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OXYGEN DEPENDENCE OF HEPATOCYTE SUSCEPTIBILITY TO MITOCHONDRIAL RESPIRATORY INHIBITORS

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Abstract—Most zone 3 specific hepatotoxins or their metabolites are mitochondrial toxins, and yet the susceptibility of hepatocytes to respiratory inhibitors at the low O_2 concentrations found in zone 3 is not known. Potassium cyanide (CN) and antimycin A (AA) were found to be 5- and 2-fold more cytotoxic at 1% than at 95% O_2, respectively. CN also inhibited the respiration of hepatocytes 36% more at 1% O_2 than at 95% O_2; however, AA inhibited the respiration to the same level at 1% and 95% O_2. CN but not AA depleted ATP levels of hepatocytes more extensively at 1% than at 95% O_2. The CN-trapping agents dihydroxyacetone, glyceraldehyde, α-ketoglutarate and pyruvate prevented CN-induced cytotoxicity more effectively at 95% O_2 than at 1% O_2. In contrast, thiosulfate was less effective in preventing CN toxicity at 95% than at 1% O_2. Hepatocyte thiocyanate formation from CN and thiosulfate was much faster at 1% than at 95% O_2, suggesting that rhodanese, the mitochondrial enzyme that forms thiocyanate from CN and thiosulfate, is more effective at 1% O_2 than at 95% O_2.

Key words: cyanide; oxygen; hepatocytes; cytotoxicity; thiosulfate

CN† toxicity is believed to result predominantly from its reversible binding to cytochrome *c* oxidase, the terminal enzyme in the mitochondrial electron transport chain, which transfers reducing equivalents from cytochrome *c* to O_2 [1–3]. Inhibition of this enzyme decreases mitochondrial O_2 utilization leading to ATP depletion. Oxygen has often been recommended as a therapeutic adjunct in CN poisoning [4, 5], although the mechanisms involved are not understood. The administration of O_2 with the combination of sodium nitrite, thiosulfate and pyruvate, a CN trap, increases the LD_{50} of CN in mice [6–8] but does not increase significantly the protective effects of thiosulfate or the methemoglobin inducer sodium nitrite [7]. Recently, it has been shown that high O_2 concentrations in combination with the CN-trapping agents α-ketoglutarate or pyruvate prevent inhibition of isolated cytochrome *c* oxidase by CN [9], and it is concluded that O_2 displaces CN from cytochrome oxidase.

Liver injury resulting from hypoxia or alcoholism is localized in the centrilobular region. Alcoholic liver injury has, therefore, been attributed to alcohol-induced hypoxia [10] as this site (zone 3) is farthest from the blood supply where the PO_2 can be as low as 1 mm Hg [11].

In the present study, we have tested the hypothesis that hepatocytes may be more susceptible to mitochondrial respiratory inhibitors at low O_2 concentrations than at high O_2 levels. If so, this could contribute to proposed mechanisms attempting to explain the centrilobular location of many hepatotoxins. Because most of the zone 3 specific hepatotoxins and/or their metabolites are mitochondrial toxins, the susceptibility of hepatocytes to respiratory inhibitors at different levels of O_2 was tested. It was found that hepatocytes were 5-fold more susceptible to CN and 2-fold more susceptible to AA at 1% O_2 than at 95% O_2. The protective effects of CN-trapping agents and sodium thiosulfate at low versus high concentrations of O_2 were also studied. It was found that O_2 increased the protective effect of α-keto acids against CN in hepatocytes but decreased the protective effect of thiosulfate.

MATERIALS AND METHODS

Chemicals

Collagenase (from *Clostridium histoliticum*) and HEPES were purchased from Boehringer–Mannheim (Montreal, Canada). CN was purchased from the Fisher Scientific Co. (Toronto, Canada). α-Ketoglutaric acid, pyruvate, and glyceraldehyde were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Trypan blue, AA, and dihydroxyacetone were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of the highest commercial grade available.

