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Metabolic mechanisms of methanol/formaldehyde in isolated rat hepatocytes: Carbonyl-metabolizing enzymes versus oxidative stress

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ABSTRACT

Methanol (CH_3OH), a common industrial solvent, is metabolized to toxic compounds by several enzymatic as well as free radical pathways. Identifying which process best enhances or prevents CH_3OH-induced cytotoxicity could provide insight into the molecular basis for acute CH_3OH-induced hepatoxicity. Metabolic pathways studied include those found in 1) an isolated hepatocyte system and 2) cell-free systems. Accelerated Cytotoxicity Mechanism Screening (ACMS) techniques demonstrated that CH_3OH had little toxicity towards rat hepatocytes in 95% O_2, even at 2 M concentration, whereas 50 mM was the estimated LC_{50} (2 h) in 1% O_2, estimated to be the physiological concentration in the centrilobular region of the liver and also the target region for ethanol toxicity. Cytotoxicity was attributed to increased NADH levels caused by CH_3OH metabolism, catalyzed by ADH1, resulting in reductive stress, which reduced and released ferrous iron from Ferritin causing oxygen activation. A similar cytotoxic mechanism at 1% O_2 was previous found for ethanol. With 95% O_2, the addition of Fe(II)/H_2O_2, at non-toxic concentrations were the most effective agents for increasing hepatocyte toxicity induced by 1 M CH_3OH, with a 3-fold increase in cytotoxicity and ROS formation. Iron chelators, desferoxamine, and NADH oxidizers and ATP generators, e.g. fructose, also protected hepatocytes and decreased ROS formation and cytotoxicity. Hepatocyte protein carbonylation induced by formaldehyde (HCHO) formation was also increased about 4-fold, when CH_3OH was oxidized by the Fenton-like system, Fe(II)/H_2O_2, and correlated with increased cytotoxicity. In a cell-free bovine serum albumin system, Fe(II)/H_2O_2 also increased CH_3OH oxidation as well as HCHO protein carbonylation. Nontoxic ferrous iron and a H_2O_2 generating system increased HCHO-induced cytotoxicity and hepatocyte protein carbonylation. In addition, HCHO cytotoxicity was markedly increased by ADH1 and ALDH2 inhibitors or GSH-depleted hepatocytes. Increased HCHO concentration levels correlated with increased HCHO-induced protein carbonylation in hepatocytes. These results suggest that CH_3OH at 1% O_2 involves activation of the Fenton system to form HCHO. However, at higher O_2 levels, radicals generated through Fe(II)/H_2O_2 can oxidize CH_3OH/HCHO to form pro-oxidant radicals and lead to increased oxidative stress through protein carbonylation and ROS formation which ultimately causes cell death.

1. Introduction

Methanol (CH_3OH) is a common industrial solvent and chemical intermediate in the production of *t*-butyl methyl ether, glycol ethers, and so forth. Methanol is readily absorbed by ingestion, inhalation and dermal contact and rapidly distributed to tissues. Although accidental or intentional consumption is the most common means of poisoning, inhalation of vapor and dermal absorption are as effective as the oral route in generating acute toxic effects [1,2]. Methanol itself is a relatively non-toxic compound, rather its metabolism to reactive intermediates accounts for CH_3OH-induced toxicities, including metabolic acidosis and ocular toxicities observed in humans [3,4]. Co-exposure to ethanol or pyrazole compounds are utilized in poison control centres to significantly inhibit CH_3OH metabolism by acting as competitive inhibitors of alcohol dehydrogenases [5,6].

Thus far, investigators have demonstrated that CH_3OH metabolism to formaldehyde (HCHO) occurs through one of at least three separate pathways (Fig. 1). The first and second involve a common intermediate, H_2O_2, which may result from NADPH-dependent electron transfer, and can contribute to the direct oxidation of CH_3OH catalyzed by catalase or the Fenton reaction, involving ferrous iron and H_2O_2, to produce hydroxyl radicals ($^{\bullet}OH$), which may react spontaneously with CH_3OH to yield HCHO. The third significant pathway for CH_3OH metabolism involves oxidation via NAD^+ catalyzed by cytosolic alcohol dehydrogenase (ADH1) [7], which is predominantly located in the centrilobular region of the liver [8]. Due to its high reactivity and covalent bind-

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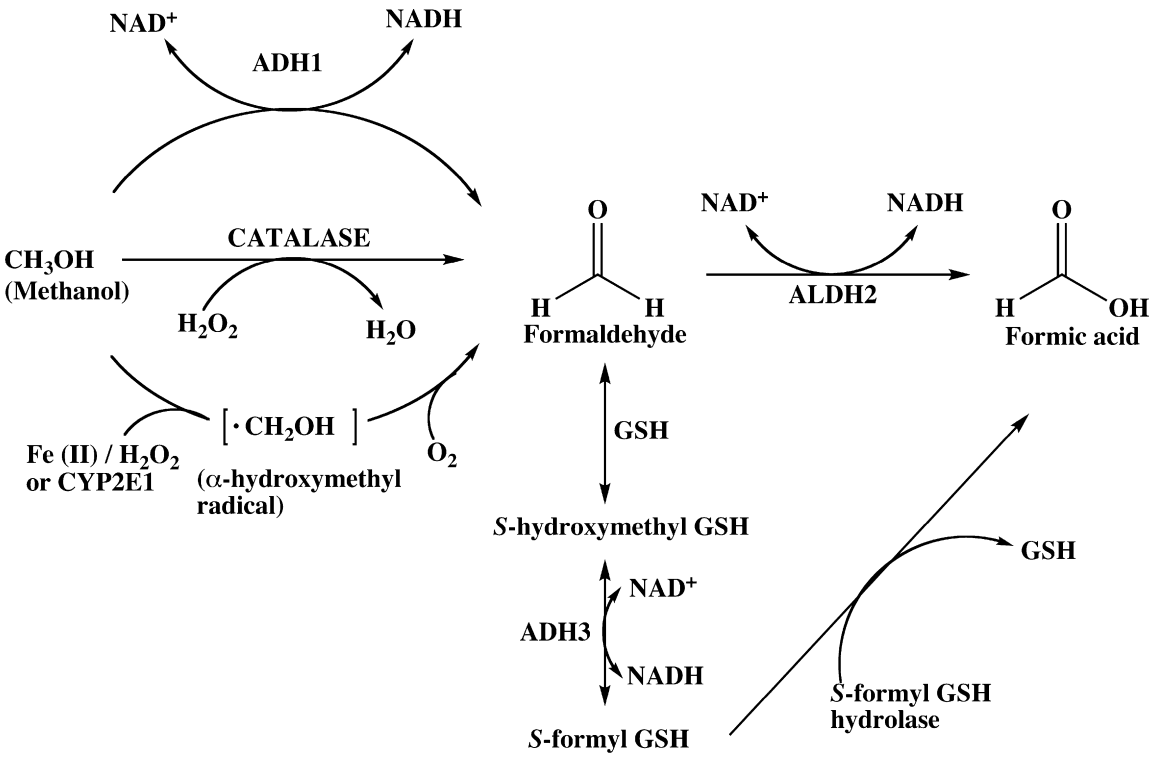

