ELSEVIER

Chemico-Biological Interactions 94 (1995) 37–48

Chemico-Biological Interactions

Desferal prevents against cell lysis induced by hydrogen peroxide to hypoxic hepatocytes: a role for free iron in hypoxia-mediated cellular injury

V. Lefebvre[1], P. Buc-Calderon*

Unité de Biochimie Toxicologique et Cancerologique, Département des Sciences Pharmaceutiques, Université Catholique de Louvain, Mounier 73, 1200 Bruxelles, Belgium

Received 7 June 1993; revision received 11 March 1994; accepted 14 March 1994

Abstract

Isolated hepatocytes incubated under hypoxic conditions were more sensitive to H_2O_2-mediated injury as compared to cells kept under aerobic conditions, but only for the highest H_2O_2 concentration tested (8 mM). At lower concentrations (2 and 4 mM) cells were still able to detoxify H_2O_2 even under hypoxic conditions. Reoxygenation of hypoxic hepatocytes did not result in a cytolytic effect, whereas reoxygenation in the presence of H_2O_2 resulted in an enhanced cytotoxicity. The duration of previous hypoxia (before H_2O_2 addition) did not affect the lytic effect induced by H_2O_2. Enzymatic activities of both catalase and glutathione peroxidase were unchanged over 2 h of incubation under hypoxic conditions. Preincubation of hepatocytes in the presence of Desferal (5 mM) resulted in the abolition of H_2O_2-mediated lytic effects. A role for free iron, released from intracellular stores and acting on H_2O_2 to yield reactive oxygen species is discussed.

Keywords: Hypoxia – reoxygenation; Iron release; Reductive stress; Hydrogen peroxide injury

* Corresponding author, Unité BCTC, UCL 7369, Av. E. Mounier 73, 1200 Bruxelles, Belgium. Tel: (02) 764 73 66; Fax: (02) 764 53 22; E-mail: Calderon@bctc.ucl.ac.be.

[1] Research Assistant of the National Fund for Scientific Research, Belgium.

SSDI 0009-2797(94)03319-4

1. Introduction

A wide variety of metabolic and biochemical changes is produced when cells are incubated under hypoxic conditions [1–7]. When hypoxia is prolonged and severe, most of the anabolic pathways are suppressed (gluconeogenesis, protein synthesis, etc), but it is rather difficult to distinguish between the intrinsic deleterious effects induced by hypoxia and the cellular adaptive responses against the hypoxic stress. Indeed, the depressed metabolism might be interpreted as an effective strategy of adaptation of cells in an adverse environment, the so-called 'metabolic arrest' [3,7] which leads to a preservation of critical cellular functions (i.e., ionic homeostasis) by decreasing metabolic rates. A similar process has been observed when cells are incubated at sublethal temperatures and further exposed to either a lethal temperature. The ability of cells to survive in such adverse conditions (thermotolerance) has also been extented to another kind of insults like H_2O_2, amino acids analogs, ethanol, etc. [9]. A depressed cellular metabolism may also lead, however, to a low cellular resistance to survive in an adverse environment, i.e., exposure of hepatocytes to 60 min of hypoxia resulted in a decreased tolerance to exposure to *tert*-butyl hydroperoxide [9]. Such enhanced susceptibility to oxidative injury has been explained as a consequence of the impaired metabolic and detoxication functions, rather than as an enhanced rate of generation of reactive species.

The reoxygenation of previously hypoxic tissues or cells enhances the metabolic impairment produced by hypoxia thus leading to a more pronounced cell death [10]. Experimental evidence suggests that such a process is mediated by the formation of reactive oxygen species, but, the elucidation of their molecular mechanisms as well as the identification of cells where they have been produced still remains controversial [10–15]. Among them, hydrogen peroxide (H_2O_2) plays an important role since it is a stable and oxidizing agent, which mediates reperfusion injury in different tissues [16–19]. Furthermore, such an H_2O_2-mediated injury has been shown to be reduced by the administration of catalase [16,20].

This work was undertaken with the aim to evaluate the sensitivity of isolated rat hepatocytes incubated under hypoxic conditions towards an oxidative stress induced by H_2O_2, an in vitro experimental model which simulates the hypoxia-reoxygenation injury. Such a model was previously used in order to characterize the deleterious effects of both hypoxia [21] and oxidative stress [22]. Moreover, increasing evidence suggests that reactive oxygen species are not generated intracellularly during reperfusion, but rather they are derived from neutrophils and Kupffer cells [11–14,23]. Therefore, to test whether hypoxic hepatocytes are still able to resist a subsequent oxidative injury, H_2O_2 was added as a bolus into cell suspensions and the viability of liver cells was monitored during the incubation. The enzymatic activities of both catalase and glutathione peroxidase, the two antioxidant enzymes which clear cells of H_2O_2 [24], were also determined under aerobic and hypoxic conditions. Finally, we evaluated a possible role for free iron by preincubating hepatocytes in the presence of desferal, a well-known iron chelator [22,25,26] which reduces H_2O_2-mediated injury [18,22]. Indeed, supporting a putative activity of iron, it has

been reported that Desferal decreased both the hepatocellular injury after a rat liver ischemia [27], and the efflux of cytosolic enzymes from isolated perfused rat liver subjected to hypoxia [15].