Isolation and incubation of hepatocytes

Hepatocytes were prepared by collagenase perfusion of the liver of male Sprague–Dawley rats (body wt 300–350 g), fed *ad lib.* on a standard chow diet and tap water, as previously described by Moldeus *et al.* [12]. Routinely, 85–90% of the freshly isolated hepatocytes excluded trypan blue (trypan blue concentration: 0.2%, w/v). The cells were suspended in Krebs–Henseleit buffer containing 12.5 mM HEPES under an atmosphere of 95% O_2 and 5% CO_2, or 1% O_2, 94% N_2 and 5% CO_2, or 10% O_2, 85% N_2 and 5% CO_2 in continuously rotating round-bottomed 50-mL flasks at 37°. The final incubation volume was 10 mL with a concentration of 10^6 cells/mL. Hepatocytes were kept under relevant atmosphere for 30 min to achieve equilibrium between gas and liquid phases before addition of chemicals.

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† Abbreviations: CN, potassium cyanide; and AA, antimycin A.

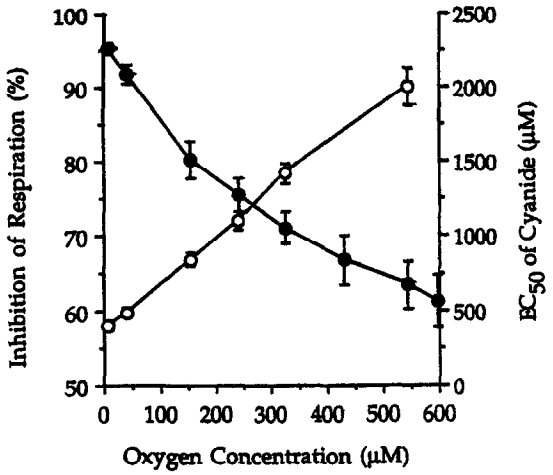


Fig. 1. Effects of oxygen concentration on inhibition of mitochondrial respiration and cytotoxicity induced by CN in isolated rat hepatocytes. For cytotoxicity (○) experiments, hepatocytes (10^6 cells/mL) were incubated with different concentrations of CN, and cell viability was measured at different time points as explained under Materials and Methods. For oxygen uptake experiments, 2 mL of hepatocytes (10^6 cells/mL) was placed in a special chamber as explained under Materials and Methods, and the oxygen level of the incubation mixture was measured in the absence and immediately after the addition of 400 μM CN (●). Each point is the mean ± SD of 3–6 separate experiments. Oxygen uptake of control hepatocytes was 12.3 ± 1.4 nmol O_2/min/10^6 cells.

Measurement of oxygen consumption

Hepatocyte respiration and the O_2 content of the incubation mixture were measured before and at different time points after the addition of CN to the hepatocytes with a Clark-type oxygen electrode (model 5300; Yellow-Spring Instrument Co., Inc., Yellow Spring, OH, U.S.A.) in a 2-mL incubation chamber maintained at 37°. Prior to oxygen consumption measurements, hepatocytes (10^6 cells/mL) were kept at 37° in Krebs–Henseleit buffer, plus HEPES (12.5 mM), pH 7.4, under 95% O_2 and 5% CO_2 or 1% O_2, 94% N_2 and 5% CO_2.

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, Milford, MA, U.S.A.) as previously described by Stocchi *et al.* [13].

Determination of thiocyanate

Thiocyanate formation from CN and thiosulfate in hepatocytes was measured according to the method described by Bowler [14]. Briefly, aliquots of hepatocytes incubated with CN and thiosulfate were taken at different time points, and proteins were precipitated with 5% trichloroacetic acid. After centrifugation, 1 mL of supernatant was added to 1 mL of ferric nitrate reagent (400 mM ferric nitrate in 1 N nitric acid), and the absorbance was measured at 470 nm.

