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Hepatocyte injury resulting from the inhibition of mitochondrial respiration at low oxygen concentrations involves reductive stress and oxygen activation

Hossein Niknahad, Sumsullah Khan, Peter J. O'Brien*

Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada, M5S 2S2

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Abstract

By correlating lactate/pyruvate ratios and ATP levels, cytotoxicity induced by the mitochondrial respiratory inhibitors or hypoxia:reoxygenation injury can be attributed not only to ATP depletion but also to reductive stress and oxygen activation. Thus hypoxia, cyanide or antimycin markedly increases reductive stress, non-heme Fe release and H_2O_2 formation in hepatocytes. Cytotoxicity was partly prevented with the ferric chelator desferoxamine, the xanthine oxidase inhibitor oxypurinol and the hydrogen peroxide scavenger glutathione. No lipid peroxidation could be detected and phenolic antioxidants had little effect. However, polyphenolic antioxidants or the superoxide dismutase mimics TEMPO or TEMPOL partly prevented cytotoxicity. Furthermore, increasing the hepatocyte NADH/NAD^+ ratio with NADH generating compounds such as ethanol, glycerol, or β-hydroxybutyrate markedly increased cytotoxicity (prevented by desferoxamine) and further increased the intracellular release of non-heme iron. Cytotoxicity could be prevented by glycolytic substrates (eg. fructose, dihydroxyacetone, glyceraldehyde) or the NADH utilising substrates acetoacetate or acetaldehyde which decreased the reductive stress and prevented intracellular iron release. These results suggest that liver injury resulting from insufficient respiration involves reductive stress which releases intracellular Fe, converts xanthine dehydrogenase to xanthine oxidase and causes mitochondrial oxygen activation. The cell's antioxidant defences are compromised and ATP catabolism contributes to oxygen activation.

Keywords: Cyanide; Antimycin A; Hypoxia:reoxygenation; ROS; Hepatocyte; Cytotoxicity

Abbreviations: CN, Cyanide; GSH, reduced glutathione; GSSG, oxidised glutathione; ROS, reactive oxygen species; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfinic acid; BPS, bathophenathroline disulfonate; NDGA, nordihydroguaiaretic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl.

*Corresponding author. Tel.: (416) 978 2716; Fax: (416) 978 8511.

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1. Introduction

Ischemic and hypoxic injury is of major clinical importance especially in organ transplantation and cardiac reperfusion. Poisoning with the mitochondrial respiratory inhibitor cyanide (CN) is a cause of death in many suicidal or homicidal attempts as well as in fire smoke inhalation [1,2]. However the cytotoxic mechanisms of either hypoxia or the mitochondrial respiratory toxins CN and antimycin A are not very well understood. One hypothesis is that membrane lysis occurs as a result of the activation of membrane phospholipases and proteases by cytosolic calcium [3,4]. The latter may be increased as a result of ATP depletion preventing normal cellular calcium homeostasis [5]. Another hypothesis is that cellular injury is caused by reactive oxygen species (ROS) generated on reoxygenation of ischemic tissue [6–8]. Under hypoxic conditions AMP catabolism results in the intracellular accumulation of hypoxanthine and xanthine. Xanthine dehydrogenase may also be converted to xanthine oxidase and form ROS on oxidising hypoxanthine and xanthine [9–11]. Recently, ROS has been implicated in cytotoxicity induced by chemical hypoxia caused by mixtures of cyanide (azide or myxothiazol) and the glycolytic inhibitor iodoacetate [12]. However, experiments using isolated mitochondria have shown that H_2O_2 is formed when respiration is blocked by antimycin as a result of the reaction of ubisemiquinone with O_2 [13]. Hepatocytes were more resistant to hypoxia:reoxygenation injury [14] than liver injury by ischemia:reperfusion so that the latter was attributed to ROS generated by Kupffer cells [15].

A marked increase in the lactate/pyruvate ratio an indicator of the free cytosolic NADH/NAD^+ ratio [16] occurs in hypoxic hepatocytes [17] or isolated perfused livers treated with cyanide [18]. It has also been proposed that an increase in the blood ketone-body ratio (β-hydroxybutyrate/acetoacetate ratio), an indicator of the free mitochondrial NADH/NAD^+ ratio [16] and redox potential should be used as an early indicator of hepatic failure [19].

In the following it is concluded that hepatocyte injury resulting from hypoxia:reoxygenation or respiratory inhibitors requires: (a) reductive stress which releases intracellular Fe and forms ROS, and (b) ATP depletion which compromises the cell's antioxidant system and supplies hypoxanthine/xanthine for xanthine dehydrogenase or xanthine oxidase.

2. Materials and methods

2.1. Chemicals

Collagenase (from *Clostridium histolyticum*), HEPES and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, PQ). Antimycin A, quercetin, TEMPOL, TEMPO, uric acid, GSH, β-hydroxybutyrate, oxypurinol, hypoxanthine, NDGA, luminol, digitonin and ellagic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Purpurogallin, caffeic acid, bathophenathroline disulfonate and trypan blue were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Desferoxamine was a gift from Ciba Geigy Canada Ltd. Other chemicals were of the highest purity grade commercially available.

2.2. Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (280–300 g) fed ad libitum on a standard chow diet and tap water. The cells were prepared by collagenase perfusion of the liver as previously described [20] and suspended (1×10^6 cells/ml) in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 12.5 mM HEPES in continuously rotating 50 ml round bottom flasks under an atmosphere of 1% O_2, 94% N_2 and 5% CO_2 for 30 min before addition of CN, antimycin and other agents. For experiments performed under hypoxic conditions, the cells were incubated for 40 min under an atmosphere of 95% N_2 and 5% CO_2 before addition of agents or cytoprotectants. The oxygen concentration in the cellular medium was <0.05% at the time that agents were added. Reoxygenation with 1% O_2, 94% N_2 and 5% CO_2 occurred after 90 min of hypoxia. GSH depleted hepatocytes were obtained by preincubation with 200 μM 1-bromoheptane as described [21].

2.3. Lactate and pyruvate assay

Lactic acid and pyruvic acid were measured in the whole aliquot with lactate dehydrogenase by following the reduction or oxidation of NADH respectively at 340 nm as described [22,23].