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–280 g were purchased from Iffa-Credo (Les Oncins, France) and housed in individual cages in a temperature- and light-controlled room. They received standard diet AO3 (UAR, Villemoisson-sur-Orge, France) and water ad libitum. Animals were fasted overnight before preparation of isolated hepatocytes.

2.2. Chemicals

Bovine serum albumin (BSA, fraction V) was from Sigma (St. Louis, MO, USA). Hydrogen peroxide (Perhydrol) was purchased from Merck (Darmstad, Germany). Desferal (desferrioxamine mesylate) was purchased from Ciba-Geigy (Switzerland). The gas mixtures O_2/CO_2 (95%/5%) and N_2/CO_2 (95%/5%) were purchased from Air Liquide (Liège, Belgium). Collagenase A (from *Clostridium histolyticum*) was from Boerhinger (Mannheim, Germany). Dulbecco's modified Eagle medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland). All other chemicals and reagents were of the purest grade available.

2.3. Preparation of isolated hepatocytes

Hepatocytes were isolated using the standard procedure described by Berry and Friend [28] and slightly modified by Krack et al. [29]. Briefly, animals were anaesthesized with an i.p. injection of pentobarbital (60 mg/kg), and cells were isolated by liver perfusion with Krebs solution containing collagenase. The yield of hepatocytes was usually in the range of 350–400 $\times$ 10^6 cells per liver, with viability varying from 85 to 95% as estimated by cell exclusion of erythrosine B. Isolated cells were then suspended in a volume of 20 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with BSA (0.3%), at a final concentration of 1 $\times$ 10^6 cells/ml of incubation. Hepatocytes were incubated at 37°C in a thermoregulated shaking waterbath (100 oscillations/min) under a continuous flow of either a mixture of O_2/CO_2 (95%/5%) for the aerobic conditions, or a mixture of N_2/CO_2 (95%/5%) for the hypoxic conditions. In order to maintain cells under these two conditions, 50 ml-rubber covered sealing flasks were used and cell suspensions were gassed through a stainless steel needle.

2.4. Assays

Hepatocyte viability was estimated by measuring the activity of lactate dehydrogenase (LDH) according to the procedure of Wrobleski and Ladue [30] both in the culture medium and in the cell pellet obtained after centrifugation as described elsewhere [29]. The results are expressed as a ratio of released activity to the total

activity. Catalase activity was measured by the method of Baudhuin [31]. GSH peroxidase activity was assayed as reported by Wendel using H_2O_2 as substrate [32]. The amount of protein was determined by the method of Lowry et al. [33] using BSA as a reference.

2.5. Statistics

Analysis of variance (two-way ANOVA with the interaction time treatment) was used to compare the concentration-response curves. The level of significance was set at $P < 0.05$.

3. Results

The time course of LDH leakage from hepatocytes incubated either under aerobic or hypoxic conditions and in the absence or in the presence of H_2O_2 is shown in Fig. 1a. In hepatocytes incubated under hypoxic conditions, cell death increased continuously reaching a value of 55% after 120 min of incubation. Conversely, LDH leakage from aerobic cells did not exceed 26% at the end of the incubation. Hypoxic conditions were also evaluated by measuring intracellular ATP content over the 120 min of incubation: while under hypoxic conditions cells contained 2.2 ± 0.5 nmol ATP/mg of protein at the end of incubation, they contained 11.3 ± 1.2 nmol ATP/mg of protein under aerobic conditions (data not shown). When H_2O_2 (8 mM) was added to the cell suspension 15 min after the onset of the incubation (indicated by the arrow), a strong increase in LDH leakage was recorded in cells incubated under hypoxic conditions. In the time interval from 15 to 30 min, the LDH leakage increased two-fold reaching 40%. At the end of the incubation (120 min) the release of LDH was greater than 75%. In aerobic cells however, the addition of H_2O_2 to the cell suspension also resulted in cell death but to a lower extent as compared to hypoxic cells. After 120 min of incubation, the release of LDH was only 38%.