Cell viability

The viability of hepatocytes was assessed by plasma membrane disruption as determined by the trypan blue (0.2%, w/v) exclusion test as well as by lactate dehydrogenase release [12]. Viability was examined immediately after isolation of hepatocytes and at different time points during the incubation.

Statistics

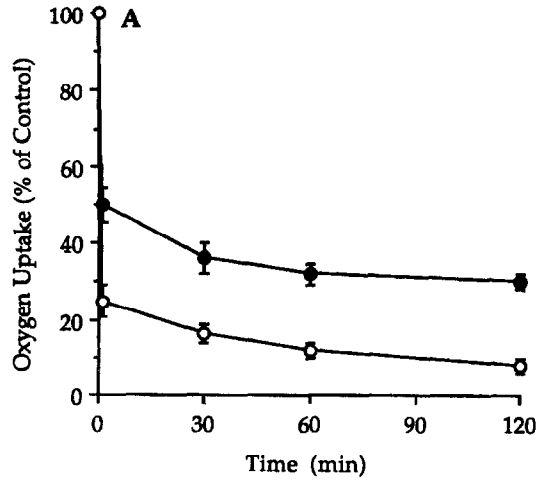
All values refer to means ± SD of at least three separate experiments. Statistically significant differences between control and experimental groups were obtained using Student's *t*-test where two groups were compared and one-way analysis of variance where more than two groups were compared. The minimal level of significance chosen was $P < 0.05$.

Table 1. Effects of oxygen levels on cytotoxicity of CN and AA in isolated rat hepatocyteAs

	Cytotoxicity (%)			
	Time (min)			
Compound	30	60	120	180
95% O_2				
None	12 ± 2	14 ± 2	16 ± 3	17 ± 3
CN, 0.4 mM	14 ± 2	17 ± 2	21 ± 2	25 ± 3
CN, 2 mM	17 ± 2	29 ± 3	51 ± 3	85 ± 4*
AA, 4 μM	14 ± 2	15 ± 2	22 ± 3	28 ± 3
AA, 10 μM	16 ± 2	31 ± 2	55 ± 3	91 ± 4*
1% O_2				
None	12 ± 2	14 ± 2	16 ± 3	17 ± 3
CN, 0.4 mM	18 ± 2	32 ± 3	56 ± 4	78 ± 5*
AA, 4 μM	17 ± 2	30 ± 3	52 ± 4	84 ± 5*

Freshly isolated hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 or 95% O_2 and 5% CO_2 as described under Materials and Methods. Cytotoxicity was determined as the percentage of cells that took up trypan blue. Values are expressed as means ± SD of 3–8 separate experiments.

* Significantly different from control untreated cells ($P < 0.001$).



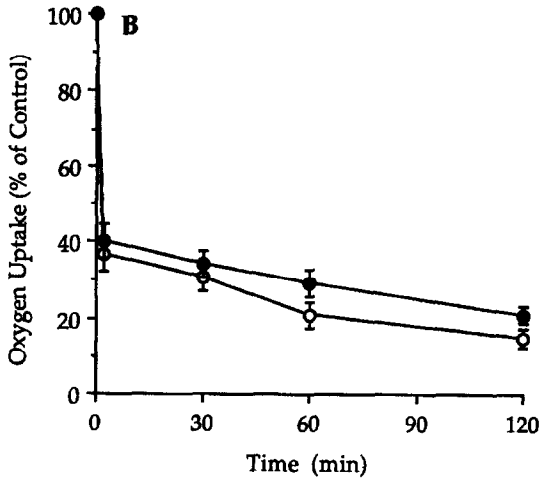


Fig. 2. Effects of oxygen levels on the rate and extension of inhibitory effects of CN or AA on hepatocyte respiration. (A) Hepatocytes (10^6 cells/mL) were incubated with 400 μM CN at 1% (○) or 95% O_2 (●), and respiration was measured at different time points using a Clark-type oxygen electrode as explained under Materials and Methods. (B) AA (4 μM) at 1% (○) or 95% (●) O_2. Other conditions were the same as for panel A. Each point is the mean ± SD of at least 3 separate experiments.