2.4. Iron release assay

Free iron (Fe^{2+}) in hepatocytes was detected by bathophenanthroline disulfonate (BPS) as described [24]. Aliquots of hepatocytes were taken at different time points and were filtered through 0.45 μm membrane filters after addition of 0.3% digitonin to permeablize the plasma membrane. BPS (1 mM) was added to 1 ml of filtrate and the absorbance was measured at 537 nm. Protein content of hepatocytes was measured according to the Lowry method.

2.5. Determination of reactive oxygen species

H_2O_2 formation was measured by a peroxidase luminol chemiluminescence method [25]. Chemiluminescence was measured using a Luminometer LB 9501-Berthold Lumat. Cyanide or antimycin A was added to 1 ml of hepatocytes (10^6 cells/ml) in Krebs-Henseleit buffer, pH 7.4 under air and in the presence of 5 units of horse-radish peroxidase and 1 mM luminol. Relative light unit (RLU) was measured every 12 s and data are expressed as RLU per second.

2.6. Determination of ATP

ATP was extracted from hepatocyte aliqouts using an alkaline extraction procedure and quanified by HPLC, using a C18 μBondapak reverse phase column (Waters Associates, Milford, MA) as previously described by Stocchi et al. [26].

2.7. Cell viability

The viability of hepatocytes were assessed by the trypan blue (0.2% w/v) exclusion test [20] as well as by the release of lactate dehydrogenase (LDH) [20]. However, since the results of trypan blue uptake test and LDH release were similar, we have reported the

Table 1
Modulation of cyanide or antimycin-induced cytotoxicity by alleviating reductive stress

Addition	% Cytotoxicity at time (min)			
	30	60	120	180
None	15 ± 2	16 ± 2	18 ± 3	20 ± 3
Cyanide 0.4 mM	18 ± 2	32 ± 3	56 ± 4	78 ± 5
+ Fructose 2 mM	15 ± 2	17 ± 2	21 ± 3	23 ± 3*
+ Dihydroxyacetone 5 mM	15 ± 2	16 ± 2	20 ± 2	22 ± 3*
+ Glycerol 10 mM	24 ± 3	49 ± 4	78 ± 4*	100*
+ β-Hydroxybutyrate 10 mM	22 ± 3	47 ± 3	80 ± 4*	100*
+ Acetoacetate 10 mM	18 ± 2	25 ± 3	36 ± 3	55 ± 4*
+ Ethanol 10 mM	22 ± 2	40 ± 3	69 ± 4	100*
+ Ethanol 10 mM + desferoxamine 1 mM	18 ± 2	23 ± 3	28 ± 3	39 ± 4*
Antimycin A 4 μM	19 ± 3	30 ± 3	52 ± 4	78 ± 6
+ Fructose 2 mM	17 ± 2	19 ± 2	22 ± 3	25 ± 3**
+ Dihydroxyacetone 5 mM	15 ± 2	17 ± 2	20 ± 2	23 ± 2**
+ Glycerol 10 mM	21 ± 2	46 ± 3	80 ± 4	100**
+ β-hydroxybutyrate 10 mM	24 ± 3	50 ± 3	82 ± 4	100**
+ Acetoacetate 10 mM	17 ± 2	22 ± 2	30 ± 3	38 ±4**
+ Ethanol 10 mM	23 ± 2	41 ± 3	77 ± 4	100**
+ Ethanol 10 mM + desferoxamine 1 mM	18 ± 2	22 ± 3	29 ± 4	40 ± 4**
+ Methylene blue 20 μM	18 ± 2	21 ± 2	28 ± 3	35 ± 3**

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 as described under Materials and methods. Cell viability was determined as the percentage of cells that took up trypan blue. Values are expressed as means of three to eight separate experiments ± SD.

*Significantly different from control CN ($P < 0.005$).

**Significantly different from control antimycin ($P < 0.005$).

results of trypan blue uptake test as the index of cell viability. Viability was examined immediately after the isolation of hepatocytes and during the incubation at various time points.

2.8. Statistics

Results are shown as means ± standard deviation (SD) of at least three different experiments with different batches of hepatocytes. The differences between control and experimental groups are examined with Analysis of Variance and significantly different results were chosen when $P < 0.05$.

3. Results

3.1. Modulating cytotoxicity by alleviating or increasing reductive stress

Cyanide and antimycin A toxicity towards isolated rat hepatocytes was dose dependent with 50% cytotoxicity occurring at about 2 h with 0.4 mM cyanide (CN) or 5 μM antimycin A under 1% oxygen (Table 1). Hypoxia:reoxygenation injury (50% cytotoxicity) required 90 min of hypoxia before reoxygenation with 1% O_2 for 30 min (Fig. 1). Cytotoxicity was considerably less at this time if hypoxia was maintained without reoxygenation (Fig. 1).

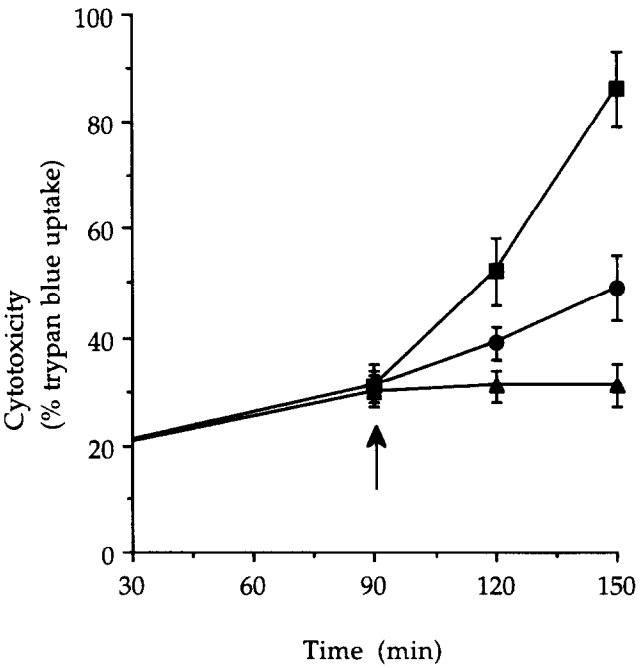

Fig. 1. Reoxygenation with 1% O_2 after hypoxia increased cytotoxicity but reoxygenation with 20% O_2 prevented cytotoxicity in isolated rat hepatocytes. Hepatocytes (10^6 cells/ml) were suspended in Krebs-Henseleit buffer, pH 7.4, at 37°C and under 95% N_2 and 5% CO_2 until the time of reoxygenation. The arrow shows the start of reoxygenation. Values are means of three separate experiments ± SD. (●) Hypoxia without reoxygenation, (■) reoxygenation with 1% O_2, (▲) reoxygenation with 20% O_2.

