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Modulating hypoxia-induced hepatocyte injury by affecting intracellular redox state

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Abstract

Hypoxia-induced hepatocyte injury results not only from ATP depletion but also from reductive stress and oxygen activation. Thus the $NADH/NAD^+$ ratio was markedly increased in isolated hepatocytes maintained under 95% N_2/5% CO_2 in Krebs–Henseleit buffer well before plasma membrane disruption occurred. Glycolytic nutrients fructose, dihydroxyacetone or glyceraldehyde prevented cytotoxicity, restored the $NADH/NAD^+$ ratio, and prevented complete ATP depletion. However, the NADH generating nutrients sorbitol, xylitol, glycerol and β-hydroxybutyrate enhanced hypoxic cytotoxicity even though ATP depletion was not affected. On the other hand, NADH oxidising metabolic intermediates oxaloacetate or acetoacetate prevented hypoxic cytotoxicity but did not affect ATP depletion. Restoring the cellular $NADH/NAD^+$ ratio with the artificial electron acceptors dichlorophenolindophenol and Methylene blue also prevented hypoxic injury and partly restored ATP levels. Ethanol which further increased the cellular $NADH/NAD^+$ ratio increased by hypoxia also markedly increased toxicity whereas acetaldehyde which restored the normal cellular $NADH/NAD^+$ ratio, prevented toxicity even though hypoxia induced ATP depletion was little affected by ethanol or acetaldehyde. The viability of hypoxic hepatocytes is therefore more dependent on the maintenance of normal redox homeostasis than ATP levels. GSH may buffer these redox changes as hypoxia caused cell injury much sooner with GSH depleted hepatocytes. Hypoxia also caused an intracellular release of free iron and cytotoxicity was prevented by desferoxamine. Furthermore, increasing the cellular $NADH/NAD^+$ ratio markedly increased the intracellular release of iron. Hypoxia-induced hepatocyte injury was also prevented by oxypurinol, a xanthine oxidase inhibitor. Polyphenolic antioxidants or the superoxide dismutase mimic, TEMPO partly prevented cytotoxicity suggesting that reactive oxygen species contributed to the cytotoxicity. The above results suggests that hypoxia induced hepatocyte injury results from sustained reductive stress and oxygen activation.

Keywords: Hypoxia; Cytotoxicity; Reductive stress; Hepatocyte; (Rat liver)

1. Introduction

Hypoxia resulting from the impairment of tissue oxygen delivery — e.g., as a result of ischemia — is a primary cause of cell injury. Other examples of hypoxic injury include patients with severe pulmonary insufficiency and consequent hypoxemia. In these patients, serum levels of hepatic enzymes may increase and ultrastructural changes appear in the centrilobular liver cells [1] probably because the centrilobular zone of the hepatic acinus has the lowest oxygen concentration. Liver injury in the isolated perfused rat liver following hypoxia also predominates in the centrilobular zone [2]. Liver injury in alcoholics also starts and predominates in the centrilobular zone [3].

Several biochemical changes have been found preceding cell injury induced by hypoxia that probably arise from the inactivation of oxidative phosphorylation in the electron-transport system. These include ATP depletion [4], increased cytosolic calcium [5] and sodium [6], phospholipase A2 activation, and membrane phospholipid degradation [7]. A marked increase in the lactate/pyruvate ratio an indicator of the free cytoplasmic $NADH/NAD^+$ ratio [8,9] also occurs in hypoxic hepatocytes [10]. It has also been proposed that an increase in the blood-ketone body ratio (acetoacetic/β-hydroxybutyric acid), an indicator of the mitochondrial $NADH/NAD^+$ ratio and redox potential should be used as an early indicator of hepatic failure [11].

Abbreviations: DCPIP, dichlorophenolindophenol; DETAPAC, diethylenetriaminepentaacetic acid; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MB, Methylene blue; MeP, methyl pyrazole; ROS, reactive oxygen species.

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In the present study, we have correlated cytotoxicity, ATP levels and the lactate/pyruvate ratio of hepatocyte injured by hypoxia. The susceptibility of hepatocytes to hypoxic cytotoxicity increased if the hepatocyte mitochondrial or cytoplasmic redox state was made more reduced by adding NADH generating glycolytic metabolites, or ethanol. In contrast, hepatocytes were more resistant to hypoxic injury if the cellular redox state was made more oxidised with NADH utilising intermediary metabolites or artificial electron acceptors. An increase in reductive stress released intracellular iron and reductively activated oxygen. Cytotoxicity, however, did not correlate with ATP levels thereby showing for the first time that prolonged reductive stress resulting in oxygen activation and *not* ATP depletion is responsible for hypoxic injury.