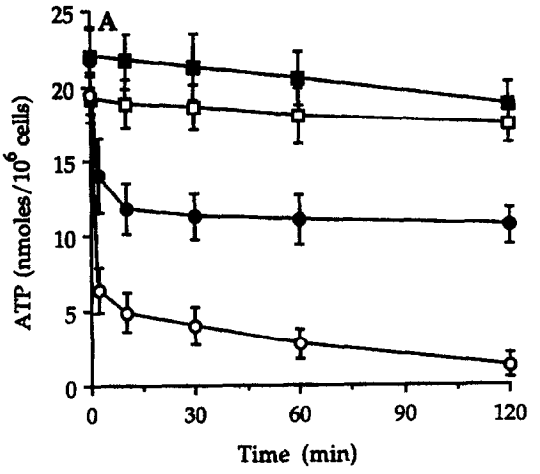
RESULTS

Effect of O_2 concentration on CN- or AA-induced cytotoxicity

Hepatocytes incubated under an atmosphere of 1% O_2 maintained their viability as well as hepatocytes maintained under 95% O_2 for at least 6 hr. However, hepatocytes incubated at 1% O_2 concentrations were 5-fold more susceptible to CN than hepatocytes incubated at high O_2 concentrations (Fig. 1), and 50% cytotoxicity (plasma membrane disruption) occurred at 2 hr with about 2 mM CN at 95% O_2 but with only 0.4 mM CN at 1% O_2 (Table 1). AA was only 2-fold more cytotoxic at 1% O_2 than at 95% O_2 (EC_{50} values for 2 hr at 95% and 1% O_2 were 10 and 4 μM, respectively) (Table 1).

Effects of CN or AA on hepatocyte respiration at different O_2 levels

Hepatocyte respiration was immediately inhibited upon addition of CN. However, respiration was more inhibited at lower concentrations of O_2 by the same concentration of CN (Fig. 1). CN (0.4 mM) inhibited about 90% of hepatocyte respiration at 1% O_2 (10.2 ± 1.1 μM O_2 in the incubation mixture) but inhibited only about 60% of respiration at 95% O_2 (590 ± 18 μM O_2) compared with hepatocyte respiration in the absence of CN. Although the effect of CN on hepatocyte respiration was immediate, hepatocyte respiration was followed up to 2 hr in order to correlate the inhibition of mitochondrial respiration with cytotoxicity and ATP depletion. As shown in Fig. 2A, the respiration of hepatocytes incubated for 2 hr with 0.4 mM CN under 1% O_2 was inhibited about 36% more than hepatocytes incubated under 95% O_2, and this difference remained constant dur-



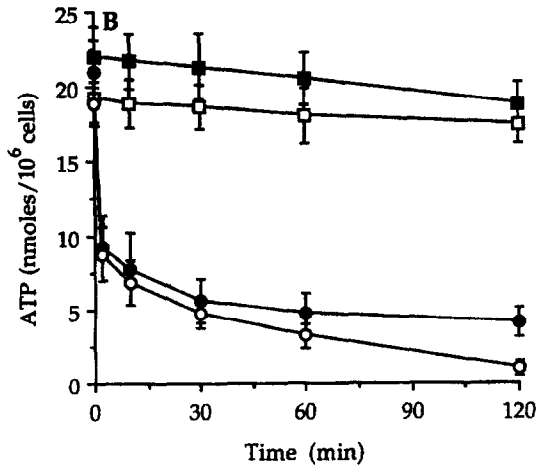


Fig. 3. Effects of 400 μM CN (A) or 4 μM AA (B) on ATP content of hepatocytes at 1% versus 95% O_2. Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer under 1% or 95% O_2. ATP was measured by an HPLC method as explained under Materials and Methods. Values are means ± SD of 3–5 separate experiments. Key: (■) control at 95% O_2, (□) control at 1% O_2, (○) CN or AA at 1% O_2, and (●) CN or AA at 95% O_2.

