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Deleterious effects of xanthine oxidase on rat liver endothelial cells after ischemia/reperfusion

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Abstract

Previous studies have demonstrated that reactive oxygen species are involved in ischemic injury. The present work was undertaken to determine in vivo the role of xanthine oxidase in the oxygen free radical production during rat liver ischemia and to examine the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) during the same period. Our results indicate a 4-fold increase in xanthine oxidase activity between 2 and 3 hours of normothermic ischemia, in parallel with a decrease in cell viability. Moderate hypothermia delays both events. Under the same conditions, the activity of oxygen radical scavenging enzymes remains unchanged. Moreover, we have compared in vitro the susceptibility of isolated liver cells to an oxidative stress induced by $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$. Our results reveal that endothelial cells are much more susceptible to reactive oxygen species than hepatocytes, probably because they lack H_2O_2-detoxifying enzymes. These findings suggest that xanthine oxidase might play a major role in the ischemic injury mainly at the level of the sinusoidal space where most endothelial cells are located.

Keywords: Xanthine oxidase; Ischemia; Reperfusion; Endothelium; Hepatocyte; (Rat); (Liver)

1. Introduction

It has been generally admitted that ischemic cell injury is mediated by reactive oxygen species [1]. Both xanthine oxidase inhibitors (allopurinol and oxypurinol) and enzymatic scavengers of O_2 free radicals (superoxide dismutase and catalase) limit the extent of post-ischemic injury, suggesting a role for xanthine oxidase-derived superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide in the process [2–5]. In healthy tissues, xanthine oxidase exists almost exclusively in a dehydrogenase form which yields uric acid by using NAD^+ as an electron acceptor. During ischemia, xanthine dehydrogenase could be converted to xanthine oxidase by limited proteolysis or sulfhydryl oxidation or both [6–8]. This conversion results in the production of $O_2^{\cdot-}$, H_2O_2 and, subsequently in the formation, of hydroxyl radicals ($^{\cdot}OH$). These reactive oxygen species can induce peroxidation of polyunsaturated fatty acids, protein oxidation and DNA strand breakage [9]. In addition, they may play an important role in recruiting and activating polymorphonuclear leukocytes [10]. These cells, once activated and attached to endothelial cells may exacerbate tissue injury via the generation of reactive oxidants and the secretion of several enzymes (i.e., myeloperoxidase, elastase, collagenase) [10].

However, the role of xanthine oxidase in the pathogenesis of ischemic cell injury is still controversial because: (i) in some cases, the time frame of conversion of xanthine dehydrogenase-xanthine oxidase is inconsistent with cell death [8,11], (ii) conflicting data have been obtained in vivo with allopurinol [2,12], (iii) allopurinol might also act by preserving the nucleotide pool during ischemia [13] or by scavenging hydroxyl radicals [14], and (iv) xanthine oxidase has been detected only in small amounts in human myocardium [15].

In the work reported here, we have investigated the change of xanthine oxidase activity induced by ischemia in rat liver, and the effects of exogenous xanthine oxidase on isolated hepatocytes and liver endothelial cells. We have also determined the activity of several antioxidant enzymes in rat liver during ischemia and in isolated liver cells in

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order to estimate whether these endogenous antioxidant enzymes are sufficient to protect cells against the oxidative stress generated by xanthine oxidase.

2. Materials and methods

2.1. In vivo experiments

Male Wistar rats weighing 200–220 g and fasted 24 h before surgery were used. Ischemia was induced under ether anesthesia by clamping the vascular pedicle of the left liver lobe with a small forceps [15]. The animals were killed and the control and ischemic hepatic lobes were removed, weighed and homogenized separately in cold Tris-HCl 0.1 M pH 8.1 buffer. Hypothermia was obtained by i.p. Nembutal (Ceva, Belgium) administration (dose ranging from 25 to 40 mg/kg body weight). In this case, animals were unconscious during the whole ischemic period in order to control body temperature with a probe (thermocouple NiCr-Ni) placed in the hepatic area and connected to a digital thermometer. Adjustments were made by placing rats under a heating lamp or on an ice bed.