2. Materials and methods

2.1. Materials

Alanine, acetoacetic acid, dihydroxyacetone, dichlorophenolindophenol (DCPIP), EGTA, fluoro-2,4-dinitrobenzene, fructose, glutathione, DL-glyceraldehyde, glutamine, β-hydroxybutyrate, iodoacetic acid, lactic acid, Methylene blue, methyl pyrazole, monensin, pyruvic acid, sorbitol, Trypan blue, and xylitol were purchased from Sigma (St. Louis, MO). Collagenase (from *Clostridium histolyticum*), Hepes, and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, PQ). HPLC grade solvents were purchased from Calden (Georgetown, Ont.). Desferoxamine was a gift from Ciba-Geigy (Canada). All other chemicals used were of analytical grade.

2.2. Isolation and incubation of hepatocytes

Freshly isolated hepatocytes were prepared from adult male Sprague–Dawley rats (280–300 g) fed ad libitum. The cells were isolated by collagenase perfusion of the liver as described by Moldeus et al. [12]. The cells ($1 \cdot 10^6$ cells/ml) were preincubated in Krebs–Henseleit bicarbonate buffer (pH 7.4) supplemented with 12.5 mM Hepes for 30 min in an atmosphere of 10% O_2/5% CO_2/85% N_2 in continuously rotating 50 ml round bottom flasks before the addition of chemicals. For experiments performed under hypoxic conditions, the cells were incubated under an atmosphere of 5% CO_2/95% N_2 following a 30 min preincubation period under aerobic conditions. Oxygen electrode measurement showed that the oxygen concentration in the cellular medium was $< 0.01\%$ (1.0–1.5 μM) 40 min after the hepatocytes were added to the flasks under the N_2 atmosphere. Glutathione depleted hepatocytes were obtained as previously described [13]. Stock solutions of chemicals and nutrients were made either in incubation buffer or in DMSO and added to the hepatocyte suspensions at the indicated time points.

2.3. Measurement of cytotoxicity

The viability of the hepatocytes were assessed by Trypan blue uptake. Viability was examined immediately after isolation, and during the incubation at various time points.

2.4. Glutathione assay

The total amount of GSH in isolated hepatocytes was measured in deproteinised samples (5% meta phosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene by HPLC as described by Reed et al. [14].

2.5. Determination of intracellular ATP

Intracellular ATP in hepatocytes was extracted using an alkaline extraction procedure and quantified by HPLC, using C18 μ Bondapak reverse-phase column (Waters, Milford) as described by Stocchi et al. [15].

Table 1
Modulation of hypoxic cytotoxicity in hepatocytes by glycolytic intermediates

Nutrient (10 mM)	% Trypan blue uptake			Lactate/pyruvate ratio	
	60 min	120 min	180 min	60 min	120 min
None	33 ± 4	51 ± 6	82 ± 7	38	47
Fructose	22 ± 3	24 ± 3	27 ± 4 *	14	15
+ monensin (10 μM)	24 ± 3	27 ± 4	32 ± 4 *	18	19
Monensin (10 μM)	45 ± 5	79 ± 5	100	51	64
Dihydroxyacetone	27 ± 4	31 ± 3	36 ± 4 *	8	9
DL-Glyceraldehyde	21 ± 3	23 ± 4	30 ± 4 *	11	9

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described in Section 2. Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of the three separate experiments (± S.D.).

* Significant difference in comparison with aerobic control ($p < 0.001$).

Lactate and pyruvate were determined using lactate dehydrogenase and values are expressed as mean from three separate experiments.

2.6. Measurement of lactate and pyruvate

Lactic acid and pyruvic acid were determined with lactate dehydrogenase by following the reduction or oxidation of NADH respectively at 340 nm as described previously [16,17].

2.7. Iron release assay

Free iron (Fe^{2+}) in hepatocytes was determined by bathophenanthroline disulfonate (BPS) assay as described [18]. Aliquots were taken at different time points and were filtered through 0.45 μm membrane filters after addition of 0.3% digitonin to render the plasma membrane permeable. BPS (1 mM) was added to 1 ml of filtrate and the absorbance was measured at 537 nm.

2.8. Statistics

Statistically significant differences between control and experimental groups were obtained using Student t-test. The minimal level of significance chosen was $p < 0.05$.

3. Results

3.1. Effect of hypoxia on cytosolic NADH / NAD$^+$ and cellular ATP levels

The hepatocyte lactate/pyruvate ratio, reflecting cytosolic NADH/NAD$^+$ ratio was markedly increased in hepatocytes maintained under hypoxia (95% N_2/5% CO_2) (Table 1) indicating that the cell became more reduced. The increased pyruvate levels could be attributed to a decrease in the rate of pyruvate oxidation by the tricarboxylic acid cycle. This increase in NADH/NAD$^+$ ratio accompanied a depletion of ATP (50% in 60 min) and preceded cytotoxicity (50% occurring at about 2 h, Figs. 1 and 2). Glutathione depleted hepatocytes were much more susceptible to hypoxic cell injury (50% cytotoxicity occurring in about 1 hour, Fig. 1).