Table 2. Protective effects of CN-trapping compounds and sodium thiosulfate against CN toxicity at 95% versus 1% oxygen

Compound	Cytotoxicity (%)			
	Time (min)			
	30	60	120	180
95% O_2				
None	12 ± 2	14 ± 2	16 ± 3	17 ± 3
CN, 2 mM	17 ± 2	29 ± 3	51 ± 3	85 ± 4*
+ Dihydroxyacetone, 3 mM	13 ± 2	15 ± 2	19 ± 2	24 ± 3†
+ Glyceraldehyde, 3 mM	14 ± 2	15 ± 2	18 ± 2	25 ± 2†
+ α-Ketoglutarate, 3 mM	15 ± 2	18 ± 2	22 ± 2	29 ± 3†
+ Pyruvate, 3 mM	14 ± 2	16 ± 2	21 ± 2	31 ± 3†
+ Thiosulfate, 3 mM	17 ± 2	31 ± 2	57 ± 4	91 ± 5*
+ Thiosulfate, 10 mM	15 ± 2	19 ± 2	28 ± 3	32 ± 3†
1% O_2				
None	12 ± 2	14 ± 2	16 ± 3	17 ± 3
CN, 2 mM	34 ± 3	56 ± 4	90 ± 4*	100*
+ Dihydroxyacetone, 10 mM	20 ± 2	29 ± 2	64 ± 3‡	81 ± 4
+ Glyceraldehyde, 10 mM	19 ± 2	32 ± 3	59 ± 3‡	77 ± 4
+ α-Ketoglutarate, 10 mM	22 ± 3	36 ± 3	72 ± 4‡	86 ± 5
+ Pyruvate, 10 mM	20 ± 2	35 ± 3	67 ± 3‡	83 ± 4
+ Thiosulfate 3 mM	13 ± 2	15 ± 2	20 ± 2	21 ± 2†
+ Thiosulfate, 10 mM	14 ± 2	15 ± 2	18 ± 2	20 ± 2†

Freshly isolated hepatocytes (10^6 cells/mL) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 or 95% O_2 and 5% CO_2 as described under Materials and Methods. CN-trapping agents or thiosulfate were added to the incubation mixture at the same time as CN. Cytotoxicity was determined as the percentage of cells that took up trypan blue. Values are expressed as means ± SD of 3–5 separate experiments.

* Significantly different from control untreated cells ($P < 0.001$).

† Significantly different from CN-treated cells ($P < 0.001$).

‡ Significantly different from CN-treated cells ($P < 0.05$).

ing incubation. However, the levels of inhibition of hepatocyte respiration by AA were not different for hepatocytes incubated at 95% or 1% O_2 (Fig. 2B).

ATP depletion by CN or AA at different O_2 levels

Addition of CN or AA to the incubation mixture caused a rapid depletion of ATP content of hepatocytes (Fig. 3). At 1% of O_2, 0.4 mM CN depleted hepatocyte ATP to about 20% by 10 min and 12% by 60 min (Fig. 3A). However, at 95% O_2, 0.4 mM CN depleted hepatocyte ATP to about 50% of control by 10 min and did not change for the rest of the incubation period (Fig. 3A). AA at a concentration that caused 50% cytotoxicity at 2 hr (4 μM) under 1% O_2 depleted the hepatocyte ATP level to about 36% at 10 min and 25% at 30 min (Fig. 3B). However, although a similar depletion of hepatocyte ATP occurred in 95% O_2 with AA after 30 min, ATP continued to decrease at 1% O_2 but not at 95% O_2 (Fig. 3B).