Fig. 1. The metabolism of methanol.

ing to proteins, lipids, RNA and DNA, HCHO have been implicated as a cause of carcinomas and is a class one human carcinogen [9,10]. HCHO toxicity as a consequence of exposure to CH_3OH-induced toxicity has been largely overlooked due to its rapid metabolism and its toxic metabolite, formate in humans. Formate is the product of HCHO oxidation by means of mitochondrial aldehyde dehydrogenase (ALDH2), however, in the cytosol, it is formed through a series of reactions involving glutathione (GSH)-dependent formaldehyde dehydrogenase (ADH3) [10]. Lastly, a multi-step pathway to CO_2 detoxifies formate; in all species studied, this detoxification is achieved through a tetrahydrofolate-dependent pathway [11,12].

Methanol metabolism occurs by several enzymatic and free radical pathways, however, these pathways have yet to be compared. Methanol or HCHO decreases antioxidant enzyme levels [13] and could contribute to hepatic injury associated with increased levels of serum aspartate aminotransferase and alanine aminotransferase in *in vivo* rat studies [14]. Attempting to identify which process best enhances or prevents CH_3OH-induced cytotoxicity could provide insight into the molecular basis for acute CH_3OH-induced hepatotoxicity in rodents. Previous research has shown that ethanol metabolism also increased hypoxia reoxygenation injury in isolated hepatocytes, as a result of increased NADH levels, increased cytosolic Fe release and increased reactive oxygen species (ROS) formation [15–17]. Also demonstrated was alcohol toxicity in the perfused liver under low oxygen supply and partial ischemia/reperfusion [18]. Alcoholic liver injury initiates and predominates in a section of the liver that is exposed to a lower oxygen concentration compared to the rest of the liver (zone III of the hepatic acinus) [19,20]. In rats, it has been demonstrated that there is no significant accumulation of blood formate after intraperioneally injecting rats with CH_3OH [21]. For this reason, it is important to also study HCHO toxicity, with respect to CH_3OH metabolism when using rat models. To better understand the potential mechanistic pathways involved in CH_3OH/HCHO hepatotoxicity in rodents, assays determining specific activities of enzymes involved in CH_3OH metabolism, specifically ADH1, ADH3, ALDH2, catalase and a Fenton-like system were performed using the Accelerated Cytotoxicity Mechanism Screening (ACMS) Technique using isolated rat hepatocytes at various oxygen concentrations [22].

2. Materials and methods

2.1. Chemicals

Type II collagenase was purchased from Worthington (Lakewood, NJ). N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) was purchased from Boehringer–Mannheim (Montreal, Canada). Methanol, formaldehyde, trichloroacetic acid (TCA), dinitrophenylhydrazine (DNPH) and all other chemicals were obtained from Sigma–Aldrich Corp. (Oakville, ON, CAN).

2.2. Animal treatment and hepatocyte preparation

Male Sprague–Dawley rats weighing 275–300 g (Charles River Laboratories) were used for experimental purposes carried out according to the guidelines of the Canadian Council on Animal Care [23]. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldeus and colleagues [24]. Isolated hepatocytes (10^6 cells/mL, 10 mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 mL round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 or 1% O_2, 94% N_2 and 5% CO_2 in a water bath of 37 °C for 30 min prior to the addition of chemicals.

2.3. Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the Trypan blue (0.1%,w/v) exclusion test [24]. Hepatocyte viability was determined every 30 min during a 3 h incubation period. Hepatocytes used were 80–90% viable before use. Fe(II)/H_2O_2 [2 μM Fe(II)/4 μM 8-

Table 1
Modulation of hepatocyte susceptibility to CH_3OH- and HCHO-induced protein carbonylation and cytotoxicity.