A different profile of cell death was observed if hepatocytes incubated for 30 min under hypoxic conditions were further reoxygenated in the presence or absence of H_2O_2 (Fig. 1b). Indeed, the reoxygenation of hypoxic hepatocytes stopped the progression of their cell death, and LDH leakage remained fairly constant at a value around 35%. Nevertheless, when such a reoxygenation was performed in the presence of H_2O_2, an enhanced cell death was observed reaching 60% of LDH leakage. Although experimental conditions were not the same as in Fig. 1a, it must be pointed out that LDH leakage was higher in cells receiving H_2O_2 during hypoxia as compared to cells which were first hypoxic and then reoxygenated.

The enhanced cell death induced by H_2O_2 was independent from the duration of the previous hypoxia. As shown in Fig. 1c, H_2O_2 at 8 mM induced more or less the same release of LDH from hepatocytes (2–2.5-fold). The addition of H_2O_2 at 15, 30 and 40 min of incubation increased the LDH leakage by 2.1-, 2.4- and 2.6-fold respectively. Lower concentrations of H_2O_2 (2 and 4 mM), induced a LDH leakage similar to that of cells incubated under hypoxic conditions but in the absence of H_2O_2 (data not shown).

The enzymatic activities of both catalase and glutathione peroxidase (the two

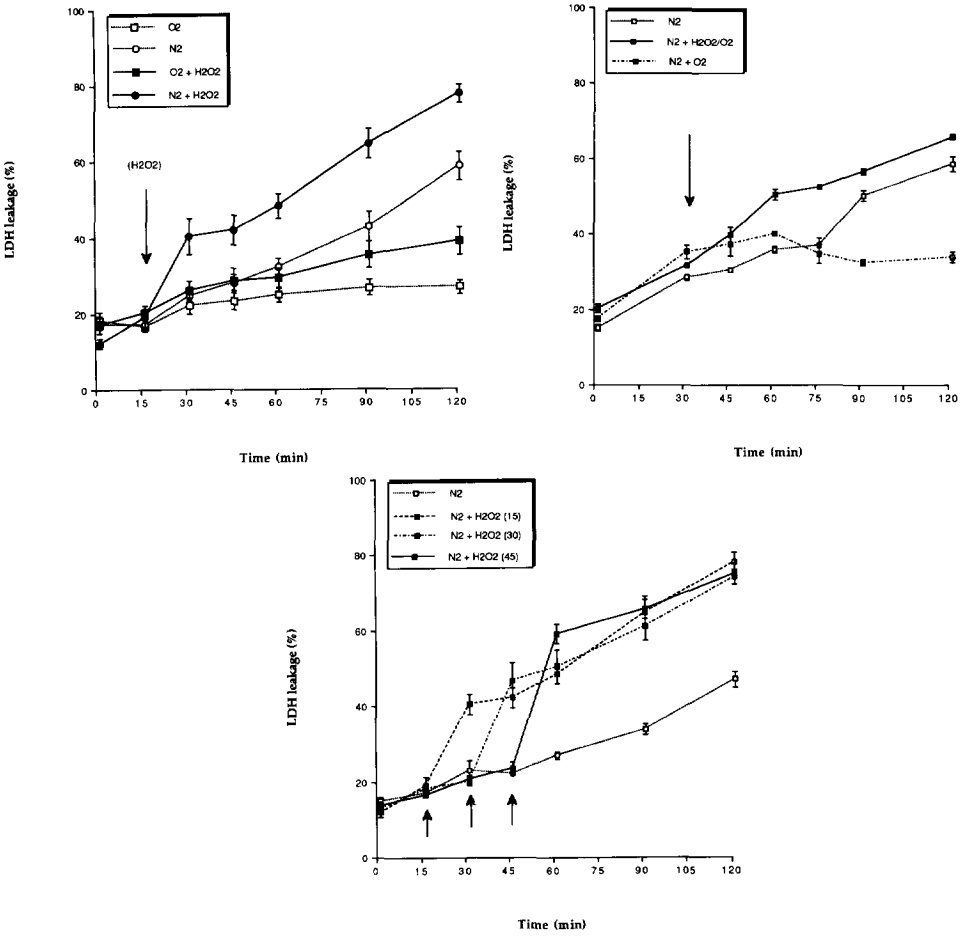

Fig. 1. (a) Hepatocytes were incubated for 120 min under aerobic conditions or hypoxic conditions. H_2O_2 at 8 mM was added into aerobic or hypoxic cell suspensions 15 min after the onset of incubation. (b) Hepatocytes were incubated for 120 min under hypoxic conditions. After 30 min of hypoxic incubation, cells were reoxygenated either in the absence or in the presence of H_2O_2 at 8 mM. (c) Hepatocytes were incubated for 120 min under hypoxic conditions. After either 15, 30 or 45 min of incubation, H_2O_2 at 8 mM was added into hypoxic cellular suspensions. At the time indicated, aliquots of the cell suspension were taken and LDH leakage was measured as described under Materials and methods. Values are means 1 ± standard error of the mean (S.E.M.) of at least three separate experiments. Arrow represents the time of H_2O_2 addition.