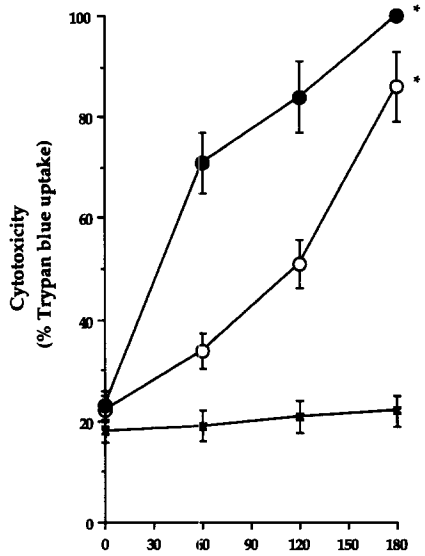

Fig. 1. Hypoxia induced cytotoxicity in isolated rat hepatocytes. Hypoxic cytotoxicity is enhanced by intracellular GSH depletion. Cell viability was determined by Trypan blue uptake at various time points. Key: (×) control, (○) hypoxia, and (●) hypoxia + GSH depletion. * Significant difference from aerobic control ($p < 0.001$). ** Significant difference from hypoxic control ($p < 0.001$).

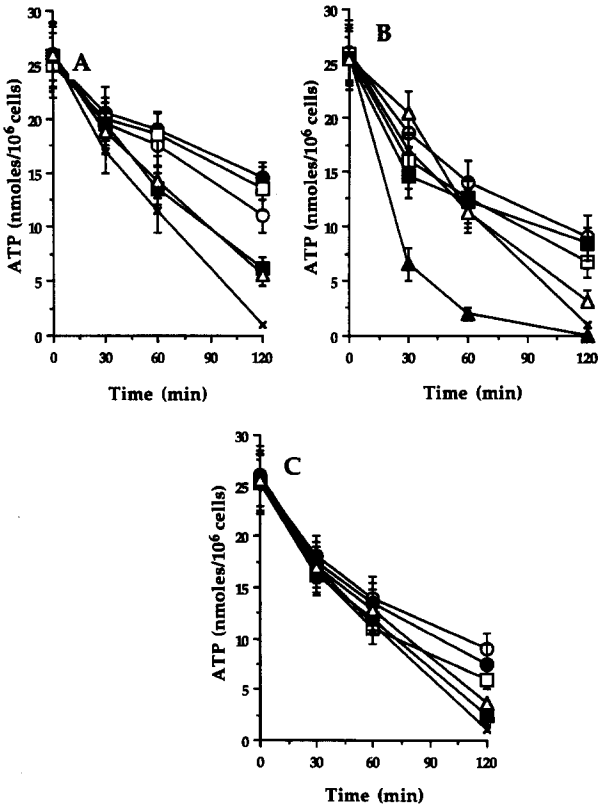

Fig. 2. Hypoxia induced ATP depletion (A) Modulation by various glycolytic nutrients. Key: (×) no addition, (●) fructose, (○) dihydroxyacetone, (□) DL-glyceraldehyde, (■) alanine, and (△) glutamine. (B) Modulation by NADH generating nutrients. Key: (×) no addition, (○) sorbitol, (□) xylitol, (■) glycerol, (△) ethanol, and (▲) β-hydroxybutyrate. (C) Modulation by NADH oxidising agents. Key: (×) no addition, (○) DCPIP, (●) MB, (□) acetaldehyde, (■) acetoacetate, and (△) oxaloacetate. ATP levels in aerobic control hepatocytes was (40±5 nmol/10^6 cells). * Significant difference from control ($p < 0.001$).

3.2. Effect of nutrients on NADH / NAD$^+$, ATP levels and cytotoxocity

As shown in Fig. 2A and Table 1, the glycolytic nutrients fructose, dihydroxyacetone, and glyceraldehyde, prevented cytotoxicity, the increase in lactate/pyruvate ratio and ATP depletion (90% at 1 hour and 40% at 2 hour). Monensin, a H^+/Na^+ ionophore did not affect the cytoprotective action of fructose (Table 1) suggesting that the protective effect of fructose resulted from restoring the

Table 3
Hypoxic cytotoxicity in hepatocytes is increased by increasing cellular reductive stress or by restoring redox homeostasis

Nutrient (10 mM)	% Trypan blue uptake at time (min)		
	60	120	180
None	35±4	54±6	83±8
Increase cytosolic NADH			
Sorbitol	46±5	75±7	100 *
Xylitol	49±6	78±8	100 *
Glycerol	43±4	71±6	100 *
Increase mitochondrial NADH			
β-Hydroxybutyrate	41±4	70±6	100 *
Alanine	31±4	62±6	100 *
Glutamine (5 mM)	34±3	67±4	100 *
Oxidise NADH			
Oxaloacetate	22±3	31±4	34±6 †
Acetoacetate	25±3	36±4	43±5 †