Effects of O_2 concentrations on the cytoprotective effect of CN-trapping agents against CN

Dihydroxyacetone, glyceraldehyde, pyruvate and α-ketoglutarate prevent CN-induced cytotoxicity by trapping CN and restoring hepatocyte respiration [8, 15, 16]. We found that these CN-trapping agents prevented CN-induced cytotoxicity more effectively at 95% O_2 than at 1% O_2 (Table 2). Dihydroxyacetone or glyceraldehyde at 3 mM concentrations was required to prevent cytotoxicity of 2 mM CN at 95% O_2, whereas more than 10 mM was required to prevent cytotoxicity at 1% O_2. Similarly, 4–5 times more pyruvate or a α-ketoglutarate was needed at 1% O_2 than at 95% O_2 to prevent cytotoxicity of 2 mM CN.

Effects of O_2 concentrations on the cytoprotective effect of thiosulfate against CN

In contrast to CN-trapping agents, the CN antidote sodium thiosulfate was less effective in preventing CN-induced cytotoxicity in hepatocytes at 95% O_2 than at 1% O_2 levels. Indeed, at 95% O_2, the CN-trapping agents are more effective than thiosulfate in preventing CN toxicity [16]. However, at 1% O_2, thiosulfate was much more effective than the CN-trapping agents (Table 2), and 3 mM thiosulfate was more effective than 10 mM dihydroxyacetone, glyceraldehyde or α-ketoglutarate in preventing the cytotoxicity of 2 mM CN (Table 2).

The rate and level of thiocyanate formation from CN and thiosulfate by hepatocytes were measured as an index of CN detoxification by thiosulfate. Thiocyanate was formed much faster at 1% O_2 than at 95% O_2 (Fig. 4). Nearly all the CN (2 mM) added to the hepatocytes appeared as thiocyanate in less than 5 min at 1% O_2, whereas at 5 min only 25% of the CN was metabolized to thiocyanate at 95% O_2 (Fig. 4). About 1.5 equimolar concentrations of thiosulfate were needed for CN detoxification at 1% O_2, and the rate of thiocyanate formation was not affected significantly by thiosulfate concentration (Fig. 4A). However, the rate and maximum level of thiocyanate formed from CN and thiosulfate at 95% O_2 were dependent on the thiosulfate concentration (Fig. 4B). Addition of the CN-trapping agents dihydroxyacetone, α-ketoglutarate, or pyruvate to hepatocytes with CN and thiosulfate did not affect significantly thiocy-