enzymes involved in the cellular metabolism of H_2O_2) were measured within cells incubated for 120 min either under aerobic or hypoxic conditions (Table 1). Catalase activity remained unchanged during the 120 min of incubation (~ 300 mU/mg protein) under these two different experimental conditions. Nevertheless, the enzymatic

Table 1
Activity of antioxidant enzymes (catalase and glutathione peroxidase) of isolated hepatocytes during hypoxia

Time (min)	GSH peroxidase (mU/mg protein)		Catalase (mU/mg protein)[a]	
	Aerobic	Hypoxic	Aerobic	Hypoxic
0	120 ± 12	119 ± 25	304 ± 45	289 ± 29
60	122 ± 8	98 ± 20	283 ± 52	294 ± 29
120	125 ± 19	97 ± 17	278 ± 19	327 ± 84

Hepatocytes were incubated for 120 min either under aerobic or hypoxic conditions. At the time indicated, aliquots of the cell suspension were taken and activities of both glutathione peroxidase and catalase were measured as described under Materials and methods. Values are means ± standard error of the mean (S.E.M.) of at least three separate experiments.
[a]Enzymatic units as defined by Baudhuin et al [22].

activity of glutathione peroxidase was slightly decreased (~20%) at the end of the incubation under hypoxic conditions (100 mU/mg protein) as compared to its initial value (120 mU/mg protein). Under aerobic conditions, glutathione peroxidase activity remained fairly constant. Statistical analysis (two-way ANOVA) indicated however, that there were no significant differences between these two conditions.

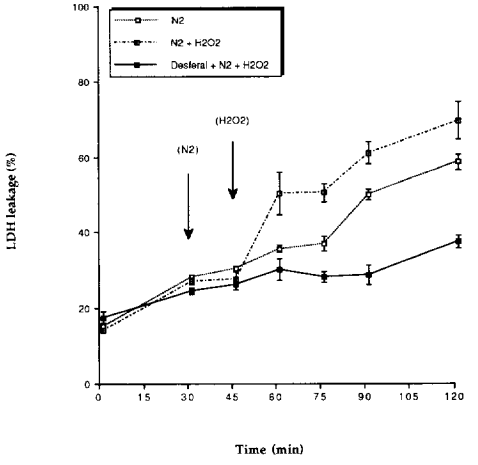

Fig. 2. Effect of Desferal on the lytic effect induced by H_2O_2 within hypoxic hepatocytes. Hepatocytes were incubated for 120 min under different experimental conditions: cells always kept under hypoxic conditions in the absence of H_2O_2; hepatocytes incubated under hypoxic conditions plus H_2O_2 (8 mM) which was added after 45 min of incubation; and cells preincubated for 30 min under aerobic conditions in the presence of 5 mM Desferal, then incubated for 15 min under hypoxic conditions, and received afterwards 8 mM of H_2O_2. At the time indicated, aliquots of the cell suspension were taken and LDH leakage was measured as described under Materials and methods. Values are means ± standard error of the mean (S.E.M.) of at least three separate experiments.

A putative role of free iron (released from intracellular stores during hypoxia) acting on H_2O_2 (Fenton reaction) was tested by using Desferal. The effect of Desferal on hepatocytes which were hypoxic at first and further exposed to H_2O_2 is shown in Fig 2. The preincubation of hepatocytes for 30 min in the presence of Desferal resulted in the abolition of the deleterious effect induced by hypoxia plus H_2O_2. Indeed, the LDH leakage was unchanged after exposure of hepatocytes to hypoxic conditions (N_2/CO_2 was applied 30 min after the onset of the incubation). Under these conditions, when H_2O_2 was added (45 min after the onset of the incubation and 15 min after hypoxia), the release of LDH was slightly increased from 22% to 27%, reaching a value of 37% at the end of the incubation. Conversely, liver cells preincubated for 30 min in the absence of Desferal, then incubated under hypoxic conditions for 15 min and further exposed to H_2O_2, showed a LDH leakage higher than 60%. Since hepatocytes which were kept under hypoxic conditions but in the absence of H_2O_2, showed a LDH leakage of about 50% at the end of the incubation, it is suggested that Desferal also protects, at least partially, against hypoxia-mediated injury.

4. Discussion

In agreement with previous reports [5–7], our results confirm that hypoxia induces cell injury which may ultimately lead to cell death. We report here that H_2O_2 is more lytic to hypoxic cells as opposed to hepatocytes incubated under aerobic conditions. Considering that hypoxic cells lose their membrane integrity and have a depressed metabolic profile, such a cytolytic effect of H_2O_2 appears as a logic consequence of the deleterious effects already induced by hypoxia [3,7,21]. Nevertheless, since H_2O_2 at either 2 or 4 mM did not enhance the LDH leakage of hypoxic cells, it may be taken as an index that hepatocytes are still able to metabolize H_2O_2.