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described in Section 2. Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of the three separate experiments (±S.D.).
* Significantly increased in comparison with hypoxic control ($p < 0.001$).
† Significantly decreased in comparison with hypoxic control ($p < 0.001$).

normal redox state (lactate/pyruvate ratio) rather than causing acidosis as a result of glycolysis. Furthermore, the NADH generating glycolytic nutrients xylitol, sorbitol and glycerol increased both hepatocyte lactate/pyruvate ratio (Table 2) and hypoxic injury without restoring ATP levels (Fig. 2B, Table 3) indicating that cytotoxicity correlated with the degree of reductive stress but not ATP depletion.

β-Hydroxybutyrate, a ketoacid nutrient which reduces mitochondrial NAD^+ also increased the lactate/pyruvate ratio and also increased hypoxic cell injury but did not prevent ATP depletion. However, hypoxic cell injury was prevented by oxaloacetate or acetoacetate which oxidised mitochondrial NADH and decreased the lactate/pyruvate ratio (Tables 2 and 3) but did not restore ATP levels (Fig. 2C). This further indicates that cytotoxicity correlated more with the degree of reductive stress than with the extent of ATP depletion. Thus, intermediary metabolites which prevented hypoxia induced reductive stress also prevented hypoxic injury.

3.3. Protection by artificial electron acceptors

Further evidence that hypoxic cell viability depends on the maintenance of normal redox homeostasis is that addition of the artificial electron acceptors, DCPIP and Methylene blue decreased the lactate/pyruvate ratio (Table 2) and prevented hypoxic toxicity (Table 4). However, ATP depletion was also partly prevented by these electron acceptors (Fig. 2C).

3.4. Effect of ethanol and acetaldehyde on NADH / NAD^+, ATP and cytotoxicity

Hypoxic cell injury was markedly increased by ethanol (Table 5). These effects on hypoxic injury did not correlate with ATP depletion as ethanol did not affect ATP depletion (Fig. 2C). However, ethanol further increased the lactate/pyruvate ratio increased by hypoxia indicating that the reductive stress induced by hypoxia was further increased by ethanol (Table 2). Decreased pyruvate levels were mainly responsible for the ethanol-induced increase in lactate/pyruvate ratio. Hypoxic cell injury was not increased by ethanol in the presence of the alcohol dehydrogenase inhibitor methyl pyrazole. Inactivation of alco-

Table 2
Modulation of cytosolic redox state (lactate/pyruvate) of isolated rat hepatocytes under hypoxia

Addition	Lactate (nmol/10^6 cells)		Pyruvate (nmol/10^6 cells)		Lactate/pyruvate ratio	
	60 min	120 min	60 min	120 min	60 min	120 min
None	630	647	18	14	35	46
Sorbitol (10 mM)	474	557	9	8	53	70
Xylitol (10 mM)	461	514	7	4	66	128
Glycerol (10 mM)	468	533	8	6	59	89
β-OHButyrate (10 mM)	612	630	11	8	56	79
Alanine (10 mM)	768	901	17	20	46	88
Glutamine (5 mM)	767	781	15	13	51	61
Oxaloacetate (10 mM)	1058	1993	158	153	7	13
Acetoacetate (10 mM)	819	946	68	73	12	13
DCPIP (0.08 mM)	830	852	27	32	31	27
MB (0.085 mM)	851	873	28	35	30	25
Ethanol (40 mM)	412	406	5	4	82	102
+MeP (0.1 mM)	573	605	13	12	44	50
Acetaldehyde (5 mM)	654	815	19	22	34	37
+MeP (0.1 mM)	631	754	17	6	37	126
Aerobic	100	111	14	14	7	8

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described in Section 2. Lactate and pyruvate were determined using lactate dehydrogenase and values are expressed as mean from three separate experiments.

Table 4
Oxidative stress induced by artificial electron acceptors dichlorophenol indophenol (DCPIP) and Methylene blue (MB) prevent hypoxic cytotoxicity

Addition	% Trypan blue uptake at time (min)		
	60	120	180
HYPOXIC			
None	36±4	51±6	86±8
DCPIP (80 μM)	33±3	36±4	44±5 *
MB (85 μM)	31±3	38±4	47±5 *
AEROBIC			
None	22±2	23±3	25±3
DCPIP (80 μM)	34±4	54±6	100 †
MB (85 μM)	36±4	51±6	85±9 †

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described in Section 2. Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of the three separate experiments (±S.D.).
* Significant difference from hypoxic control ($p < 0.001$).
† Significant difference from hypoxic control ($p < 0.001$).

hol dehydrogenase in hepatocytes also prevented the ethanol induced increase in lactate/pyruvate ratio. These results on hypoxic injury therefore correlate with effects on redox homeostasis rather than ATP levels.