However, reoxygenation with 20% or 95% O_2 prevented cytotoxicity from occurring and longer periods of hypoxia were required (Fig. 1). Also higher concentrations of CN or antimycin A were required to cause cytotoxicity under 20% or 95% oxygen (data not shown).

Glycerol, β-hydroxybutyrate and sorbitol which increase the NADH/NAD^+ ratio in hepatocytes also markedly increased the susceptibility of hepatocytes to hypoxia:reoxygenation, CN or antimycin induced cytotoxicity (Tables 1and 2). However, the glycolytic nutrients fructose (Fig. 2A–C) or dihydroxyacetone which supply ATP without increasing the NADH/NAD^+ ratio were cytoprotective (Tables 1and 2). Cytoprotection by dihydroxyacetone against cyanide cytotoxicity was previously shown to involve cyanide trapping [27].

Cyanide or antimycin treatment increased the lactate concentration of the cell two fold indicating increased glycolysis. However, the lactate/pyruvate ratio was increased 3 to 4 fold (Table 3) indicating NADH generation. Cellular lactate/pyruvate ratio was measured to determine the free NADH/NAD^+ ratio [16]. Although lactate/pyruvate ratio correlated somewhat with the total NADH/NAD^+ ratio as measured by HPLC (data not shown), the results for lactate/pyruvate ratio are given so as to reflect the cytosolic

Table 2
Modulation of hypoxia: reoxygenation cytotoxicity by alleviating reductive stress

Addition	% Cytotoxicity at time (min)		
	90	120	150
Hypoxia/reoxygenation (1% O_2)	31 ± 4	52 ± 6	86 ± 8
+ Fructose 2 mM	18 ± 2	20 ± 2	21 ± 3*
+ Dihydroxyacetone 5 mM	17 ± 2	19 ± 2	23 ± 3*
+ Sorbitol 10 mM	40 ± 4	71 ± 4	100*
+ β-Hydroxybutyrate 10 mM	38 ± 3	75 ± 4	100*
+ Acetoacetate 10 mM	25 ± 3	36 ± 3	43 ± 4*

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under an atmosphere of 95% N_2, 5%CO_2 for 90 min and reoxygenated with 1% O_2, 94% N_2, 5% CO_2 as described under Materials and methods. Cell viability was determined as the percentage of cells that took up trypan blue. Values are expressed as means of at least three different experiments ± SD.

*Significantly different from hypoxia/reoxygenation ($P < 0.001$).

free NADH/NAD^+ ratios. As shown in Tables 1 and 3, the cellular NADH generators ethanol, glycerol or sorbitol further increased the lactate/pyruvate ratio which further increased cytotoxicity (Tables 1 and 3). On the other hand, the cellular NADH oxidant acetoacetate or the artificial electron acceptor methylene blue normalised the lactate/pyruvate ratio of antimycin treated cells (Tables 1 and 3) and prevented cytotoxicity.

Table 3
Modulation of cytosolic redox state (lactate/pyruvate) of isolated rat hepatocytes treated with cyanide or antimycin A

Addition	Lactate ($nmol/10^6$ cells)		Pyruvate ($nmol/10^6$ cells)		Lactate/pyruvate ratio	
	30 min	60 min	30 min	60 min	30 min	60 min
None	660	641	70	58	9.4	10.9
Cyanide 0.4 mM	1342	1432	40	35	33.6	40.9
+ Ethanol 20 mM	1211	1230	27	24	44.9	51.3
+ Glycerol 10 mM	1147	1316	29	23	39.6	57.2
+ Fructose 10 mM	2185	2795	156	224	14	12.5
+ Acetoacetate 10 mM	1732	2166	97	112	17.8	19.3
Antimycin A 4 μM	1327	1467	42	41	31.6	35.8
+ Ethanol 20 mM	1203	1265	32	24	37.6	52.7
+ Glycerol 10 mM	1184	1237	21	16	56.4	77.3
+ Fructose 10 mM	1771	2560	131	187	13.5	13.7
+ Acetoacetate 10 mM	1639	2203	178	148	9.2	14.9
+ MB[a] 20 μM	560	615	29	53	19.3	11.6

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2. Lactate and pyruvate were determined using lactate dehydrogenase. Values are expressed as mean of two different experiments.

[a] Methylene blue.