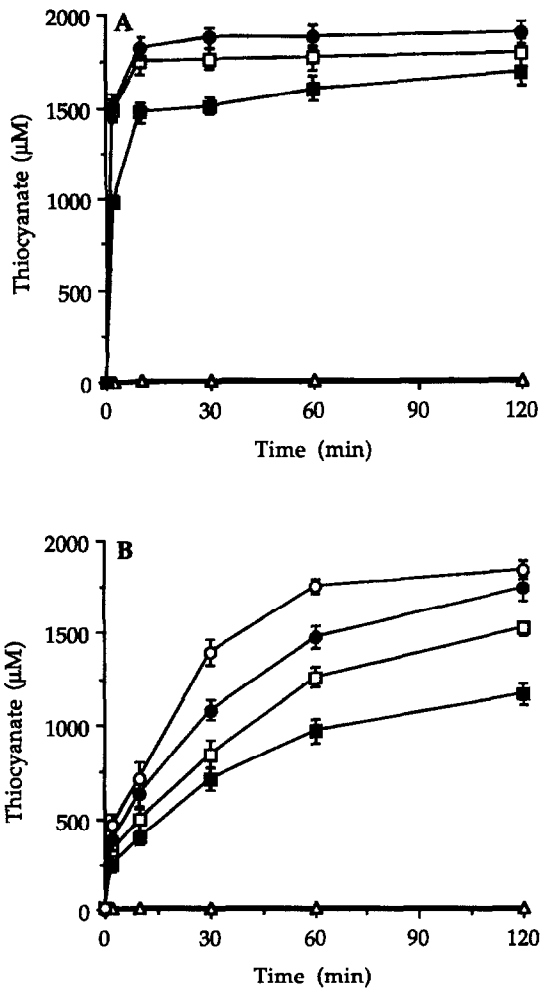


Fig. 4. Effects of oxygen levels on thiocyanate formation from CN and thiosulfate by hepatocytes. Hepatocytes (10^6 cells/mL) were incubated under 1% (A) or 95% (B) oxygen. CN was added first and thiosulfate last. Samples were taken at the marked time points following the addition of thiosulfate and tested for thiocyanate formation as explained under Materials and Methods. Values are means ± SD of 3–6 experiments. Key: (△) no addition or CN 2 mM, (■) CN 2 mM + thiosulfate 2 mM, (□) CN 2 mM + thiosulfate 3.5 mM, (●) CN 2 mM + thiosulfate 5 mM, and (○) CN 2 mM + thiosulfate 10 mM.

anate formation at 95% or 10% O_2 (data not shown) but slightly decreased the rate of thiocyanate formation at 1% O_2 (Fig. 5), suggesting that these CN-trapping compounds compete with thiosulfate at 1% O_2.

DISCUSSION

CN toxicity generally is believed to be the result of cytochrome oxidase inhibition [2, 3, 17], and oxygen is also known to be helpful in combination with some CN antidotes in preventing CN poisoning *in vivo* [6, 7]. However, the mechanism of the protective effect of O_2 against CN lethality is not known. One theory is that high O_2 concentrations result in an increase in the physically dissolved tissue O_2 tension, which then activates CN-insensitive respiratory pathways, and this pathway bypasses the CN blockade of tissue O_2 utilization [7]. Another theory could be that at high O_2 concentrations, cytochrome *c* oxidase is mostly in its fully oxidized form and does not bind CN as readily as the partially reduced $a^{2+} \cdot a^{3+}$ form [18, 19], which would be expected to predominate at a low oxygen concentration. The partially reduced form is believed to undergo a conformational change, which increases the accessibility of a_3 to CN [20, 21]. However, it has been shown that at the 1% O_2 concentration (10.2 μM O_2), used in our studies, the activity of cytochrome oxidase is 80% of that at 95% O_2 and the P_{50} value for oxidation-reduction of cytochrome oxidase is 3.2 μM O_2 [22].

In the present study, we have shown that hepatocytes were more susceptible to cyanide at 1% O_2 than at 95% O_2. Mitochondrial respiration also was inhibited more readily at 1% than at 95% O_2, suggesting that cytochrome oxidase is less inhibited by CN at high O_2 concentrations. Hepatocyte ATP levels also were depleted more readily by CN at 1% than at 95% O_2. Cytochrome oxidase, in its fully reduced state, forms a complex with a stoichiometry of one CN molecule per enzyme [23], and O_2 and CN are known to bind to the same site of cytochrome oxidase [24]. Our data suggest that CN competes with O_2 in binding to cytochrome oxidase; therefore, high O_2 levels prevent CN from binding to cytochrome oxidase, and CN is more effective at low O_2 concentrations. Recently, it has been suggested that O_2 displaces CN from cytochrome oxidase as the combination of high O_2 concentration and α-ketoglutarate or pyruvate effectively protects cytochrome oxidase against CN poisoning [9]. It also has been suggested that O_2 is able to oxidize the fully reduced cytochrome oxidase-CN complex and produce a partially reduced oxidase-CN complex [23], implying that O_2 either promotes CN dissociation or is able to oxidize the CN-bound cytochrome oxidase directly.