Hypoxic cell injury was prevented by acetaldehyde (Table 5) and acetaldehyde only partly prevented ATP depletion (Fig. 2C). However, acetaldehyde markedly decreased the lactate/pyruvate ratio. By contrast, hypoxic cell injury in alcohol dehydrogenase inactivated hepatocytes was increased by acetaldehyde as was the lactate/pyruvate ratio (Table 2) and could be attributed to

Table 5
Modulation of hypoxia induced cytotoxicity by ethanol and acetaldehyde

Addition	%Trypan blue uptake at time (min)		
	60	120	180
HYPOXIC			
None	34±4	52±6	87±8 *
Ethanol (40 mM)	63±6	81±7 †	100 †
+methyl pyrazole (0.1 mM)	35±3	48±5	63±6
+allopurinol (0.5 mM)	61±5	78±7	100
Acetaldehyde (5 mM)	31±4	39±5	54±5 †
+methyl pyrazole (0.1 mM)	71±7	89±8	100
AEROBIC			
None	20±2	21±3	23±3
Ethanol (40 mM)	23±3	24±3	31±4
+methyl pyrazole (0.1 mM)	22±2	25±3	33±4
Acetaldehyde (5 mM)	25±3	29±3	37±4
+methyl pyrazole (0.1 mM)	23±3	26±3	29±4

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described Section 2. Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of the three separate experiments (±S.D.).
* Significant difference in comparison with aerobic control ($p < 0.001$).
† Significant difference in comparison with hypoxic control ($p < 0.001$).

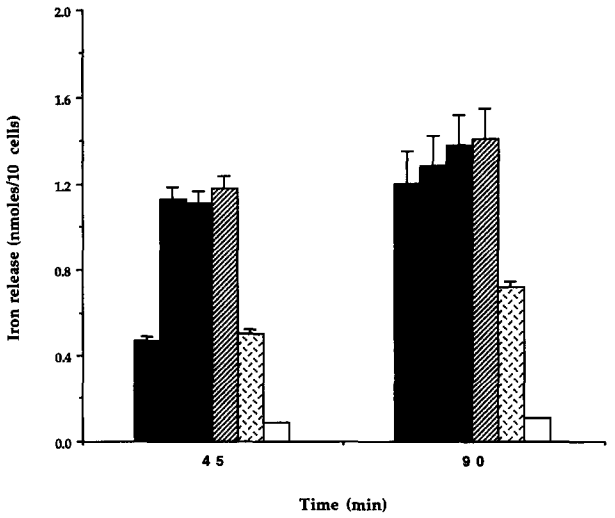

Fig. 3. Increase in intracellular Fe^{2+} under hypoxia. Aliquots were taken at 45 and 90 min and Fe^{2+} was measured as described in Section 2. Values are expressed as mean±SD of three separate experiments. Bars from left to right indicate, respectively: hypoxia; +ethanol (20 mM); +xylitol (10 mM); +sorbitol (10 mM); +dihydroxyacetone (10 mM); and +desferoxamine (1 mM).

mitochondrial NADH generation via aldehyde dehydrogenase.

3.5. Iron release from intracellular stores by hypoxia

Hypoxia caused the intracellular release of free iron in hepatocytes (Fig. 3). Furthermore, NADH generating nutrients sorbitol or xylitol and ethanol increased the amount of iron release, whereas dihydroxyacetone decreased the amount of iron released under hypoxia (Fig. 3).

3.6. Cytoprotection by polyphenolic antioxidants and desferoxamine

As shown in Table 6, hypoxic injury was effectively prevented by the polyphenolic antioxidants purpurogallin, caffeic acid, quercetin or ellagic acid and the superoxide dismutase mimic TEMPO but not by the phenolic antioxidants butylated hydroxyanisole or butylated hydroxytoluene. The ferric ion chelator desferoxamine or the xanthine oxidase inhibitor oxypurinol also markedly protected hypoxic injury, whereas hypoxanthine increased hypoxic cell injury.

3.7. Effect of extracellular calcium on hypoxia induced cell injury

As shown in Fig. 4A, hypoxic cell injury was also increased if extracellular calcium was increased to 5 mM. This Ca^{2+} enhanced hypoxic cell injury was prevented by the addition of the Ca^{2+} chelators EGTA and DETAPAC (Fig. 4B). However, EGTA had little effect on hypoxic injury when extracellular Ca^{2+} was 0.5 mM.