Our results also show that the duration of hypoxia did not influence the sensitivity of hypoxic cells towards H_2O_2. For instance, no enhanced cell death was observed whatever the duration of hypoxia (i.e., 15, 30, or 45 min). In addition, both catalase and glutathione peroxidase activities were unchanged during the hypoxic incubation. Therefore, it is unlikely that the enhanced susceptibility of hypoxic cells might be associated with a progressive impairment of cellular antioxidant defences, although it has been reported that hypoxia decreases the intracellular content of reduced glutathione [34].

In an attempt to reproduce ischemic conditions where lactate accumulates, Kowalski et al. [35] have recently reported that addition of lactate protected cells in a concentration-dependent manner, most probably by supplying reducing equivalents for peroxide reduction. Indeed, intracellular acidosis (resulting from anaerobic glycolysis) has been reported to have a beneficial effect on cell survival during hypoxia [36]. Nevertheless, while such a low pH avoids the activation of degradative enzymes, intracellular acidosis may lead to iron mobilization from storage proteins (i.e., ferritin) thus releasing a 'catalytically active' iron inside the cells. In addition to intracellular acidosis as a possible mechanism to explain iron mobilization within cells, it has been reported that iron may be released from ferritin

by the enzyme xanthine oxidase in both the presence and in the absence of O_2 [37]. This enzyme is formed from xanthine dehydrogenase by proteolytic conversion during hypoxia [38]. However, while these mechanisms (pH and xanthine oxidase) might contribute in some way to iron mobilization from intracellular stores, it appears that reductive stress (due to the tremendous amount of NADH accumulated during hypoxia), is the main mechanism which can explain how iron is released from ferritin. Indeed, NADH by reducing Fe^{3+} to Fe^{2+} can release small amounts of iron from ferritin in vitro, an effect which is more efficient in the presence of flavine nucleotides [39].

Another proposed mechanism to explain iron mobilization from storage proteins during hypoxia, is that proteases acting on metalloenzymes may release iron within cells. Such a possibility is however unlikely since proteolysis, at least during the early phase of hypoxia, is inhibited [21]. The release and accumulation of 'free' iron represents then, a tremendous potential danger for the cell, since the exogenously added H_2O_2 provides the second substrate needed for free radical production by the Fenton reaction [22,40,41].

We reported previously that H_2O_2-mediated lytic effect to hepatocytes are strongly enhanced in the presence of iron salts [22]. Under these experimental conditions, the preincubation of hepatocytes with Desferal abolished not only the cytolytic effects produced by the association of iron salts and H_2O_2, but also by H_2O_2 alone [22]. On the other hand, iron chelation by Desferal prevents injury in rat liver tissues after ischemia alone or with subsequent reperfusion [27] as well as hypoxia-mediated damage to isolated perfused rat livers [15]. These results suggested the existence of an intracellular source of iron which was available for H_2O_2, but its identification is quite difficult. Ferritin is the main iron-storage protein, and obviously the principal target for iron release. Indeed, while some mechanisms have been proposed to explain such a mobilization from ferritin [37,39], iron leakage from metalloproteins rarely occurs, since as already discussed, proteolysis is rather inhibited during early phase of hypoxia [21].

The preincubation of hepatocytes in the presence of Desferal (5 mM) totally prevents against H_2O_2-mediated cytolysis. On the basis of this result, as well as our previous report [22], we conclude that free iron is needed to express the lytic effect of H_2O_2 under hypoxic conditions. The molecular mechanism(s) leading to cytotoxicity is (are) still unknown, but it appears unlikely that lipid peroxidation in particular, may play a crucial role. Indeed, under aerobic conditions, oxidative degradation of membrane phospholipids appears already as a minor and secondary mechanism contributing to cytotoxicity of H_2O_2 [22]. Moreover, the propagation step of a lipid peroxidation reaction:

$$(L^{\cdot} + O_2 \rightarrow LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \rightarrow L^{\cdot} \rightarrow O_2 \rightarrow LOO^{\cdot}, \text{ etc})$$

will be strongly minimized at low pO_2. Then, it is rather difficult that lipid peroxidation may be involved in the cytotoxicity by H_2O_2 to hypoxic hepatocytes.