Treatment	Cytotoxicity (% Trypan blue uptake)			ROS formation (FI units)	Protein carbonylation (nmoles/10^6 cells)	HCHO concentration (mM)
Time (min)	60	120	180	90	90	90
Control (95% O_2)	19 ± 1	22 ± 1	26 ± 1	89	5.3 ± 0.1	0
+ 1 M CH_3OH	36 ± 2	43 ± 4	46 ± 3	122	6.3 ± 0.2	0
+ H_2O_2 generating system	35 ± 5	63 ± 2	72 ± 4	175	26.5 ± 4.2[b]	0.9 ± 0.1[b]
+ 0.5 mM Desferoxamine	36 ± 7	39 ± 6[c]	52 ± 8[c]	135	25.9 ± 3.2	0.8 ± 0.1
+ 0.25 mg/mL catalase	33 ± 2	40 ± 4[c]	43 ± 4[c]	121	27.4 ± 0.7[c]	1.0 ± 0.2[c]
+ Fenton-type system	58 ± 3[b]	77 ± 2[b]	100[b]	329	52.1 ± 0.9[b]	1.5 ± 0.2[b]
+ 0.5 mM Desferoxamine	34 ± 2[d]	41 ± 7[d]	46 ± 6[d]	146	30.7 ± 4.4[d]	0.8 ± 0.2
+ 10 mM Fructose	40 ± 4	59 ± 3[d]	69 ± 1[d]	481	27.2 ± 2.1[d]	0.8 ± 0.3
+ 4 mM HCHO	38 ± 4[a]	53 ± 5[a]	71 ± 6[a]	206	40.6 ± 5.1[a]	2.6 ± 0.3[a]
+ Fenton-type system	86 ± 4[e]	100[e]	100[e]	344	69.1 ± 7.2[e]	3.7 ± 0.3[e]
+ 0.5 mM Desferoxamine	38 ± 6[f]	48 ± 5[f]	53 ± 7[f]	110	11.2 ± 2.1[f]	0[f]
+ 10 mM Fructose	46 ± 3[f]	56 ± 4[f]	60 ± 5[f]	200	17.6 ± 3.4[f]	0.1 ± 0.1[f]
+ 4 mM sodium borohydride	37 ± 3[f]	45 ± 4[f]	57 ± 4[f]	149	9.9 ± 1.6[f]	0.1 ± 0.2[f]
Control (1% O_2)	27 ± 2	31 ± 2	37 ± 3			
+ 50 mM CH_3OH	39 ± 5[g]	47 ± 2[g]	52 ± 3[g]			NA[*]
+ 0.5 mM Desferoxamine	39 ± 5	43 ± 6	45 ± 1			NA[*]
+ 10 mM Fructose	20 ± 3[g]	34 ± 5[g]	35 ± 4[g]			NA[*]

H_2O_2 generating system: glucose 10 mM and glucose oxidase 0.5 U/mL. Fenton-type system: 2 μM Fe(II)/4 μM HQ/H_2O_2 generating system did not have any significant effect on protein carbonylation. All modulating chemicals were nontoxic concentrations and did not have any significant effect on protein carbonylation or ROS.

[*] NA: HCHO concentrations were undetectable with the method (NASH assay) used in this study. Means ± SE for three separate experiments are given.

[a] Significant as compared to control hepatocytes (95% O_2) ($P < 0.05$).

[b] Significant as compared to 1 M CH_3OH treated hepatocytes ($P < 0.05$).

[c] Significant as compared to 1 M CH_3OH + H_2O_2 generating system treated hepatocytes ($P < 0.05$).

[d] Significant as compared to 1 M CH_3OH + Fenton-type system treated hepatocytes ($P < 0.05$).

[e] Significant as compared to 4 mM HCHO treated hepatocytes ($P < 0.05$).

[f] Significant as compared to 4 mM HCHO + Fenton-type system treated hepatocytes ($P < 0.05$).

[g] Significant as compared to control hepatocytes (1% O_2) ($P < 0.05$).

hydroxyquinoline (HQ) + H_2O_2 generating system (10 mM glucose; 0.5 U/mL glucose oxidase)] was used as an iron-mediated Fenton-like system. GSH-depleted hepatocytes were obtained by preincubating the cells with 200 μM 1-bromoheptane for 30 min [25]. Alcohol dehydrogenase (ADH1)-inhibited hepatocytes were obtained by preincubating the cells with 100 μM 4-methylpyrazole for 30 min. Aldehyde dehydrogenase (ALDH2)-inhibited hepatocytes were obtained by preincubating cells with inhibitors (5 mM chloral hydrate, 100 μM disulfiram, 200 μM crotonaldehyde) for 15 min and 200 μM cyanamide for 45 min. The concentrations of inhibitors/modulators used were nontoxic.

2.4. Measurement of formaldehyde metabolism

Formaldehyde formation was determined colorimetrically according to the method by Nash [26]. Aliquots of hepatocyte suspension (1 mL) were withdrawn at different time points and trichloroacetic acid (TCA; 30%, w/v, 56 μL) were added to the samples to stop the reaction and lyse the cells. The mixture was centrifuged at 500 rpm for 1 min to precipitate the cells. The supernatant (500 μL) was added to an equal volume of NASH's reagent (mixture of 4 M ammonium acetate, 1 M acetic acid, 1 M acetyl acetone and Millipore water). Both intracellular and extracellular HCHO was measured. The solution was vortexed and incubated for 60 min at 37 °C with shaking. The formaldehyde levels of the mixture were determined at 412 nm using a SpectraMax Plus384.

2.5. Carbonylation assay

The total protein-bound carbonyl content was measured by derivatizing the protein carbonyl adducts with 2,4-dinitrophenylhydrazine (DNPH). An aliquot of hepatocyte suspension (0.5 mL) at different time points was added to an equivalent volume (0.5 mL) of 0.1% DNPH (w/v) in 2.0 N HCl and allowed to incubate for 1 h at room temperature. The reaction was terminated and the total cellular protein precipitated by the addition of an equivalent of 1.0 mL volume of TCA (20%, w/v). Cellular protein was pelleted by centrifugation at 10,000 rpm, and the supernatant was discarded. Excess unincorporated DNPH was extracted three times using an excess volume (0.5 mL) of ethanol:ethyl acetate (1:1) solution. Following extraction, the recovered cellular protein was dried under a stream of nitrogen and dissolved in 1 mL of Tris-buffered 8.0 M guanidine–HCl, pH 7.2. The resulting solubilized hydrazones were measured at 370 nm. The concentration of DNPH derivatized protein carbonyls was determined using an extinction coefficient of 22,000 M^{-1} cm^{-1} [27].