Table 6
Prevention of hypoxia induced cytotoxicity by radical scanvengers or a ferric ion chelators

Addition	%Trypan blue uptake at time (min)		
	60	120	180
None	36±4	53±6	88±8
Caffeic acid (0.3 mM)	23±3	24±4 *	35±4 *
Purpurogallin (0.2 mM)	21±3	24±4 *	41±4 *
Quercetin (0.1 mM)	20±3	22±4 *	33±4 *
Ellagic acid (0.3 mM)	21±4	23±3 *	40±5 *
TEMPO (0.1 mM)	24±3	32±4 *	43±5 *
Ascorbate (0.3 mM)	24±3	29±4 *	37±4 *
BHA (0.05 mM)	35±4	54±5	86±8
BHT (0.03 mM)	34±4	56±6	84±7
Desferoxamine (1 mM)	24±3	36±4	54±6 *
Hypoxanthine (5 mM)	47±4	75±7	100
Oxypuranol (0.05 mM)	23±3	26±3 *	41±4 *

Hepatocytes (10^6 cells/ml) were incubated in Krebs–Henseleit buffer (pH 7.4) at 37°C. Cells were maintained under hypoxic environment as described in Section 2. Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of the three separate experiments (±S.D.).

* Significant difference in comparison with hypoxic control ($p < 0.001$).

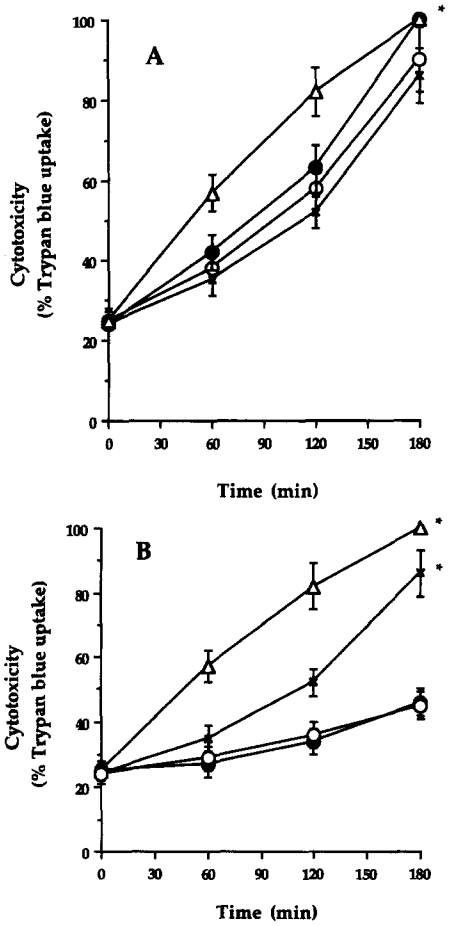

Fig. 4. Extracellular calcium increased hypoxia induced cytotoxicity in isolated rat hepatocytes. Calcium chelators DETAPAC and EGTA delayed the cytotoxicity. Key: (A) — (×) no addition, (○) 1 mM Ca^{2+}, (●) 2 mM Ca^{2+}, and (△) 5 mM Ca^{2+}; (B) — (×) no addition, (△) 5 mM Ca^{2+}, (●) 5 mM Ca^{2+} + DETAPAC (1 mM), and (○) 5 mM Ca^{2+} + EGTA (1 mM).

4. Discussion

Cytotoxicity caused by hypoxia is generally attributed to the consequences of drastic ATP depletion resulting because ATP utilisation outweighs ATP biosynthesis [19–21]. Recent studies have also reported that fructose prevents hepatocyte injury induced by 'chemical hypoxia' by enhancing the production of glycolytic ATP [22–26]. In the present study, we found that glycolytic substrates such as dihydroxyacetone or glyceraldehyde also prevented hepatocyte hypoxic injury. Lactate formation was increased and ATP levels were not completely depleted. This would appear to suggest that the depletion of ATP below a minimal level is a critical event in the induction of irreversible cell injury [27,28] and that glycolytic substrates are cytoprotective towards hypoxic cell injury by generating ATP. Alternatively, acidosis resulting from fructose induced lactate formation has also been suggested as a major protecting factor in 'chemical hypoxia' [29]. However, fructose still protected against hypoxic cell injury in the presence of monensin, a H^+/Na^+ ionophore which equalises intracellular and extracellular pH [29] suggesting that acidosis was not involved in the cytoprotective mechanism.

Other mechanisms seem to be more important for the maintenance of cell viability. A marked increase in the cellular NADH/NAD^+ ratio occurred in hepatocytes maintained under hypoxic conditions. Furthermore, the NADH generating glycolytic nutrients such as xylitol, sorbitol or glycerol increased the susceptibility of hepatocytes to hypoxic injury even though ATP depletion was partly prevented. The further increase in the cellular NADH/NAD^+ ratio presumably occurs as a result of the action of cytosolic xylitol, sorbitol and glycerol dehydrogenases respectively [30–33]. Furthermore, the increase in hypoxic injury by xylitol and sorbitol could be eliminated by the addition of pyruvate to reoxidise the cytosolic NADH generated (data not shown). Increasing the mitochondrial NADH/NAD^+ ratio with β-hydroxybutyrate, alanine and glutamine also increased the susceptibility of hepatocytes to hypoxic injury (Tables 2 and 3). Although β-hydroxybutyrate increased the rate of ATP depletion (Fig. 2B), alanine and glutamine slightly prevented ATP depletion (Fig. 2A). Chance proposed that one of the factors contributing to hypoxic injury could be a reductive stress created by an accumulation of reducing equivalents [34]. The present study provides direct evidence for this hypothesis. Ballard also demonstrated an increase in the cytosolic and mitochondrial NADH/NAD^+ ratio in the livers of rats exposed to hypoxia [35].