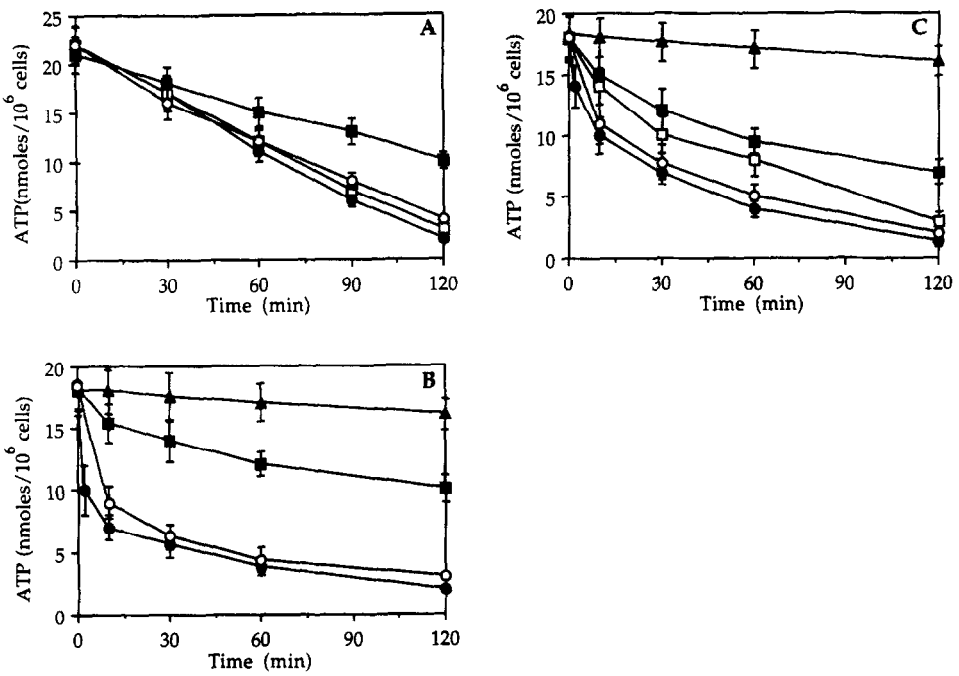

Fig. 2. Modulation of hypoxia:reoxygenation-, cyanide-, or antimycin-induced ATP depletion by various glycolytic, NADH generating or NADH oxidising nutrients in isolated rat hepatocytes. ATP was measured as explained in Materials and methods. Reoxygenation was with 1% O_2 after 90 min of hypoxia. Values are expressed as mean ± SD of at least three different experiments. (A): (●) hypoxia:reoxygenation, (■) + fructose 10 mM, (O) + acetoacetate 10 mM, (□) + glycerol 10 mM; (B): (▲) control 1% O_2, (●) cyanide 0.4 mM, (■) + fructose 10 mM, (O) + acetoacetate 10 mM; (C): (▲) control 1% O2, (●) antimycin A 4 μM, (■) + fructose 10 mM, (O) + acetoacetate 10 mM, (□) + sorbitol 10 mM.

However, acetoacetate did not restore ATP depletion induced by hypoxia, CN or antimycin (Fig. 2A–C). Fructose, a glycolytic nutrient, also normalized the lactate/pyruvate ratio (Table 3) but only partially delayed ATP depletion (Fig. 2) although cytotoxicity was prevented (Tables 1–3).

3.2. Cytoprotection by polyphenolic antioxidants

Hypoxia:reoxygenation injury was effectively prevented by the superoxide dismutase mimics TEMPO and TEMPOL and the polyphenolic antioxidants purpurogallin, caffeic acid, quercetin and NDGA (Table 4). CN or antimycin toxicity was also prevented by quercetin, uric acid, NDGA, TEMPO and TEMPOL (Tables 5 and 6). The phenolic antioxidants BHA and BHT did not affect antimycin cytotoxicity or hypoxia: reoxygenation injury (Tables 4 and 6), however, they slightly protected hepatocytes against cyanide toxicity (Table 5). No malondialdehyde formation was detected indicating that lipid peroxidation was not detectable by this method.

Table 4
Modulation of hypoxia:reoxygenation cytotoxicity by antioxidants, desferoxamine, GSH or oxypurinol

Addition	% Cytotoxicity at time (min)		
	90	120	150
Hypoxia/reoxygenation (1% O_2)	31 ± 4	52 ± 6	86 ± 8
+ Desferoxamine 1 mM	19 ± 3	25 ± 3	56 ± 5[a]
+ GSH 2 mM	20 ± 2	23 ± 3	58 ± 5[a]
+ Quercetin 100 μM	19 ± 2	22 ± 3	58 ± 4[a]
+ TEMPO 100 μM	22 ± 3	36 ± 4	51 ± 5[a]
+ TEMPOL 300 μM	21 ± 2	33 ± 4	48 ± 4[a]
+ Caffeic acid 300 μM	22 ± 3	25 ± 4	36 ± 6[a]
+ Purpurogallin 100 μM	21 ± 3	27 ± 4	34 ± 6[a]
+ Ellagic acid 300 μM	22 ± 2	24 ± 3	50 ± 4[a]
+ Ascorbate 300 μM	24 ± 3	28 ± 3	34 ± 4[a]
+ BHA 50 μM	32 ± 4	53 ± 6	76 ± 8[a]
+ BHT 30 μM	29 ± 4	57 ± 5	78 ± 8[a]
+ Oxypurinol 50 μM	18 ± 2	22 ± 3	40 ± 4[a]
+ Hypoxanthine 5 mM	53 ± 4	83 ± 5	100[a]
+ GSH depleted hepatocytes[b]	33 ± 3	95 ± 5	100[a]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under an atmosphere of 95% N_2, 5% CO_2 for 90 min and reoxygenated with 1% O_2, 94% N_2, 5% CO_2 where indicated as described under Materials and methods. Cell viability was determined as the percentage of cells taking up trypan blue. Values are expressed as means of at least three different experiments ± SD.

[a] Significantly different from hypoxia/reoxygenation ($P < 0.005$).

[b] GSH depleted hepatocytes were obtained as described in Materials and methods.

3.3. Cytoprotection by desferoxamine and GSH

Desferoxamine (1 mM) markedly delayed injury by hypoxia:reoxygenation (Table 4), CN (Table 5) and antimycin (Table 6). GSH (1–2 mM) also partially delayed hypoxia or chemical hypoxia cytotoxicity (Tables 4–6). On the other hand, depleting hepatocyte GSH beforehand made the hepatocytes much more sensitive to hypoxia:reoxygenation (Table 4), CN (Table 5) or antimycin (Table 6).

3.4. Involvement of xanthine dehydrogenase/oxidase in cytotoxicity

The xanthine oxidase inhibitor oxypurinol also protected hepatocytes from CN, antimycin or hypoxia:reoxygenation injury (Tables 4–6). Furthermore, the addition of hypoxanthine increased cytotoxicity in all cases which was prevented by oxypurinol (Tables 4–6).