Xanthine oxidase activity was immediately measured on the supernatants, obtained by high speed centrifugation of homogenates (39 000 rpm, 40 min, in the n°40 rotor of the Spinco ultracentrifuge), by following uric acid production ($\varepsilon_{292\ nm} = 9.65\ M^{-1}\ cm^{-1}$) in a reaction medium containing 100 μM Tris-HCl (pH 8.1) and 60 μM xanthine [17]. The total activity of xanthine dehydrogenase plus xanthine oxidase was obtained after addition of 0.13 mM NAD^+. Superoxide dismutase activity (Cu-Zn SOD) was measured at room temperature, on the same supernatants, by assessing the inhibition of the rate of epinephrine oxidation by superoxide radicals [18]. The reaction medium contained 100 μM epinephrine, 200 μM EDTA, 20 mM triethanolamine hydrochloride (pH 9.0) and xanthine oxidase ($\varepsilon_{480\ nm} = 4020\ M^{-1}\ cm^{-1}$). Catalase and glutathione peroxidase were assayed on the homogenates as described respectively in [19] and [20]. Catalase activity was determined at 4°C in a reaction medium containing 200 μM imidazole (pH 7.0), 0.04% Triton X-100, 0.1% bovine serum albumin and 0.005% H_2O_2. The reaction was stopped by the addition of 7.8 mM $TiOSO_4$ which combines with the residual H_2O_2 to give rise to a yellow compound. Absorbance was read at 420 nm. Catalase unit was defined as the amount of enzyme which, under experimental conditions, decreases the log $[H_2O_2]$ by 1 in one minute. Selenium-dependent glutathione peroxidase was measured at room temperature by following the NADPH disappearance ($\varepsilon_{340\ nm} = 6.22\ M^{-1}\ cm^{-1}$) in a reaction mixture consisting of 0.09 mM *tert*-butyl hydroperoxide, 0.8 mM reduced glutathione (GSH), 1.1 U/ml glutathione reductase, 0.07 mM NADPH and 0.5 mM EDTA/ 30 mM potassium phosphate (pH 8.0). Proteins were estimated by the method of Lowry et al. [21] using bovine serum albumin as standard.

Data were expressed as means ± S.E.M. Statistical comparisons of values were made by analysis of variance (ANOVA, BMDP 8V, BMDP Statistical Software, Los Angeles, CA) followed by a Student's paired *t*-test; $p < 0.05$ was considered significant.

2.2. Cell isolation and culture

Isolated hepatic cells were essentially prepared by the method described by Seglen [22]. After liver perfusion with collagenase, parenchymal (PC) and non-parenchymal cells (NPC) were separated from an initial cellular suspension by differential centrifugation (16 g, 2 min). Purified endothelial cells (EC) were obtained by centrifugal elutriation in 2 steps [23]. The first step aimed at discarding the remaining parenchymal cells (rotor speed: 1500 rpm, flow rate: from 10 to 25 ml/min). The second one allowed us to separate endothelial cells from Kupffer and fat-storing cells (rotor speed: 2500 rpm, flow rate: from 13 to 22 ml/min). Endothelial cells were then sedimented at 335 g for 5 min. All centrifugation was carried out at 4°C in a medium containing 0.34 mM Na_2HPO_4, 0.35 mM KH_2PO_4, 0.8 mM $MgSO_4$, 40 mM Hepes, 137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$ (pH 7.6) plus 1% bovine serum albumin (E Buffer). Dead endothelial cells were discarded by isopycnic centrifugation in a solution of Nycodenz (density = 1.15 g/ml) [23].

Monolayers of PC and EC were maintained overnight in collagen-coated dishes containing Dulbecco's modified Eagle's medium plus 10% foetal calf serum (FCS). The average number of cells grown per cm^2 was $0.21 \cdot 10^6$ for PC and $0.42 \cdot 10^6$ for EC. The endothelial cell identity was confirmed by the observation of the characteristic sieve plates using scanning electron microscopy.

