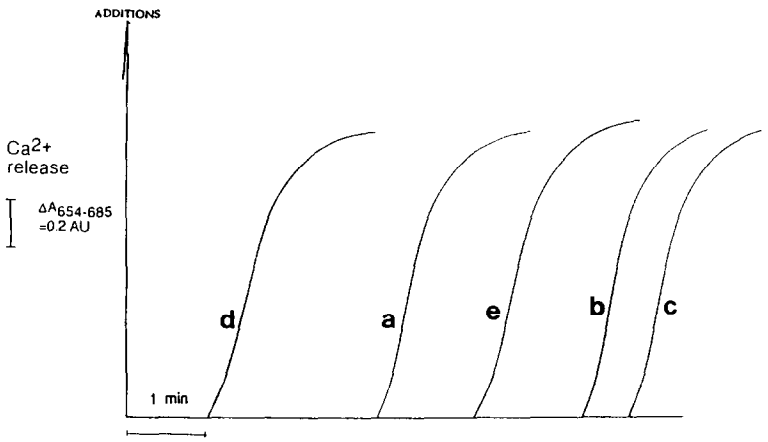

Fig. 3. CAA-induced mitochondrial Ca^{2+} release. (a) CAA (0.3 mM), (b) CAA (0.3 mM) + DTT (5 mM), (c) control mitochondria, (d) GSH-depleted mitochondria + CAA (0.3 mM), and (e) GSH-depleted mitochondria.

DTT fully restored protein thiols if added 40 min after CAA and prevented cytotoxicity, suggesting that the cytotoxic process is initiated when CAA forms reversible protein thiol adducts with CAA. DTT was unable to restore hepatocyte GSH levels, suggesting that CAA forms irreversible GSH conjugates. GSH-depleted hepatocytes were more sensitive to CAA, suggesting that GSH conjugates were not cytotoxic and that GSH detoxified CAA.

CAA has two potential sites of reaction with cell nucleophiles: the α chlorine can be displaced by a typical SN2 type reaction and the carbonyl group can participate in the formation of both reversible and irreversible adducts. The major urinary excretion metabolite of chloroethanol and chloroacetate in rats was identified as thiodiglycolic acid [12, 24–26], indicating that the sulfhydryl of GSH displaces the α chlorine of CAA and chloroacetate to form initially the intermediate *S*-carboxymethylglutathione. GSH probably forms irreversible adducts with CAA because DTT could not restore hepatocyte GSH levels, thus ruling out potential, reversible thiol adducts with the carbonyl moiety of CAA. Protein thiols were fully restored by DTT 40 min after CAA addition, suggesting that protein thiols may react with the carbonyl moiety of CAA to form reversible adducts such as hemithioacetals or thioacetals.

The earliest cytotoxic effects found were an inhibition of hepatocyte respiration, partial collapse of mitochondrial membrane potential and ATP depletion following addition of CAA to isolated hepatocytes. These effects of CAA were more marked in GSH-depleted hepatocytes. The antioxidant DPPD or the iron chelator desferoxamine did not prevent these toxic effects, suggesting that lipid peroxidation was not responsible for CAA-induced mitochondrial toxicity.

DTT prevented hepatocyte ATP depletion and cytotoxicity induced by CAA. In addition, DTT stopped further CAA-induced ATP depletion if added up to 40 min after initial exposure to CAA. DTT also restored state IV respiration of isolated hepatocytes inhibited by the addition of CAA. The antidotal mechanisms of DTT may be 2-fold. First, it is possible that DTT stabilizes reversible Schiff bases formed between CAA and noncritical protein amino groups. DTT may also displace reversible adducts between critical mitochondrial thiols and CAA to form a cyclic, noncytotoxic mixed thioacetal. Cysteine and analogues such as penicillamine have also been used to prevent acetaldehyde-induced *in vivo* toxicity [27].

CAA caused calcium release from Ca^{2+}-loaded isolated mitochondria particularly if their GSH was depleted prior to addition of CAA. Furthermore, DTT prevented Ca^{2+} release, suggesting that mitochondrial toxicity could be attributed to reversible protein adduct formation with CAA. Calcium uptake is known to be regulated by an antiport that is noncompetitively inhibited by ruthenium red [28]. Ruthenium red delayed CAA toxicity in hepatocytes which suggests that calcium cycling may be an important event in CAA-induced mitochondrial toxicity. Taken together, these results further suggest that the primary cytotoxic lesion caused by CAA is mitochondrial damage which caused a decrease in hepatocyte ATP levels.

Lipid peroxidation as determined by MDA formation was induced by 0.5 mM CAA after a lag period of 60–90 min. The lag period was shorter at higher concentrations of CAA. Some lipid peroxidation occurred at subtoxic concentrations of CAA. Acetaldehyde-induced lipid peroxidation has been attributed to oxygen activation when xanthine oxidase utilises acetaldehyde as a substrate [29, 30]. Furthermore oxypurinol, a xanthine oxidase inhibitor, protected against CAA toxicity, suggesting that CAA is a substrate for xanthine oxidase and that superoxide radicals formed by the enzyme may be involved in CAA-induced lipid peroxidation. Another suggestion is that hepatocytes oxidise aldehydes to aldehyde peroxy radicals, peracids or

hydroxyl radicals, which initiate extensive lipid peroxidation [31].

CAA toxicity was delayed with antioxidants such as DPPD or iron chelators such as desferoxamine. GSH depletion was unaffected, which suggests that lipid peroxidation does not contribute to GSH depletion. Removal of oxygen also prevented lipid peroxidation and delayed cytotoxicity induced by CAA.

In summary, the CAA-induced loss in hepatocyte viability can be attributed to protein thiol depletion, ATP depletion and lipid peroxidation. ATP depletion may be initiated by reversible adducts formed with CAA and mitochondrial protein thiols. Dithiothreitol added some time after CAA restored protein thiols, hepatocyte respiration, stopped further ATP depletion, and prevented cytotoxicity. Preventing lipid peroxidation with antioxidants or desferoxamine delayed cytotoxicity without restoring protein thiols, hepatocyte respiration or stopping ATP depletion.

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