3.5. Effect of ethanol on hypoxia:reoxygenation or chemical hypoxia toxicity

Increasing the hepatocyte cytosolic NADH/NAD^+ ratio with ethanol also markedly increased hepatocyte susceptibility to hypoxia:reoxygenation, CN or antimycin (Tables 1 and 7). This increased injury by ethanol was prevented by desferoxamine (Tables 1 and 7) which suggests that the increased reductive stress caused increased release of iron from protein bound stores. As shown in Table 7 the increase in hypoxia:reoxygenation toxicity by ethanol was prevented by oxypurinol, desferoxamine, TEMPO and quercetin. However,

Table 5
Modulation of cyanide-induced cytotoxicity by antioxidants, GSH, chelating iron or oxypurinol

Addition	% Cytotoxicity at time (min)			
	30	60	120	180
None	15 ± 2	16 ± 2	18 ± 3	20 ± 3
Cyanide 0.4 mM	18 ± 2	32 ± 3	56 ± 4	78 ± 5
+ Desferoxamine 1 mM	19 ± 2	21 ± 2	22 ± 2	32 ± 3[a]
+ GSH 1 mM	20 ± 2	23 ± 2	26 ± 3	37 ± 3[a]
+ Quercetin 100 μM	19 ± 2	24 ± 3	35 ± 4	49 ± 5[a]
+ TEMPO 100 μM	20 ± 2	22 ± 3	37 ± 4	58 ± 4[a]
+ TEMPOL 300 μM	18 ± 2	22 ± 3	35 ± 3	52 ± 4[a]
+ Caffeic acid 300 μM	20 ± 3	26 ± 3	41 ± 5	63 ± 5
+ NDGA 30 μM	19 ± 2	22 ± 3	28 ± 4	37 ± 4[a]
+ Uric acid 300 μM	21 ± 2	23 ± 3	33 ± 4	56 ± 4[a]
+ BHA 50 μM	19 ± 3	23 ± 3	28 ± 4	38 ± 5[a]
+ BHT 30 μM	20 ± 2	27 ± 4	35 ± 5	49 ± 6[a]
+ Oxypurinol 50 μM	18 ± 2	20 ± 2	23 ± 2	31 ± 3[a]
+ Hypoxanthine 1 mM	28 ± 3	56 ± 4	87 ± 5	100[a]
+ Hypoxanthine 1 mM + oxypurinol 50 μM	23 ± 3	34 ± 3	51 ± 4	66 ± 5[b]
+ GSH depleted hepatocytes	24 ± 2	38 ± 3	74 ± 4	100[a]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 as described under Materials and methods. Cell viability was determined as the percentage of cells that took up trypan blue. Values are expressed as means of three to eight separate experiments ± SD.

[a] Significantly different from control CN ($P<0.005$)

[b] Significantly different from CN + hypoxanthine ($P < 0.005$).

GSH depleted hepatocytes were obtained as described in Materials and methods. GSH depletion did not affect the viability of control untreated hepatocytes.

no malondialdehyde formation was detected and the phenolic antioxidants BHA and BHT did not affect cytotoxicity.

3.6. Iron release from intracellular stores by cyanide or antimycin A

Cyanide or antimycin A caused the intracellular release of free iron which doubled hepatocyte free iron levels (Fig. 3A,B). Increasing the reductive stress with ethanol increased the amount of iron released from intracellular stores and increased the susceptibility of hepatocytes to CN or antimycin (Fig. 3A,B). Similar results also were observed with the NADH generators sorbitol or glycerol (data not shown). Ofsetting the reductive stress with fructose (Fig. 3A,B) or dihydroxyacetone (data not shown), on the other hand, inhibited the release of iron by cyanide or antimycin A.

3.7. Formation of reactive oxygen species by cyanide or antimycin A

Cyanide or antimycin A markedly increased H_2O_2 formation by hepatocytes as determined by peroxidase-catalysed luminol chemiluminescence (Fig. 4). Addition of CN to hepatocytes immediately increased chemiluminescence from ~25 RLU/s to ~80 RLU/s which decreased to a plateau level of about 47 RLU/s after 4 min (Fig. 4). The peak of

Table 6
Modulation of antimycin A-induced cytotoxicity by polyphenolic antioxidants, desferoxamine, GSH or oxypurinol

Addition	% Cytotoxicity at time (min)			
	30	60	120	180
None	14 ± 2	16 ± 2	19 ± 3	22 ± 4
Antimycin A 4 μM	19 ± 3	30 ± 3	52 ± 4	78 ± 6
+ Desferoxamine 1 mM	19 ± 2	22 ± 3	25 ± 3	50 ± 4[a]
+ GSH 1 mM	17 ± 2	19 ± 2	23 ± 2	47 ± 4[a]
+ Quercetin 200 μM	23 ± 3	25 ± 3	30 ± 4	53 ± 5[a]
+ TEMPO 100 μM	23 ± 2	29 ± 3	35 ± 4	49 ± 6[a]
+ TEMPOL 300 μM	20 ± 2	26 ± 3	33 ± 3	46 ± 5[a]
+ NDGA 30 μM	22 ± 2	24 ± 2	38 ± 3	50 ± 4[a]
+ Uric acid 300 μM	21 ± 3	30 ± 2	37 ± 3	55 ± 4[a]
+ BHA 50 μM	21 ± 2	34 ± 4	50 ± 3	76 ± 5
+ BHT 30 μM	23 ± 3	30 ± 3	54 ± 4	80 ± 6
+ Oxypurinol 50 μM	18 ± 2	21 ± 2	26 ± 3	53 ± 4[a]
+ Hypoxanthine 5 mM	26 ± 3	43 ± 4	92 ± 5	100[a]
+ Hypoxanthine 5 mM + Oxypurinol 50 μM	23 ± 3	30 ± 4	49 ± 4	66 ± 5[b]
+ GSH depleted hepatocytes	27 ± 2	48 ± 3	83 ± 4	100[a]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under 1% O_2, 94% N_2 and 5% CO_2 as described under Materials and methods. Cell viability was determined as the percentage of cells that took up trypan blue. Values are expressed as means of three to eight different experiments ± SD.

[a] Significantly different from antimycin alone ($P < 0.005$).

[b] Significantly different from antimycin + hypoxanthine ($P < 0.005$).

GSH depleted hepatocytes were obtained as described under Materials and methods. GSH depletion did not affect the viability of control untreated cells.

chemiluminescence emission after addition of antimycin was lower than that of CN and reached a plateau level of 35 RLU/s after 2 min (Fig. 4).