Since the enhanced cytotoxicity of H_2O_2 during hypoxia is devoid of a satisfactory explanation at a molecular level, the following scheme is proposed (Fig. 3):

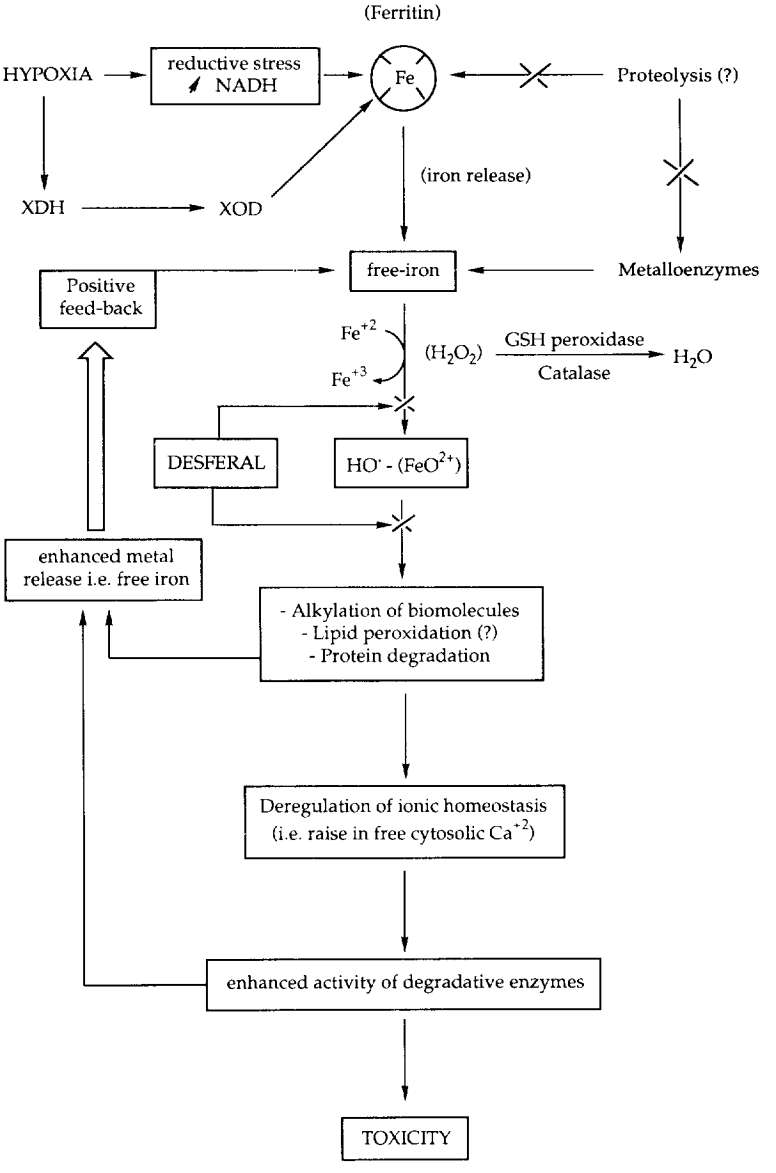

Fig. 3. Increased susceptibility of hypoxic hepatocytes to deleterious effects of H_2O_2. A progressive decrease in pO_2 levels induces the activation of glycogenolysis with formation of lactic acid and NADH accumulation. The reductive stress and the low pH increases iron release from ferritin (xanthine oxidase and proteolysis also contributed but in a minor way). In the presence of H_2O_2, iron ions catalyse the formation of hydroxyl radicals ($OH^{\cdot}$) and/or other reactive oxygen species, which by destroying biomolecules leads ultimately to cell toxicity.

hypoxia increases the release of iron from ferritin by either intracellular acidosis (low pH), by catalytic action of xanthine oxidase, or by reductive stress (enhanced NADH formation). A minor contribution, at least in the early phase of hypoxia, may proceed by proteolytic destruction of metalloenzymes. When free iron accumulates within cells and H_2O_2 overwhelms the detoxification metabolic ability of both catalase and glutathione peroxidase, highly reactive oxygen species are produced by the Fenton reaction. Once generated, they destroy essential biomolecules leading to an increased metal release and a loss of cellular functions (i.e., ionic pumps). Impairment of calcium homeostasis, for instance, will result in the activation of degradative enzymes. Such a proteolysis triggers positive feed-back reactions thus, increasing the release of transition metals. Finally, cellular toxicity occurs. Desferal either by chelating iron or acting as a free radical scavenger, is able to prevent against the deleterious effects induced by H_2O_2 to hypoxic hepatocytes. Intracellular acidosis by anaerobic glycolysis and formation of reactive oxygen species during reperfusion (wherever they are being produced), are two unavoidable phenomena during organ transplantation procedures. On the basis of previous results [15,27] and those reported herein, we support the use of Desferal during in situ liver perfusion before organ removal from liver donors. Such a procedure may chelate iron (wherever the intracellular source may be) yielding it inaccessible for H_2O_2 which is overproduced during reperfusion.