The cytoprotection by fructose, dihydroxyacetone or glyceraldehyde could also be attributed to a restoration of cellular redox homeostasis as the hypoxia induced increase in cellular NADH/NAD^+ ratio was prevented. The intermediary metabolites, oxaloacetate or acetoacetate which oxidise mitochondrial or cytosolic NADH [30,36] also

alleviated the reductive stress and prevented hypoxic cell injury even though ATP depletion was not affected. Cytoprotection by oxaloacetate or acetoacetate is therefore best explained in terms of affecting reductive stress rather than ATP depletion. The artificial electron acceptors, Methylene blue or DCPIP have previously been shown to oxidise cellular NADH [30,36], and therefore would be expected to counteract the reductive stress caused by hypoxia. This seems to be the case as hypoxic cell injury was prevented and ATP depletion was partially prevented and the cellular $NADH/NAD^+$ ratio was decreased.

A characteristic feature of alcoholic liver injury is the predominance of lesions in the privenular (centrilobular) zone of the hepatic acinus where hepatic oxygen concentrations are lowest. Centrilobular necrosis was shown to occur in ethanol-fed rats and it was hypothesised that ethanol causes hypoxic injury by increasing oxygen consumption [37]. An alternative hypotheses was that ethanol further increased the reductive stress caused by the low centrilobular oxygen levels. In support of this the hepatic venous lactate/pyruvate ratio was markedly increased when ethanol was given to baboons [38]. The present study provides additional evidence for the latter hypothesis. Hypoxic cytotoxicity in isolated hepatocytes was also increased in the presence of ethanol. Furthermore, the hepatocyte $NADH/NAD^+$ ratio increased by hypoxia was further increased by ethanol whereas ATP depletion was not affected. This again suggests that reductive stress is a cause of hypoxic cytotoxicity. The alcohol dehydrogenase inhibitor, methyl pyrazole, prevented these effects of ethanol on hypoxic cytotoxicity suggesting that the reductive stress was due to ethanol metabolism. A recent in vivo study suggested that the continuous administration of Methylene blue in a liquid diet to rats decreased the redox shift caused by ethanol and prevented ethanol induced hypoxic injury [39]. By contrast, acetaldehyde decreased the cytosolic $NADH/NAD^+$ ratio and prevented hypoxic cytotoxicity, an effect also prevented by inactivating alcohol dehydrogenase with methyl pyrazole.

Desferoxamine prevented hypoxic injury to perfused rat livers [40]. In the present study, desferoxamine prevented hypoxic injury. Furthermore, the intracellular nonbound iron was increased with the concomitant increase in the lactate/pyruvate ratio in hepatocytes under hypoxia and more free iron was released when the reductive stress was further increased with the NADH generators ethanol, sorbitol, or xylitol. This suggests that reductive stress is responsible for intracellular iron release. Lefebvre and Buc-Calderon [41] suggested that hypoxia can cause iron release but did not give direct evidence except that desferoxamine was cytoprotective in hypoxic injury. In vitro studies also suggest that NADH can release Fe^{2+} from ferritin by reducing Fe^{3+} [42].

The polyphenolic antioxidants purpurogallin, caffeic acid, quercetin which scavenge radicals [43,44] and complex iron [45] also partly prevented hypoxic cell injury. This suggests that reactive oxygen species (ROS) are formed during hypoxia even though oxygen concentrations were very low. The antioxidants propylgallate and trolox C were also reported to protect isolated perfused rat liver against hypoxic injury [46,47]. Under hypoxia, the utilization of ATP as an energy source results in its being hydrolysed to ADP and then AMP. The latter then undergoes catabolism to hypoxanthine and xanthine which produces ROS with the help of xanthine oxidase formed from xanthine dehydrogenase during hypoxia [48]. Oxypurinol also prevented hypoxic injury, whereas hypoxanthine increased hypoxic injury. Hypoxanthine may also act by increasing cytosolic NADH via xanthine dehydrogenase. The ROS (e.g., OH radicals) formed when free iron is released could modify the plasma membrane by oxidizing amino acids of membrane proteins to form carbonyls and/or oxidise tyrosine to form bityrosine intermolecular or intramolecular cross-links [49]. Recently, oxidative damage to brain protein as measured by the protein carbonyl content was found to increase significantly during the reperfusion phase following global ischemia [50].