4. Discussion

It is generally believed that ATP depletion is a major factor in the molecular mechanism of cytotoxicity that results if mitochondrial respiration is prevented e.g. using hypoxic conditions or inactivating cytochrome oxidase with cyanide or inactivating the cytochrome *b*-c1 complex with antimycin A_1. Mitochondrial and cytosolic free NADH/NAD^+ ratios are increased in the liver of hypoxic rats [28] or in rats treated with cyanide [29].

There is currently no apparent consensus regarding the sequence of events and parameters responsible for cell injury. Disruption of the plasma membrane as a result of phospholipase A_2 or protease activation by increased cytosolic calcium is one hypothesis [30]. In the present study we have shown the existence of another cytotoxic pathway that results from prolonged reductive stress. Thus cytotoxicity but not ATP depletion could be prevented by NADH oxidising nutrients such as acetoacetate or artificial electron acceptors. Furthermore, cytotoxicity was enhanced by NADH generating nutrients such as lactate, sorbitol, and glycerol whereas cytotoxicity was prevented by nutrients which supplied

Table 7
Ethanol-enhanced hypoxia:reoxygenation injury in isolated rat hepatocytes and cytoprotection by antioxidants or desferoxamine

Addition	% Cytotoxicity at time (min)		
	90	120	150
Hypoxia/reoxygenation	31 ± 4	55 ± 6	84 ± 7
+ Ethanol 30 mM	78 ± 7	100[a]	100
+ Ethanol 30 mM + methyl pyrazole 100 μM	41 ± 5	60 ± 5[b]	85 ± 8
+ Ethanol 30 mM + hypoxanthine 5 mM	100	100	100
+ Ethanol 30 mM + oxypurinol 50 μM	33 ± 4	43 ± 4[b]	61 ± 6
+ Ethanol 30 mM + desferoxamine 200 μM	26 ± 3	38 ± 3[b]	57 ± 5
+ Ethanol 30 mM + BHA 50 μM	36 ± 2	54 ± 6[b]	84 ± 6
+ Ethanol 30 mM + TEMPO 100 μM	31 ± 3	40 ± 5[b]	52 ± 6
+ Ethanol 30 mM + TEMPOL 300 μM	30 ± 3	39 ± 4[b]	55 ± 5
+ Ethanol 30 mM + quercetin 100 μM	30 ± 3	38 ± 5[b]	49 ± 5
+ Acetaldehyde 10 mM	32 ± 3	38 ± 4[a]	48 ± 5[a]
+ Acetaldehyde 10 mM + methylpyrazole 100 μM	44 ± 4	54 ± 5[a]	87 ± 6
Control (1% O_2)	15 ± 3	16 ± 3	19 ± 3
Ethanol 30 mM (1% O_2)	15 ± 2	17 ± 3	20 ± 3

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37°C. Cells were maintained under hypoxic environment and reoxygenated with 1% O_2 at 90 mins as described under Materials and methods. Cell viability was determined as the percentage of cells which did not exclude trypan blue. Values are means of at least three experiments ± SD.

[a] Significantly different from hypoxia reoxygenation alone ($P < 0.001$).

[b] Significantly different from hypoxia reoxygenation + ethanol ($P < 0.001$).

glycolytic ATP with-out increasing the cytosolic free NADH/NAD^+ ratio e.g. fructose and dihydroxyacetone.

Cyanide or antimycin caused marked reductive stress as reflected by an increase in the cytosolic free NADH/NAD^+ ratio. Cyanide or antimycin cytotoxicity was also increased by the NADH generating nutrients sorbitol or glycerol and prevented by the NADH oxidising nutrient acetoacetate or the artificial electron acceptor methylene blue. However, sorbitol partly prevented ATP depletion. A marked increase in β-hydroxybutyrate/acetoacetate ratio reflecting the mitochondrial free NADH/NAD^+ ratio [16] was also noticed in hepatocytes exposed to cyanide or antimycin A (data not shown). Furthermore, fructose, a glycolytic substrate that does not affect the cytosolic free NADH/NAD^+ ratio in normal cells normalised the cytosolic free NADH/NAD^+ ratio in cyanide or antimycin treated cells and prevented cytotoxicity. This suggests that cell viability is dependent on the maintenance of normal cellular redox potentials. On the other hand, investigators have found that cytoprotection by fructose requires a functioning mitochondrial ATP synthase and have concluded that fructose acts by supplying ATP [31,32].

The ferric iron chelator, desferoxamine partly prevented CN or antimycin-induced hepatocyte injury. Desferoxamine also prevented the further increase in cytotoxicity when NADH generating nutrients were present. Cyanide-induced injury to the isolated perfused rat liver was also prevented by desferoxamine [33]. Furthermore, we also found that intracellular nonbound Fe was doubled after addition of CN or antimycin, in parallel with the