CN-trapping agents, which bind reversibly to free CN [8, 16, 25], were much more effective in preventing CN

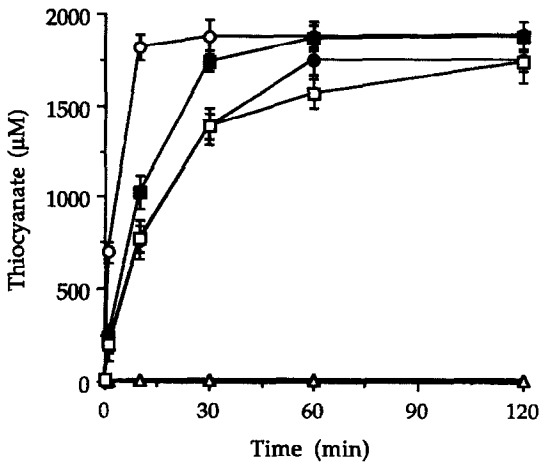


Fig. 5. Effects of CN-trapping agents on thiocyanate formation from CN and thiosulfate. Hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer at 1% O_2 and a 10 mM concentration of CN-trapping agents for 5 min before addition of thiosulfate. Key: (△) no addition, (○) 2 mM CN and 5 mM thiosulfate, (●) + 10 mM dihydroxyacetone, (■) + 10 mM α-ketoglutarate, or (□) + 10 mM pyruvate. Values are means ± SD of at least 3 experiments. Other conditions were the same as for Fig. 4.

toxicity and restoring hepatocyte respiration at high O_2 levels than at low O_2 levels. This suggests that CN is either displaced or loosened from its binding site at high O_2 levels, making it more available for CN-trapping agents that bind to CN, and further decreases the amount of free CN. This results in the pulling of the equilibrium between free CN and CN reversibly bound to cytochrome oxidase, resulting in the reactivation of cytochrome oxidase as CN is released from its binding site. The keto acid CN-trapping agents can permeate the hepatocyte membrane as we have shown that they normalized the lactate:pyruvate ratio and ATP levels of hypoxic [26] or AA-treated hepatocytes [27].

Other mechanisms may also be involved in the protective effects of oxygen on CN toxicity as O_2 also decreased, to a lesser extent, AA toxicity in hepatocytes. Thus, AA was 2-fold more toxic under 1% than under 95% O_2; however, the inhibition of hepatocyte respiration or the extent of ATP depletion by AA was similar under 1% and 95% O_2. This suggests that other mechanisms, in addition to ATP depletion, contribute to the cytotoxic mechanisms including compromising the cellular antioxidant defense mechanisms against reactive oxygen species [27]. Other hepatotoxic respiratory inhibitors were also more toxic under 1% than under 20% or 95% O_2 and could explain why hepatic necrosis is localized in the zone with lowest PO_2 [28].

Unlike the CN-trapping agents, thiosulfate was more effective in preventing CN toxicity in hepatocytes at low O_2 concentrations than at high O_2 levels. We found that CN was more effectively converted to thiocyanate by hepatocytes in the presence of thiosulfate at low O_2 levels, suggesting that rhodanese is less effective at high O_2 levels than at low O_2 levels. Previously, it was reported that rhodanese activity was similar in the brain or liver of mice treated with CN in air or oxygen atmospheres [29]. However, we found that rhodanese was more active at 1% or 10% O_2 than at 95% O_2. The inactive hepatocyte rhodanese found at 95% O_2 was reactivated by incubation of hepatocytes at 1% O_2 (data not shown), suggesting that inactivation of rhodanese by O_2 was reversible. The mechanism of inhibition of rhodanese at high O_2 concentrations is not known; however, it is possible that at high O_2 levels superoxide and hydrogen peroxide formation inactivates rhodanese [30].

This study, therefore, suggests that CN is much more toxic at low than at high O_2 concentrations probably because O_2 displaces CN from cytochrome *c* oxidase, which restores mitochondrial respiration and ATP levels. The CN-trapping agents dihydroxyacetone, glyceraldehyde, α-ketoglutarate, and pyruvate were more effective in preventing CN toxicity at high O_2 concentrations, whereas thiosulfate was more effective at low O_2 concentrations. The higher susceptibility of hepatocytes to AA at 1% O_2 than at 95% O_2, which was not accompanied by higher ATP depletion or inhibition of mitochondrial respiration, suggests that mechanisms other than ATP depletion may also contribute to the higher susceptibility of hepatocytes to mitochondrial respiratory inhibitors at low O_2 levels.

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