2.6. Reactive oxygen species (ROS) formation

Hepatocyte ROS formation was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes and is hydrolyzed to form a non-fluorescent dichlorofluorescin (DCF). DCF then reacts with 'ROS' to form the highly fluorescent dichlorofluorescein and effluxes the cell. ROS formation was assayed by withdrawing 1 mL samples, which were then centrifuged for 1 min at 50 × *g*. The cells were resuspended in Krebs–Heinseleit buffer and 1.6 μM DCFD was added. The cells were incubated at 37 °C for 10 min, and the fluorescence intensity was measured at 490 nm excitation and 520 nm emission wavelengths [28].

2.7. Statistical analysis

Statistical analysis was performed by a one-way ANOVA (analysis of variance) test, and employing Tukey's post hoc test to assess significance.

Table 2
Methanol induced bovine serum albumin protein carbonylation through oxidation by Fenton system and catalase.

Treatment	Protein carbonylation (μM)			HCHO concentration (mM)		
Time (min)	60	90	120	60	90	120
Control	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	0	0	0
+ 30 mM CH_3OH	5.5 ± 0.2	5.5 ± 0.2	5.5 ± 0.2	0	0	0
+ Fenton-type system	8.6 ± 0.1[a,b]	8.6 ± 0.1[a,b]	8.6 ± 0.1[a,b]	0.2 ± 0.1[a,b]	0.4 ± 0.1[b]	0.5 ± 0.1[a,b]
+ 0.05 mg/mL catalase/H_2O_2 generating system	7.0 ± 0.3[a,b]	7.0 ± 0.3[a,b]	7.0 ± 0.3[a,b]	0.6 ± 0.1[a,b]	0.8 ± 0.1[a,b]	1.0 ± 0.1[a,b]
+ 2 mM hydroxylamine	6.1 ± 0.3[c]	6.1 ± 0.3[c]	6.1 ± 0.3[c]	0[c]	0[c]	0[c]
+ 200 μM azide	6.0 ± 0.5[c]	6.0 ± 0.5[c]	6.0 ± 0.5[c]	0[c]	0[c]	0[c]

Agents were incubated with bovine serum albumin (2 mg/mL) in 0.1 mol/L phosphate buffer (pH 7.4) at 37 °C. HCHO assay (NASH assay) control: phosphate buffer solution 0.1 mol/L. Fenton-type system: 200 μM Fe(II)/200 μM EDTA/1 mM H_2O_2. Modulating chemicals did not have any significant effect on protein carbonylation.
[a] Significant as compared to control hepatocytes ($P < 0.05$).
[b] Significant as compared to 30 mM CH_3OH treated hepatocytes ($P < 0.05$).
[c] Significant as compared to 30 mM CH_3OH + catalase/H_2O_2 treated hepatocytes ($P < 0.05$).

3. Results

3.1. CH_3OH-induced cytotoxicity due to reductive stress (high cellular NADH) and protein oxidation

Methanol had little toxicity towards rat hepatocytes in 95% O_2 (carbogen) even at 2 M concentration (results not shown), whereas 50 mM CH_3OH was the estimated LC_{50} (at 2 h) at 1% O_2 (Table 1). Cytotoxicity was markedly increased by Fe(II)/H_2O_2, which were the most effective nontoxic agents for increasing the toxicity of 1 M CH_3OH in 95% O_2, an increase of approximately 3-fold. However, this cytotoxicity was inhibited by pretreatment (30 min preincubation) of hepatocytes with desferoxamine (Desferal), an iron chelator, in both the H_2O_2 generating system and the Fe(II)/H_2O_2 Fenton-like system. Desferoxamine also decreased the ROS formation. Fructose, an NADH oxidizer and ATP supplier, also protected CH_3OH-treated hepatocytes from Fe(II)/H_2O_2 toxicity. The amount of HCHO formed through CH_3OH metabolism in 1% O_2 was too low to measure by the NASH assay.

Protein carbonylation was increased 4-fold when hepatocytes were incubated with CH_3OH in the presence of Fe(II)/H_2O_2. Similar results were obtained with Fe(II)/H_2O_2 increasing HCHO cytotoxicity 2–4-fold, which correlated with hepatocyte protein carbonylation. The Schiff base reductant, sodium borohydride (equimolar concentration) rescued hepatocytes from HCHO-induced protein carbonylation by reducing the amount of intracellular HCHO.

The addition of catalase in the presence of a H_2O_2 generating system with CH_3OH treated hepatocytes increased HCHO formation and protein carbonylation approximately 4-fold, however, there was no increase in hepatocyte cytotoxicity, indicating the inability of catalase to permeate the hepatocyte and therefore acts extracellularly to catalyze CH_3OH oxidation by H_2O_2.

3.2. Bovine serum albumin protein carbonylation by CH_3OH oxidation to HCHO in vitro

Methanol induced bovine serum albumin protein carbonylation occurred through oxidation to HCHO in the presence of the Fenton reagent and catalase/H_2O_2 (Table 2). Ferrous iron and H_2O_2 increased protein carbonylation approximately 1.6-fold, whereas catalase/H_2O_2 only increased bovine serum albumin protein carbonylation approximately 1.3-fold. The increase in protein carbonylation of CH_3OH + Fe(II)/H_2O_2 is mostly due to HCHO and radicals as it had less HCHO formation compared to CH_3OH + catalase/H_2O_2. Catalase inhibitors hydroxylamine and azide both decreased bovine serum albumin protein carbonylation and the HCHO concentration to undetectable amounts.

Also demonstrated in Fig. 2, HCHO induced protein carbonylation was inhibited by an equimolar concentration of glutathione (GSH) added after 60 min of incubation. Glutathione conjugates are formed with HCHO thereby inhibiting protein carbonylation.