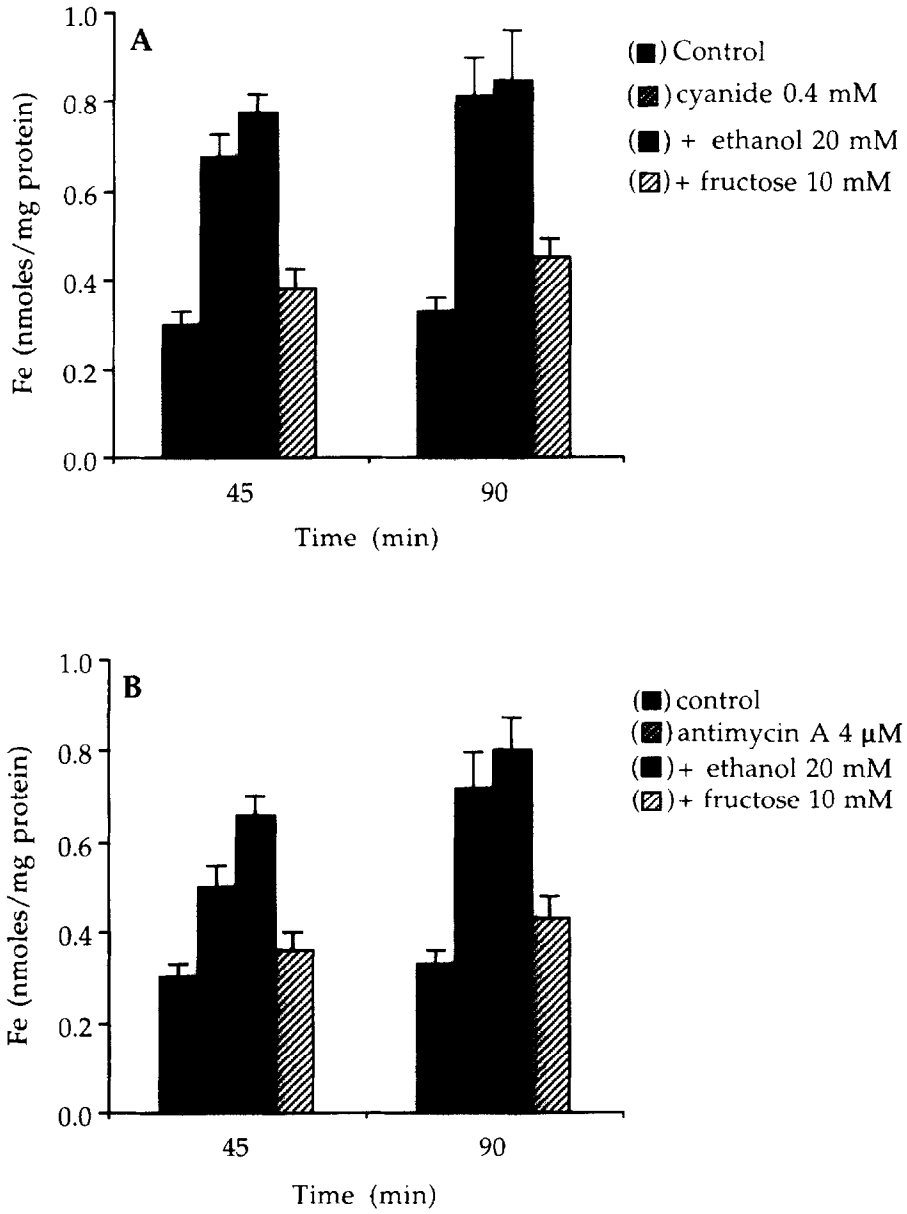

Fig. 3. Increased Fe^{2+} release from intracellular stores by cyanide (A) or antimycin (B) in isolated rat hepatocytes. Aliqouts were taken at 45 and 90 min and Fe^{2+} was measured as explained under Materials and methods. Values are expressed as mean ± SD of three separate experiments.

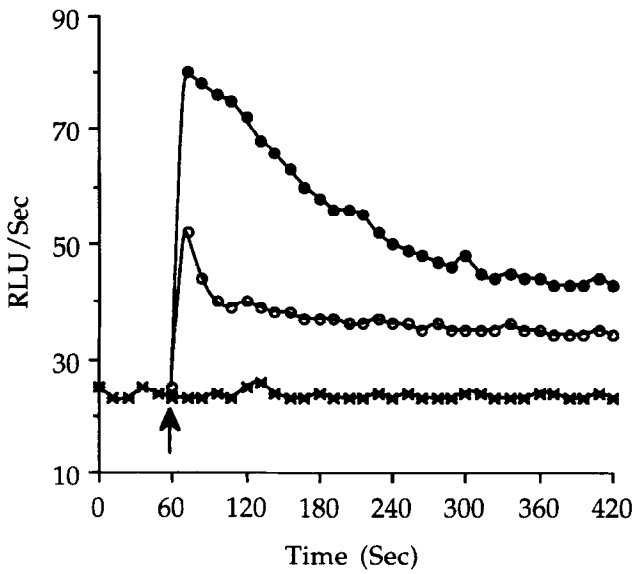

Fig. 4. Luminol chemiluminescence in isolated rat hepatocytes was increased by cyanide or antimycin A treatment. Cyanide 1 mM (●) or antimycin A 10 μM (O) was added to 1 ml of hepatocytes (10^6 cells/ml) incubated with 1 mM luminol (x) and relative light unit (RLU) was measured every 12 s as explained under materials and methods. Arrow shows the time of addition of cyanide or antimycin A.

increased cytosolic free NADH/NAD^+ ratio. More iron was released when the reductive stress was further increased with the NADH generators ethanol, sorbitol or glycerol. An increase in non protein bound iron was also found preceding ischemia: reperfusion injury of rat kidneys [34].

The released ferrous iron may contribute to the formation of reactive oxygen species because TEMPO or TEMPOL which are nitroxide free radicals and superoxide dismutase mimics [35] partly prevented cyanide or antimycin induced cytotoxicity. Addition of CN or antimycin to hepatocytes immediately increased H_2O_2 formation as determined by chemiluminescence. The iron released as a result of reductive stress may further increase ROS formation from H_2O_2 via the Fenton reaction. Isolated mitochondria in vitro also formed ROS in the presence of antimycin and the site of ROS formation was attributed to oxygen reduction by ubisemiquinone [13] or reduced cytochrome *b*566 [36]. Mitochondria may also be a source of reactive oxygen species after reoxygenation of hypoxic hepatocytes [25]. The polyphenolic antioxidants pyrogallol, purpurogallin, NDGA, quercetin, and caffeic acid which scavenge superoxide radicals [37,38] and complex iron [39] also partly prevented cell injury. The antioxidant uric acid which forms ferric complexes [40] and ascorbate which scavenges superoxide radicals [41] were also partly cytoprotective. However, no malonaldehyde formation was detected suggesting that lipid peroxidation was not critical to the cytotoxic mechanism. Furthermore, phenolic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene which are excellent at preventing lipid peroxidation but poor at scavenging superoxide radicals [37] were only slightly cytoprotective.