Acknowledgements

The authors express their gratitude to Prof. R. Verbeek for his valuable advice, and to Mr. M. Cheddin for his excellent technical assistance. This work was supported by grant no. 3.4528.91 from the Fund for Medical Scientific Research (Belgium).

References

1 J.L. Farber, F.R. Chien and S.J.R. Mittnacht, The pathogenesis of irreversible cell injury in ischemia, Am. J. Pathol., 102 (1981) 271–281.
2 G. Van den Berghe, M.F. Vincent and F. Bontemps, Pathways and control of adenine nucleotide catabolism in anoxic rat hepatocytes, Biomed. Biochim. Acta, 48 (1989) 5–10.
3 P.W. Hochachka, Defense strategies against hypoxia and hypothermia, Science, 231 (1986) 234–241.
4 I. Anundi and H. de Groot, Hypoxic liver cell death: critical pO_2 and dependence of viability on glycolysis, Am. J. Physiol., 257 (1989) G58–64.
5 J.P. Kehrer, D.P. Jones, J.J. Lemasters and H. Jaeschke, Mechanisms of hypoxic cell injury, Toxicol. Appl. Pharmacol., 106 (1990) 165–178.
6 A.W. Harman, A.L. Nieminen, J.J. Lemasters and B. Herman, Cytosolic free magnesium, ATP, and blebbing during chemical hypoxia in cultured rat hepatocytes, Biochem. Biophys. Res. Commun., 170 (1990) 477–483.
7 P. Buc-Calderon, V. Lefebvre and M. Van Steenbrugge, Inhibition of protein synthesis in isolated hepatocytes as an immediate response to oxygen limitation, in: P.W. Hochachka, G. Lutz, T. Sick, M. Rosenthal and G. van den Thillart (Eds.), Surviving hypoxia: Mechanisms of Control and Adaptation, CRC Press, Boca Raton, 1993, pp 271–280.
8 YR. Donati, D.O. Slosman and B.S. Polla, Oxidative injury and the heat-shock response, Biochem. Pharmacol., 40 (1990) 2571–2577.

9 D.L. Tribble, D.P. Jones and D.E. Edmonson, Effect of hypoxia on *tert*-butyl hydroperoxide-induced oxidative injury in hepatocytes, Mol. Pharmacol., 34 (1988) 413–420.
10 J.T. Flaherty and M.L. Weisfeldt, Reperfusion injury, Free Rad. Biol. Med., 5 (1988) 409–419.
11 H. Jaeschke, C.V. Smith and J.R. Mitchell, Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver, J. Clin. Invest., 81 (1988) 1240–1246.
12 J. Metzger, S.P. Dore and B.H. Lauterburg, Oxidant stress during reperfusion of ischemic liver. No evidence for a role of xanthine oxidase, Hepatology, 8 (1988) 580–584.
13 A. Koo, H. Komatsu, G. Tao, M. Inoue, P.H. Guth and N. Kaplowitz, Contribution of no-reflow phenomenon to hepatic injury after ischemia reperfusion: evidence for a role for superoxide anion, Hepatology, 15 (1991) 507–514.
14 H. Jaeschke, A. Farhood, A.P. Bautista, Z. Spolarics, J.J. Spitzer and C.W. Smith, Functional inactivation of neutrophils with a Mac-1 (CD11b/CD18) monoclonal antibody protects against ischemia-reperfusion injury in rat liver, Hepatology, 17 (1993) 915–923.
15 M Younes and O. Strubelt, The involvement of reactive oxygen species in hypoxic injury to rat liver, Res. Commun. Chem. Pathol. Pharmacol., 59 (1988) 369–381.
16 S.R. Jolly, W.J. Kane, M.B. Bailie, G.D. Abrams and B.R. Lucchesi, Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase, Circ. Res., 54 (1984) 277–285.
17 J.H. Jackson, C.W. White, N.B. Parker, J.W. Ryan and J.E. Repine, Dimethylthiourea consumption reflects H_2O_2 concentration and severity of acute lung injury, J. Appl. Physiol., 59 (1985) 1995–1998.
18 J.M. Brown, L.S. Terada, M.A. Grosso, G.J. Whitman, S.E. Velasco, A. Patt, A.H. Harken and J.E. Repine, Hydrogen peroxide mediates reperfusion injury in the isolated rat heart, Mol. Cell. Biochem., 84 (1988) 173–175.
19 M.S. Paller, Hydrogen peroxide and ischemic renal injury: effect of catalase inhibition, Free Rad. Biol. Med., 10 (1991) 29–34.
20 C.L. Myers, S.J. Weiss, M.M. Krish and M. Schlafer, Involvement of hydrogen peroxide and hydroxyl radical in the 'oxygen paradox' reduction of creatine kinase release by catalase, allopurinol or deferoxamine, but not by superoxide dismutase, J. Mol. Cell. Cardiol., 17 (1985) 675–684.
21 V. Lefebvre, M. Van Steenbrugge, V. Beckers, M. Roberfroid and P. Buc-Calderon, Adenine nucleotides and inhibition of protein synthesis in isolated hepatocytes incubated under different Po_2 levels, Arch. Biochem. Biophys., 304 (1993) 322–331.
22 I. Latour, J.L. Pregaldien and P. Buc-Calderon, Cell death and lipid peroxidation in isolated hepatocytes incubated in the presence of hydrogen peroxide and iron salts, Arch. Toxicol., 66 (1992) 743–749.
23 M. Okuda, H.C. Lee, B. Chance and C. Kumar, Glutathione and ischemia reperfusion injury, Free Rad. Biol. Med., 12 (1992) 271–279.
24 D.P. Jones, L. Eklöw, H. Thor and S. Orrenius, Metabolism of hydrogen peroxide in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogeneously generated H_2O_2, Arch. Biochem. Biophys., 210 (1981) 505–516.
25 B.H. Halliwell, Protection against tissue damage in vivo by desferrioxamine: what is its mechanism of action ? Free Rad. Biol. Med., 7 (1989) 645–651.
26 S.K. Jonas, P.A. Riley and R.L. Wilson, Hydrogen peroxide cytotoxicity. Low temperature enhancement by ascorbate or reduced lipoate, Biochem. J., 264 (1989) 651–655.
27 R. Omar, I. Nomikos, G. Piccorelli, J. Savino and N. Agarwal, Prevention of postischaemic lipid peroxidation and liver cell injury by iron chelation, Gut, 30 (1989) 510–514.
28 M.N. Berry and D.S. Friend, High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study, J. Cell Biol., 43 (1969) 506–520.
29 G. Krack, O. Gravier, M. Roberfroid and M. Mercier, Subcellular fractionation of isolated hepatocytes. A comparison with liver homogenates, Biochim. Biophys. Acta, 632 (1980) 619–629.
30 F. Wrobleski and J. Ladue, Lactic dehydrogenase activity in blood, Proc. Soc. Exp. Biol. Med., 90 (1955) 210–213.
31 P. Baudhuin, H. Beaufay, Y. Rahman-Li, O.Z. Selliger, R. Wattiaux, P. Jacques and C. de Duve, Tissue fractionation studies. Intracelullar distribution of monoamineoxydase, aspartate aminotrans-