3.3. Cytotoxic pathways of HCHO metabolism in hepatocytes

As demonstrated in Table 1, the cytotoxicity of HCHO treated hepatocytes was significantly increased 2–4-fold in the presence of Fe(II)/H_2O_2. Cytotoxicity was further prevented by the iron chelator desferoxamine. Cytotoxicity was also inhibited by an equimolar concentration of sodium borohydride, a Schiff base carbonyl reductant.

Fig. 3a–c illustrates that the toxicity of 2.5 mM HCHO was greatly increased when metabolizing enzymes were inhibited. Inhibition of ALDH2 (Fig. 3a) by cyanamide, disulfiram or chloral hydrate generated the greatest HCHO-induced cytotoxicity as compared to ADH1 and ADH3 inhibition. The alcohol aversion therapy drug, cyanamide (active component in Temposil and Dipsan), had the greatest effect on cytotoxicity with an approximate 4-fold increase in toxicity, followed by chloral hydrate and disulfiram (Antabuse). A comparison of the effectiveness of the various enzyme inhibitors at inhibiting HCHO metabolism showed that ALDH2 is the major cytotoxic detoxifying pathway, followed by ADH3, and lastly ADH1 in isolated rat hepatocytes.

3.4. Inhibition of CH_3OH/HCHO metabolizing enzymes and the effect on hepatocyte protein carbonylation and HCHO metabolism

As shown in Fig. 4, isolated hepatocytes treated with HCHO and HCHO metabolizing enzyme inhibitors resulted in an increase

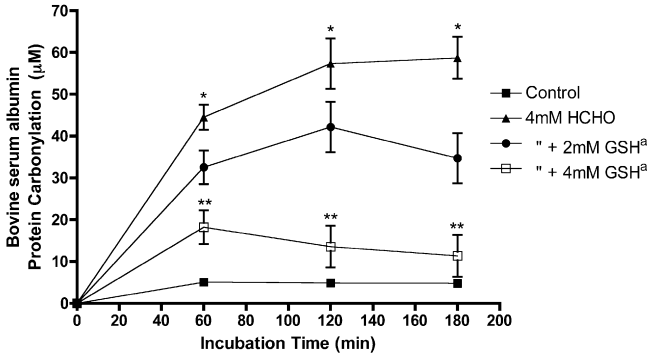

Fig. 2. Formaldehyde induced bovine serum albumin protein carbonylation *in vitro* and protection by glutathione Agents were incubated with bovine serum albumin (2 mg/mL) in 0.1 mol/L phosphate buffer (pH 7.4) at 37 °C. [a]GSH, glutathione, added 60 min following other agents. *Significant as compared to control ($P < 0.05$). **Significant as compared to 4 mM HCHO with bovine serum albumin (BSA) ($P < 0.05$).

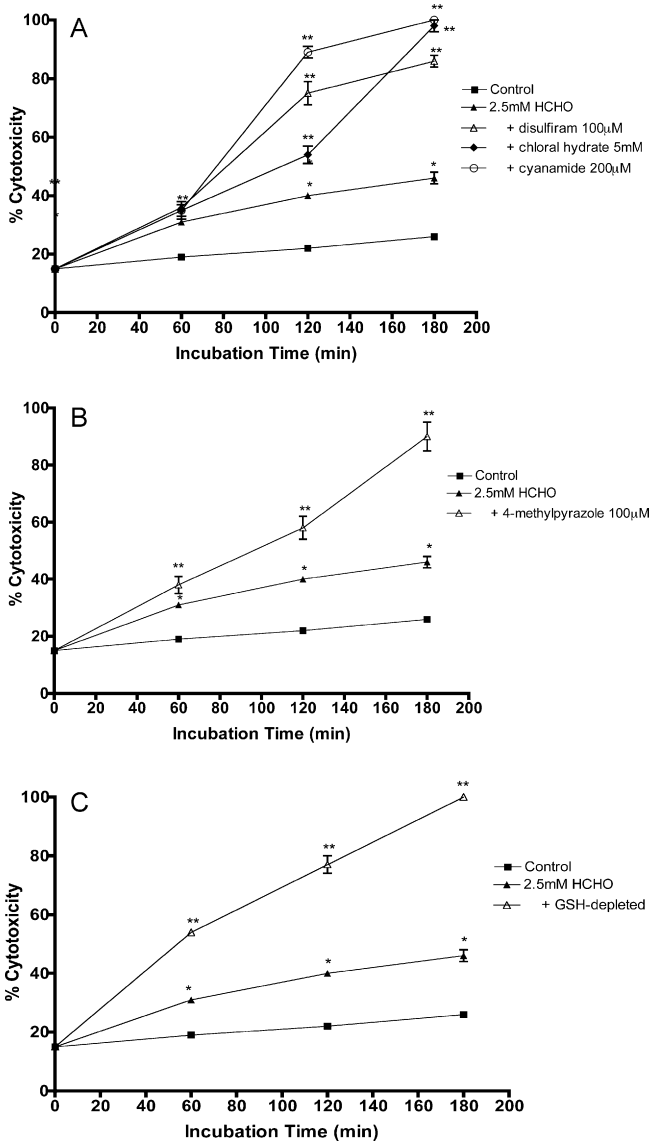

Fig. 3. Modulation of HCHO-induced cytotoxicity by inhibitors of the HCHO metabolizing enzyme systems in 95% O_2. (A) ALDH2 inhibitors; cyanamide, disulfiram, chloral hydrate, (B) ADH1 inhibitor; 4-methylpyrazole and (C) ADH3 inhibition; GSH-depleted hepatocytes and their effect on HCHO cytotoxicity. GSH-depleted hepatocytes were prepared by pre-incubation of hepatocytes for 30 min with 200 μM 1-bromoheptane before the addition of other agents. All modulating chemicals were nontoxic concentrations to hepatocytes. Means ± SE for three separate experiments are given. *Significant as compared to control hepatocytes ($P < 0.05$). **Significant as compared to 2.5 mM HCHO treated hepatocytes ($P < 0.05$).