Although hepatocytes were previously found to be sensitive to hypoxic injury [42], they were apparently more resistant to hypoxia:reoxygenation injury [14] than was perfused liver [15]. Because of this the site of ROS formation was postulated to be Kupffer cells [15] or xanthine oxidase formed from xanthine dehydrogenase which may have leaked from injured hepatocytes [11,14]. However, the reoxygenation oxygen concentration used by these investigators (usually 95% O_2 or in some cases 20% O_2) rapidly restores hepatocyte ATP levels [10] unless the hepatocytes have sustained sufficient hypoxic injury such that the mitochondrial respiratory chain is irreversibly damaged. The present study, however, shows that hepatocytes after only 90 mins of hypoxia are highly susceptible to hypoxia: reoxygenation cytotoxicity if reoxygenated with 1% O_2, an O_2 concentration which probably approximates that found at the zone 3 site of necrosis induced by hypoxia [43,44] but which only partly restored ATP (15–25%) (results not shown). Reoxygenation with 20% O_2 prevented further hypoxic injury and ATP was rapidly restored. However, 150 mins of hypoxia caused 50% cytotoxicity which was further increased on reoxygenation with 20% oxygen (results not shown). Hypoxia:reoxygenation injury with 1% O_2 was also increased by NADH generating nutrients and prevented by NADH oxidising nutrients. This injury was also prevented with desferoxamine, TEMPO, TEMPOL, purpurogallin, pyrogallol and quercetin but not with BHA and BHT. Desferoxamine was reported to prevent hypoxia: reoxygenation injury in perfused liver [8] and to improve functional and metabolic recovery of isolated perfused hearts subjected to global ishemia [45]. The antioxidants propyl gallate and trolox C were also reported to protect isolated perfused rat liver against hypoxic injury [46,47]. Reperfusion damage in isolated rat hearts subjected to regional ischemia was also prevented by TEMPO [35]. Furthermore, Okuda et al. [48] have directly demonstrated an increase in ROS formation when ischemic rat livers were reperfused. The lack of detectable malondialdehyde formed during hypoxia:reoxygenation or chemical hypoxia toxicity and the lack of cytoprotection by the antioxidants BHA or BHT suggests that membrane phospholipids are not major cytotoxic targets for ROS.

Cytotoxicity induced by hypoxia:reoxygenation, cyanide or antimycin was partially prevented by oxypurinol, a xanthine oxidase inhibitor [49]. Cytotoxicity was also markedly enhanced by hypoxanthine, an effect prevented by oxypurinol. This suggests that xanthine oxidase activity contributes to cytotoxic ROS formation or that xanthine dehydrogenase activity increases reductive stress. To distiguish these two possibilities, the changes in xanthine dehydrogenase and xanthine oxidase activities induced by the respiratory inhibitors or hypoxia: reoxygenation need to be measured. Allopurinol also prevented hypoxia: reoxygenation or cyanide toxicity in perfused fasted rat livers [34,50]. Hypoxia has also been shown to convert some xanthine dehydrogenase to xanthine oxidase [8–11], however, it was suggested that extracellular conversion occurred following enzyme leakage from injured cells [14].

Added GSH delayed hypoxia:reoxygenation, CN or antimycin toxicity probably by reacting with extracellular ROS. Another possibility is that extracellular GSH prevents the leakage of intracellular GSH from hepatocytes, previously shown with cyanide and antimycin [51], as membrane permeability may increase as a result of ATP depletion and/or reductive stress. Furthermore, GSH depleted hepatocytes were also much more susceptible to injury. Hepatocyte hydrogen peroxide detoxification systems involve catalase and GSH/GSH peroxidase in conjunction with NADPH/GSH reductase. The latter system in conjunction with superoxide dismutase will also detoxify superoxide radicals [52]. The hepatocytes ability to detoxify ROS is probably compromised during hypoxia or chemical

hypoxia as the synthesis of GSH and NADPH (via mitochondrial transhydrogenase) is ATP dependent [44,53]. Other investigators have also found that exogenous GSH prevents hypoxia and CN-induced damage to isolated perfused rat liver [54].

Ethanol at nontoxic concentrations markedly increased cytotoxicity induced by hypoxia:reoxygenation, cyanide or antimycin. Ethanol-induced cytotoxicity was prevented by desferoxamine, oxypurinol, TEMPO, and quercetin but was enhanced by hypoxanthine. The ethanol-increased cytotoxicity was prevented by the alcohol dehydrogenase inhibitor methyl pyrazole. By contrast, acetaldehyde, the ethanol metabolite formed by alcohol dehydrogenase prevented hypoxia:reoxygenation cytotoxicity, an effect prevented by methyl pyrazole. Furthermore, the hepatocyte lactate/pyruvate ratio was markedly enhanced by ethanol and decreased by acetaldehyde (results not shown). This indicates that ethanol increases cytotoxicity by further increasing the hepatocyte NADH/NAD^+ ratio thereby increasing reductive stress [27]. Desferoxamine prevented the ethanol/hypoxia cytotoxicity indicating that the increased reductive stress may have mobilised more iron from intracellular stores. Such a mechanism was recently suggested for hepatic necrosis induced by the much more toxic allyl alcohol [55]. Other investigators have shown that ethanol is hepatotoxic under conditions of ischemia:reperfusion [56]. Furthermore, alcohol-induced liver injury in vivo occurs in zone 3 of the liver with the lowest oxygen tension and has been attributed to hypoxia as a result of an ethanol-induced increased hepatocyte oxygen consumption not accompanied by increased oxygen delivery [57]. However, others concluded that ethanol toxicity involves chemical hypoxia as a result of reductive stress [58] or mitochondrial damage in zone 3 hepatocytes [59]. As ethanol in vivo also increases hepatic hypoxanthine and xanthine levels and allopurinol prevented lipid peroxidation, xanthine oxidase was suggested as a source of ROS in ethanol-induced hepatic injury [60].

Taken together the results of the present study suggest that the mechanism of hepatocyte injury resulting from inhibition of mitochondrial respiration includes a cytotoxic pathway that arises partly from an energy deficit but also from reductive stress which releases non protein bound iron from intracellular pools and induces cytotoxic ROS formation. ATP utilisation resulting in AMP catabolism provides hypoxanthine and xanthine for xanthine dehydrogenase or xanthine oxidase which could increase reductive stress by increasing NADH or forming ROS respectively. ATP levels are also expected to compromise the ROS detoxification capacity of hepatocytes by inhibiting GSH and NADPH synthesis. These theories could explain why cytotoxicity induced by hypoxia:reoxygenation, cyanide or antimycin was partly prevented by desferoxamine, oxypurinol, superoxide scavenging antioxidants and GSH whereas cytotoxicity was increased by NADH generators including ethanol.

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