Cultured cells were loaded with ^{51}Cr (3 μCi/ml) for a minimum of 3 h before exposure to different sources of reactive oxygen molecules. Then, 500 μM xanthine + 18 mU/ml xanthine oxidase or 23 mM glucose + 30 mU/ml glucose oxidase prepared in 2 ml of FCS-deprived medium were added to the monolayers. At various times, aliquots of media were removed for ^{51}Cr and lactate dehydrogenase activity (LDH) measurements. After a maximum of 5 h of treatment, 0.1% Triton X-100 was added to obtain the total LDH activity or the total amount of ^{51}Cr. The data were expressed as the percent of ^{51}Cr or enzymatic activity released in the media at the various sampling times. In some experiments, superoxide dismutase (100 U/ml) and/or catalase (72 U/ml) were added at the same time as xanthine oxidase.

Lactate dehydrogenase (LDH) activity was determined by recording the NADH disappearance at 340 nm in a reaction medium containing 1 mM pyruvate, 0.1 mM NADH and 50 mM Tris-HCl (pH 7.4) [24]. For the endothelial cells, LDH activity was measured by a more

sensitive method. It involved following the NADH appearance associated with lactate oxidation using a SCF-500 TM model spectrofluorimeter (SLM Instruments). The reaction medium contained 0.13 M lactate, 44 mM sodium pyrophosphate (pH 8.8) and 0.67 mM NAD^+ [25].

2.3. Chemicals

Xanthine oxidase (EC 1.2.3.2 from cow milk, grade III), superoxide dismutase (EC 1.15.1.1 from bovine erythrocytes), catalase (EC 1.11.1.6 from bovine liver), glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*), glutathione reductase (EC 1.6.4.2. from baker's yeast), GSH, pyruvate, lactate, NAD^+ and NADH were purchased from Sigma (St. Louis, MO, USA). TBHP was from Merck (Darmstadt, Germany). 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) and $FeCl_3$ were obtained from Janssen (Beerse, Belgium). Desferal was from Ciba-Geigy (Groot Bijgarden, Belgium). Nycodenz and ^{51}Cr were purchased respectively from Nycomed (Norway) and from NEN, Dupont de Nemours (Dreiech, Germany).

3. Results

3.1. In vivo experiments on the liver

In these experiments, left liver lobe ischemia was performed on rats maintained at normal temperature or subjected to hypothermia as described in [16]. Indeed, it is known that a decrease of rat body temperature by a few degrees markedly increases the resistance of liver to ischemia [26]. Table 1 reports the xanthine oxidase (XO) or xanthine dehydrogenase plus xanthine oxidase (XD + XO) activity in the rat left liver lobe deprived of blood supply for increasing times, as compared with the unligated lobes used as control. At normal body temperature, the activities of xanthine oxidase in the ischemic and control lobes are quite similar during the first two hours. Later, xanthine oxidase activity is markedly increased in the ischemic lobe after 3 h and becomes 4-times higher than the corresponding control lobes. This increase results from the conversion of xanthine dehydrogenase to xanthine oxidase, since the XO + XD activity is not significantly changed during the same period. Hence, from 20% after 0 h of ischemia, the ratio XO/(XO + XD) reaches 42 and 55% in the hepatic lobes that had been subjected to 3 h and 4 h of ischemia, respectively. When rat temperature is maintained at 30°C during the entire ischemic period, the increase in xanthine oxidase activity is delayed by 1 h (Table 1). The ANOVA analysis confirmed that blood deprivation has a significant effect on xanthine oxidase activity ($p < 0.02$) but not on XO + XD activity ($p = 0.11$). These results strengthen the hypothesis that xanthine oxidase is implicated in cell death induced by ischemia. Previous reports have indicated that ischemia has to be maintained for 2–3 h to irreversibly alter liver tissue and that hypothermia delays this point of no return [26]. It is noteworthy that, as it was observed for the release of lysosomal enzymes under the same experimental conditions [26], a decrease in temperature of only 2–3°C is sufficient to prevent an increase of xanthine oxidase in ischemic liver (Fig. 1).