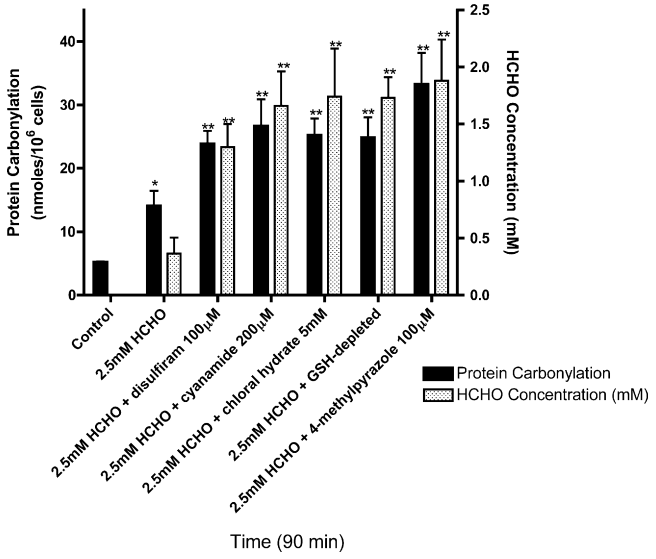

Fig. 4. Modulation of HCHO metabolism and protein carbonylation by inhibitors of the HCHO-metabolizing enzyme systems (90 min) in 95% O_2. GSH-depleted hepatocytes were prepared by pre-incubation of hepatocytes for 30 min with 200 μM 1-bromoheptane before the addition of other agents. All modulating chemicals had no significant effect on protein carbonylation. Means ± SE for three separate experiments are given. *Significant as compared to control hepatocytes ($P < 0.05$). **Significant as compared to 2.5 mM HCHO treated hepatocytes ($P < 0.05$).

in hepatocyte protein carbonylation, a marker of protein oxidation. Inhibition of ADH by competitive inhibitor 4-methylpyrazole, used as an antidote in CH_3OH or ethylene glycol poisoning, formed the greatest amount of protein carbonylation, with a 3-fold increase as compared to HCHO treated hepatocytes. Inhibition of ADH also prevented HCHO metabolism by 75.2% (1.88 mM HCHO un-metabolized). Increased HCHO concentrations correlated with increased HCHO-induced protein carbonylation in hepatocytes.

4. Discussion

The toxic effects observed with CH_3OH poisoning have been attributed to its metabolite, formate, formed by several enzymatic and free radical pathways. However, these pathways have yet to be compared in their ability to either detoxify or generate cytotoxicity in hepatocytes. Using the ACMS technique, this study investigated the molecular mechanisms involved in CH_3OH hepatotoxicity, utilizing assays to determine specific activities of enzymes involved in CH_3OH metabolism, specifically ADH1, ADH3, ALDH2, catalase and a Fenton-like system. Hepatic cytotoxicity was also determined at various O_2 concentrations.

In this study, CH_3OH cytotoxicity towards isolated rat hepatocytes occurred more readily at lower, more physiological O_2 levels, and was the most effective means for increasing toxicity. Hepatocyte susceptibility to CH_3OH at low O_2 suggests cytotoxicity was due to reductive stress, high NADH/NAD^+ ratio, caused by CH_3OH metabolism. Similar results were obtained in previous literature demonstrating that hepatocytes were much more susceptible to ethanol toxicity at lower O_2 concentrations (1%) as a result of its metabolism by alcohol/aldehyde dehydrogenases, which catalyzes NAD^+ reduction to NADH [17]. Normal cellular functions depend on a balanced redox environment [17]. Models of ischemia/hypoxia reperfusion injury have demonstrated that severe reductive stress develops leading to increased cellular NADH levels, which reduces Fe(III) in Ferritin thereby enabling Fe(II) to be released which reacts with H_2O_2 to form cytotoxic Fenton radicals [15,17]. Decreased O_2 levels of approximately 1% are the estimated physiological concentration in the centrilobular region of the liver, and the target where alcohol liver injury begins and predominates. Reductive stress and Fenton's reaction could occur *in vivo* as immune cells also release H_2O_2/Fe during inflammation [17].

The addition of Fe(II)/H_2O_2 were the most effective nontoxic agents for increasing the toxicity of 1 M CH_3OH in 95% O_2, suggesting that the toxicity of CH_3OH in the Fenton-type system is mainly due to HCHO formation and radicals generated or the presence of inflammation. The inflammatory model, H_2O_2 generating system, increased cytotoxicity 2-fold, while in the presence of Fe(II)/H_2O_2 there was a 3-fold increase. The protection *in vitro* by desferoxamine with nontoxic H_2O_2 generating system and CH_3OH treated hepatocytes suggests the involvement of iron in the oxidation of CH_3OH in this hepatocyte system.

In a cell-free system, CH_3OH induced bovine serum albumin protein carbonylation occurred through oxidation to HCHO in the presence of Fe(II)/H_2O_2 and catalase/H_2O_2, however levels of protein carbonyl content were lower, possibly due to the lower concentrations of HCHO formed. The increased protein carbonyl levels with CH_3OH + Fe(II)/H_2O_2 is mostly due to HCHO and radicals as it had less HCHO formation as compared to CH_3OH + catalase/H_2O_2. The lack of inhibition by catalase with hepatocytes could be because it does not permeate the hepatocyte and acts extracellularly. Catalase works well in a cell-free system, but not in hepatocytes as the catalase is located in peroxisomes and little is in the cytosol. Addition of extracellular catalase, therefore, had no effect on CH_3OH-induced hepatocyte cytotoxicity.