The maintenance of extracellular GSH is a critical component in protection against oxidative and chemically induced damage to receptors, transport systems and enzymes in the plasma membrane. Addition of GSH in the media protected against oxidative injury without transport into the cells and without degradation to yield cysteine. In the absence of added GSH, the cells release GSH to maintain this extracellular pool [51]. Previously, it was shown that cyanide and antimycin caused the efflux of GSH from isolated hepatocytes [52]. In the present study, the hypoxia induced efflux of intracellular GSH was partly prevented by fructose, dihyroxyacetone, but was enhanced by sorbitol and ethanol (data not shown). The addition of extracellular GSH partly prevented hypoxic injury by reacting with extracellular ROS [53]. GSH depleted cells were highly susceptible to hypoxic injury indicating that intracellular GSH acts as a redox buffer to prevent hypoxic injury.

Gasbarrini et al. [4] reported that in isolated rat hepatocytes, anoxia increases intracellular Ca^{2+} in two distinct phases. The first phase may involve mitochondrial Ca^{2+} release. However, removing extracellular Ca^{2+} during anoxia prevents both the rise in intracellular Ca^{2+} and plasma membrane damage [4]. In the present study, hypoxia induced cytotoxicity was increased with increasing concentrations of extracellular calcium. This suggests that an influx of extracellular calcium disrupts the plasma membrane by activating Ca^{2+}-dependent enzymes such as phospholipase A_2, proteinases, etc. [54,55].

On the basis of our present study, we propose the following sequence of events that results in hypoxic injury (Fig. 5). The lack of oxygen and thus mitochondrial dysfunction inhibits NADH oxidation and results in a more reduced cellular redox state ($NADH/NAD^+$). Mitochondrial ATP formation is inhibited and cellular ATP levels

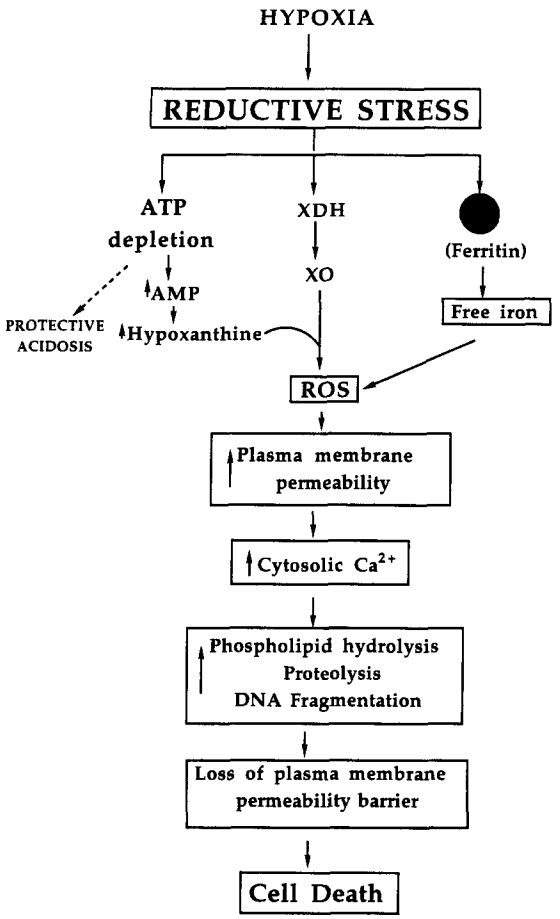

Fig. 5. Proposed mechanism of hypoxic cell death.

are decreased by ATP utilising cellular functions. Although the glycolytic nutrients fructose, dihydroxyacetone and glyceraldehyde seemed to prevent hypoxic injury by partly preventing ATP depletion, they also normalised the hepatocyte NADH/NAD^+ ratio. Addition of the cytosolic NADH generating ethanol or the glycolytic substrates sorbitol, xylitol, glycerol further increased the reductive stress caused by prolonged hypoxia and increased the release of intracellular iron. The mitochondrial NADH generating substrates β-hydroxybutyrate, alanine or glutamine also increased hypoxic injury. Hypoxia may also convert xanthine dehydrogenase to xanthine oxidase [47] which could be responsible for reactive oxygen species formation and cause a loss of membrane integrity. This occurred in spite of the partial prevention of ATP depletion by sorbitol, xylitol, glycerol, alanine, glutamine and the lack of an effect of ethanol on ATP depletion. By contrast, the NADH oxidising intermediary metabolites, oxaloacetate or acetoacetate, acetaldehyde or artificial electron acceptors prevented hypoxic injury and normalised the hepatocyte NADH/NAD^+ ratio. ATP depletion was, however, not affected by oxaloacetate, acetoacetate and was partly prevented by acetaldehyde. The cell's susceptibility to hypoxic injury may therefore depend less on the cell's capacity to maintain energy status and more on the cell's capacity to maintain redox homeostasis.

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