Exogenous superoxide dismutase, alone or in combination with catalase, as well as several free radical scavengers, have often been found to be beneficial in improving ischemia-reperfusion injury [3–5]. Therefore, we have

Table 1
Activities of xanthine oxidase activity (XO), xanthine oxidase + xanthine dehydrogenase activity (XO + XD) and ratio XO(XO + XD) in rat liver subjected to ischemia at 37 or 30°C

Time	37°C			30°C		
(h)	spec. act. (mU/mg prot.)		ratio XO/(XO + XD)	spec. act. (mU/ mg prot.)		ratio XO/(XO + XD)
	XO	XO + XD	(%)	XO	XO + XD	(%)
0	0.30 ± 0.03	1.48 ± 0.20	20.8 ± 1.2	0.25 ± 0.08	1.49 ± 0.35	16.3 ± 1.2
	0.33 ± 0.05	*1.61 ± 0.28*	*20.5 ± 1.7*	*0.24 ± 0.04*	*1.53 ± 0.18*	*15.2 ± 1.3*
1	0.30 ± 0.02	1.70 ± 0.29	18.4 ± 2.6	0.30 ± 0.03	1.67 ± 0.03	18.1 ± 2.0
	0.31 ± 0.04	*2.05 ± 0.16*	*15.0 ± 1.4*	*0.34 ± 0.02*	*2.19 ± 0.17*	*15.5 ± 0.1*
2	0.25 ± 0.05	1.43 ± 0.22	17.0 ± 1.9	0.34 ± 0.03	2.05 ± 0.13	16.5 ± 0.5
	0.24 ± 0.08	*1.68 ± 0.16*	*14.6 ± 4.9*	*0.38 ± 0.11*	*2.26 ± 0.37*	*15.8 ± 3.6*
3	1.04 ± 0.17 [a]	2.23 ± 0.24	47.7 ± 8.4 [a]	0.48 ± 0.14	2.09 ± 0.43	22.1 ± 6.7
	0.29 ± 0.04	*1.81 ± 0.15*	*16.0 ± 2.3*	*0.51 ± 0.03*	*2.85 ± 0.19*	*18.2 ± 2.9*
4	1.24 ± 0.23 [a]	1.92 ± 0.13	63.1 ± 7.8 [b]	0.46 ± 0.04 [a]	1.73 ± 0.17	26.6 ± 2.5 [a]
	0.29 ± 0.03	*1.62 ± 0.16*	*18.7 ± 3.6*	*0.30 ± 0.04*	*1.82 ± 0.26*	*16.9 ± 2.3*
5				0.85 ± 0.02 [b]	1.69 ± 0.04	50.4 ± 0.9 [b]
				0.33 ± 0.06	*1.91 ± 0.43*	*17.2 ± 0.4*
6				1.17 ± 0.35	1.85 ± 0.45	62.0 ± 8.4 [a]
				0.29 ± 0.05	*1.57 ± 0.18*	*18.2 ± 1.6*

Measurements were performed on the non-sedimentable fraction of the ischemic lobes (plain) and the corresponding control lobes (*italic*) in the absence (XO) or presence of 0.13 mM NAD^+ (XO + XD). Results are expressed as means ± S.E.M. ($n = 4$ at 37°C and 3 at 30°C).
[a] $= p < 0.03$.
[b] $= p < 0.01$.

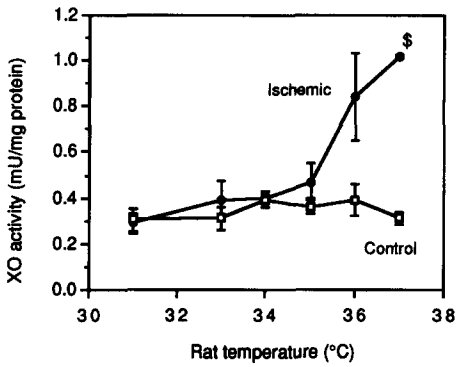

Fig. 1. Relationship between rat temperature during the ischemic period and the increase of the specific activity of xanthine oxidase. Ischemia was maintained for 4 hours after i.p. Nembutal administration. Measurements were performed on the non-sedimentable fraction of the ischemic and the corresponding control lobes in absence of 0.13 mM NAD^+. Results are expressed as means ± S.E.M. ($n = 3$) ($ = $p < 0.01$).

decided to investigate the influence of ischemia on the endogenous activity of superoxide dismutase, catalase and glutathione peroxidase. As shown in Table 2, these antioxidant enzymes are not significantly affected by ischemia ($p = 0.50$, 0.29 and 0.09, respectively), suggesting that they are still able to remove the reactive oxygen species delivered by xanthine oxidase after 4 h of ischemia.