Formaldehyde induced bovine serum albumin protein carbonylation in a cell-free system was prevented with an equimolar concentration of glutathione (GSH) added after 60 min of incubation. It is likely that HCHO formed GSH conjugates, as GSH-depleted hepatocytes were found to be more susceptible to HCHO toxicity. Glutathione detoxifies HCHO enzymatically, as well as non-enzymatically [29,30], which prevents damage to proteins. Bovine serum albumin undergoes protein carbonylation by HCHO (Section 3.2); these levels were also increased significantly compared to levels observed with hepatocytes.

As shown in Fig. 3a–c, 2.5 mM HCHO toxicity was greatly increased when HCHO metabolizing enzymes were inhibited. The addition of 2.5 mM HCHO alone to hepatocytes was not cytotoxic, as it was further oxidized to formate, catalyzed by ALDH2, as well as reduced back to CH_3OH catalyzed by ADH1. When these metabolizing enzymes were inhibited, there was an accumulation of HCHO in the hepatocyte, demonstrated in Fig. 4, thus causing cytotoxicity. Inhibition of ALDH2 by cyanamide, disulfiram or chloral hydrate generated the most HCHO-induced cytotoxicity. The alcohol aversion therapy drug, cyanamide (active component in Temposil and Dipsan), had the greatest effect on cytotoxicity with an approximate 4-fold increase in toxicity, followed by chloral hydrate (at 3 h) and disulfiram (Antabuse). The ALDH2 inhibitor cyanamide was more effective than disulfiram at increasing HCHO most likely because cyanamide had higher efficacy than disulfiram. A comparison of the effectiveness of the various enzyme inhibitors at inhibiting HCHO metabolism shows that ALDH2 was the major cytotoxic detoxifying pathway in hepatocytes. Hepatocyte susceptibility to HCHO was also increased when hepatocyte GSH was depleted prior to HCHO addition; ADH3 is a GSH-dependent dehydrogenase, which detoxifies HCHO through conjugation, which ultimately forms formate. It should be acknowledged that hepatocytes were more susceptible to HCHO partly because GSH detoxifies H_2O_2 catalyzed by GSH peroxidase and GSH reductase.

4-Methylpyrazole, an ADH1 inhibitor and commonly used as an antidote in CH_3OH or ethylene glycol poisoning, also markedly increased formaldehyde cytotoxicity; this result confirms the role of ADH1 in HCHO reduction back to CH_3OH. However, the role of ADH1 in HCHO detoxification was not as significant as ALDH2 or GSH pathways.

Previous research has shown that the mechanisms of HCHO-induced toxicity in hepatocytes involves lipid peroxidation and mitochondrial toxicity [10]. Fig. 4 demonstrated that hepatocytes treated with HCHO and HCHO metabolizing enzyme inhibitors resulted in an increase in hepatocyte protein carbonylation, as seen with DNPH-derivatized protein carbonyl content. Competitive inhibition of ADH1 by methylpyrazole markedly increased protein carbonylation, with a 3-fold increase as compared to HCHO treated hepatocytes. Also observed was the highest concentration of HCHO, with only 24.8% HCHO metabolized. Various inhibitors of ALDH2 were also shown to inhibit HCHO metabolism, however to a lesser extent, followed by GSH depleted hepatocytes, which noticeably increased cytotoxicity, but had the least effect on HCHO metabolism. These results suggest that HCHO-induced toxicity in hepatocytes does not involve protein modification; nonetheless, increased HCHO concentrations correlated with increased HCHO-induced protein carbonylation in hepatocytes. Additionally, ADH1 was the main enzymatic pathway that prevents HCHO-induced protein carbonylation.

In this hepatocyte model, CH_3OH toxicity in 95% O_2 was only demonstrated if a Fenton-type system or a H_2O_2-generating system was present. However, when the O_2 content was decreased to 1%, CH_3OH toxicity occurred as a result of reductive stress. Several interventions, including iron chelators, fructose, or sodium borohydride decreased cytotoxicity. Previous studies from this lab [15–17] demonstrated that ethanol metabolism increased hypoxia reoxygenation injury in isolated hepatocytes, as a result of increased NADH levels, increased cytosolic Fe release and increased reactive oxygen species formation. The results presented suggest that reductive stress (high NADH:NAD^+ ratio) was caused by CH_3OH metabolism in hepatocytes catalyzed by alcohol/aldehyde dehydrogenases. The cytoprotective effects of desferoxamine against CH_3OH cytotoxicity suggest that CH_3OH caused intracellular release of free iron from Ferritin, which can then react in a Fenton-type reaction to increase the overall HCHO concentration and ultimately generate cytotoxicity and ROS formation, leading to cell death. Using Accelerated Cytotoxicity Mechanism Screening (ACMS) in hepatocytes under low versus high oxygen conditions, CH_3OH toxicity was increased by the following conditions/treatments: low oxygen concentration > Fenton-type system > H_2O_2-generating system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