ferase, alanine aminotransferase, D amino acid oxidase and catalase in rat liver tissue, Biochem. J., 92 (1964) 179–184.

32 A. Wendel, Glutathione peroxidase, Methods Enzymol., 77 (1981) 325–333.

33 O. Lowry, N. Rosebrough, L. Farr and R. Randall, Protein measurement with the Folin reagent, J. Biol. Chem., 183 (1951) 265–275.

34 X. Shan, T.Y. Aw, R. Shapira and D.P. Jones, Oxygen dependence of glutathione synthesis in hepatocytes, Toxicol. Appl. Pharmacol., 101 (1989) 261–270.

35 D.P. Kowalski, T.Y. Aw, Y. Park and D.P. Jones, Postanoxic oxidative injury in rat hepatocytes: lactate-dependent protection against *tert*-butyl hydroperoxide, Free Rad. Biol. Med., 12 (1992) 205–212.

36 R.T. Currin, G.J. Gores, R.G. Thurman and J.J. Lemasters, Protection by acidotic pH against anoxic cell killing in perfused rat liver: evidence for a pH paradox, FASEB J., 5 (1991) 205–210.

37 P. Biemond, A.J.G. Swaak, C.M. Beindorff and J.F. Koster, Superoxide dependent and-independent mechanisms of iron mobilization from ferritin by xanthine oxidase. Implications for oxygen-free-radical-induced tissue destruction during ischaemia and inflammation, Biochem. J., 239 (1986) 169–173.

38 E. Della Corte and F. Stirpe, The regulation of rat liver xanthine oxidase, Biochem. J., 126 (1972) 739–745.

39 H. Jaeschke, C. Kleinwaechter and A. Wendel, NADH-dependent reductive stress and ferritin-bound iron in alkyl alcohol-induced lipid peroxidation in vivo: the protective effect of vitamin E, Chem.-Biol. Interact., 81 (1992) 57–68.

40 R.A. Floyd, Direct demonstration that ferrous ion complexes of di- and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide, Arch. Biochem. Biophys., 225 (1983) 263–270.

41 B.H. Halliwell and J.M.C. Gutteridge, Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts, Arch. Biochem. Biophys., 246 (1986) 501–514.