[1] L. Fishbein (Ed.), Methanol. Environmental Health Criteria 196, World Health Organization, Geneva, 1997.
[2] M.B. Kadiiska, R.P. Mason, Acute methanol intoxication generates free radicals in rats: an ESR spin trapping investigation, Free Radic. Biol. Med. 28 (2000) 1106–1114.
[3] D. Jacobsen, K.E. McMartin, Methanol and ethylene glycol poisonings. MECHANISM of toxicity, clinical course, diagnosis, and treatment, Med. Toxicol. 1 (1986) 309–334.
[4] J. Liesivuori, H. Savolainen, Methanol and formic acid toxicity: biochemical mechanisms, Pharmacol. Toxicol. 69 (1991) 157–163.
[5] R. Blomstrand, H. Ostling-Wintzell, A. Lof, K. McMartin, B.R. Tolf, K.G. Hedstrom, Pyrazoles as inhibitors of alcohol oxidation and as important tools in alcohol research: an approach to therapy against methanol poisoning, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 3499–3503.
[6] G. Magnusson, J.A. Nyberg, N.O. Bodin, E. Hansson, Toxicity of pyrazole and 4-methylpyrazole in mice and rats, Experientia 28 (1972) 1198–1200.
[7] C. Harris, S.W. Wang, J.J. Lauchu, J.M. Hansen, Methanol metabolism and embryotoxicity in rat and mouse concept uses: comparisons of alcohol dehydrogenase (ADH1), formaldehyde dehydrogenase (ADH3), and catalase, Reprod. Toxicol. 17 (2003) 349–357.
[8] D. Meško, R. Pullmann, Blood-Plasma-Serum, in: V. Marks, D. Meško, T. Cantor (Eds.), Differential Diagnosis by Laboratory Medicine: A Quick Reference for Physicians, Springer-Verlag, Berlin Deidelberg, 2002, p. 52.
[9] R.K. Upreti, M.Y.H. Farooqui, A.E. Ahmed, G.A.S. Ansari, Toxicokinetics and molecular interaction of [^{14}C]-formaldehyde in rats, Arch. Environ. Contam. Toxicol. 16 (1987) 263–273.
[10] S. Teng, K. Beard, J. Pourahmad, M. Moridani, E. Easson, R. Poon, P.J. O'Brien, The formaldehyde metabolic detoxification enzyme systems and molecular

cytotoxic mechanism in isolated rat hepatocytes, Chem. Biol. Interact. 130–132 (2001) 285–296.
[11] M.A. Medinsky, D.C. Dorman, J.A. Bond, O.R. Moss, D.B. Janszen, J.I. Everitt, Pharmacokinetics of methanol and formate in female cynomolgus monkeys exposed to methanol vapors, Res. Rep. Health Eff. Inst. (June (77)) (1997) 1–30 (discussion 31–38).
[12] J.T. Eells, K.A. Black, C.E. Tedford, T.R. Tephly, Methanol toxicity in the monkey: effects of nitrous oxide and methionine, J. Pharmacol. Exp. Ther. 227 (1983) 349–353.
[13] E. Skrzydlewska, J. Farbiszewski, Diminished antioxidant defence potential of liver, erythrocytes and serum from rats with sub-acute methanol intoxication, Vet. Hum. Toxicol. 38 (1996) 429–433.
[14] N.J. Parthasarathy, R.S. Kumar, S. Manikandan, R.S. Devi, Methanol-induced oxidative stress in rat lymphoid organs, J. Occup. Health 48 (2006) 20–27.
[15] S. Khan, P.J. O'Brien, Modulating hypoxia-induced hepatocyte injury by affective intracellular redox state, Biochem. Biophys. Acta 1269 (1995) 153–156.
[16] N. Hossein, S. Khan, P.J. O'Brien, Hepatocyte injury resulting from the inhibition of mitochondrial respiration at low oxygen concentrations involves reductive stress and oxygen activation, Chem. Biol. Interact. 98 (1995) 27–44.
[17] S. Khan, P.J. O'Brien, Role of the cellular redox state in modulating acute ethanol toxicity in isolated hepatocytes, Clin. Biochem. 32 (1999) 585–589.
[18] M. Younes, H. Wagner, O. Strubelt, Enhancement of acute ethanol hepatoxicity under condition s of low oxygen supply and ischemia/reperfusion, Biochem. Pharmacol. 38 (1989) 3573–3581.
[19] H. De Groot, T. Noll, Oxygen gradients: the problem of hypoxia, Biochem. Soc. Trans. 15 (1987) 363–365.
[20] M. Kessler, H. Lang, E. Sinagowitz, Homeostasis of oxygen supply in liver and kidney, in: D.F. Bruley, H.J. Bicher (Eds.), O_2 Transport to Tissue Part A, 1973, pp. 351–360.
[21] K.E. McMartin, A.B. Makar, G. Martin-Amat, M. Palese, T.R. Tephley, Methanol poisoning. The role of formic acid in the development of metabolic acidosis in the monkey and the reversal by 4-methylpyrazole, Biochem. Med. 13 (1975) 319.
[22] P.J. O'Brien, A.G. Siraki, Accelerated cytotoxicity mechanism screening using drug metabolising enzyme modulators, Curr. Drug Metab. 6 (2005) 101–109.
[23] CCAC (Canadian Council on Animal Care), Guide to the Care and Use of Experimental Animals, Bradda Printing Services, ON, Canada, Ottawa, 1993.
[24] P. Moldeus, J. Hogberg, S. Orrenius, Isolation and use of liver cells, Methods Enzymol. 52 (1978) 60–71.
[25] S. Khan, P.J. O'Brien, 1-Bromoalkanes as new potent nontoxic glutathione depleters in isolated rathepatocytes, Biochem. Biophys. Res. Commun. 179 (1991) 436–441.
[26] T. Nash, The colourimetric estimation of formaldehyde by means of the Hantzsch reaction, Biochemistry 55 (1953) 416–421.
[27] D.P. Hartley, D.J. Kroll, D.R. Petersen, Prooxidant-initiated lipid peroxidation in isolated rat hepatocytes: detection of 4-hydroxynonenal- and malondialdehyde-protein adducts, Chem. Res. Toxicol. 10 (1997) 895–905.
[28] H. Possel, H. Noack, W. Augustin, G. Keilhoff, G. Wolf, 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation, FEBS Lett. 416 (1997) 175–178.
[29] S. Naylor, R.P. Mason, J.K. Sanders, D.H. Williams, G. Moneti, Formaldehyde adducts of glutathione. Structure elucidation by two-dimensional n.m.r. spectroscopy and fast-atom-bombardment tandem mass spectrometry, Biochem. J. 249 (1988) 573–579.
[30] R. Bateman, D. Rauh, K.M. Shokat, Glutathione traps formaldehyde by formation of a bicyclo[4.4.1]undecane adduct, Org. Biomol. Chem. 5 (2007) 3363–3367.