3.2. Effect of reactive oxygen species on isolated liver cells

To explore the possible deleterious effect of xanthine oxidase on liver cells, we incubated isolated hepatocytes in the presence of xanthine and xanthine oxidase, a system frequently used to study the cytotoxic effects initiated by $O_2^{\cdot-}$ and H_2O_2. Hepatocytes were selected because they represent the major cellular component of the liver mass and volume and are mainly responsible for specific liver metabolic activity. The cells ($2 \cdot 10^6$/dish) were first loaded with ^{51}Cr (3 μCi/ml), treated with xanthine/xanthine oxidase or glucose/ glucose oxidase, then at various times, medium samples were taken to evaluate ^{51}Cr and lactate dehydrogenase released during the treatment. As illustrated in Fig. 2, hepatocytes withstand such an oxidative stress. Little or no ^{51}Cr (or LDH, data not shown) release is observed when these cells are exposed to the xanthine oxidase system, even though the production of the reactive hydroxyl radicals is stimulated by the addition of chelated Fe^{3+} [9]. In this case, only 18% of ^{51}Cr are released by the cells versus 10% for control cells. It is noteworthy that this resistance to an oxidative stress is also observed when the glucose/glucose oxidase system is used and when the cellular density drops to $1 \cdot 10^6$/dish (data not shown).

Table 2
Specific activities of Cu-Zn SOD, catalase and glutathione peroxidase in rat liver subjected to ischemia at 37°C

Ischemia (h)		Cu-Zn SOD (U/mg protein)	Catalase (U/mg protein)	Glutathione peroxidase (mU/mg protein)
0 h	Ischemic	19.8 ± 6.8	0.25 ± 0.04	0.33 ± 0.03
	Control	*20.2 ± 5.2*	*0.24 ± 0.01*	*0.33 ± 0.05*
1 h	Ischemic	15.6 ± 4.5	0.22 ± 0.04	0.25 ± 0.06
	Control	*15.6 ± 4.0*	*0.25 ± 0.02*	*0.30 ± 0.06*
2 h	Ischemic	19.7 ± 5.2	0.30 ± 0.05	0.28 ± 0.04
	Control	*19.6 ± 4.9*	*0.33 ± 0.03*	*0.31 ± 0.06*
4 h	Ischemic	16.7 ± 18.9	0.29 ± 0.10	0.31 ± 0.06
	Control	*16.1 ± 5.5*	*0.30 ± 0.07*	*0.35 ± 0.06*

Measurements were performed on the non-sedimentable fraction (SOD) or homogenates (catalase and glutathione peroxidase) of the ischemic lobes and the corresponding control lobes at 4°C (for catalase) and room temperature (for the others). Values are means ± S.E.M. of 3 independent experiments. No significant difference was observed.

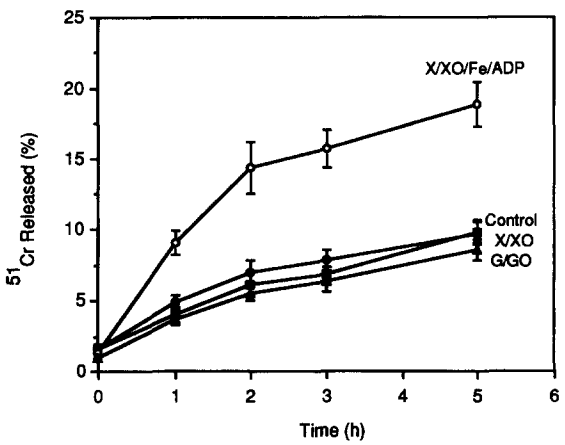

Fig. 2. Viability of hepatocytes exposed to an oxidative stress. The ^{51}Cr-preloaded cells (3 μCi/ml) were incubated for a maximum of 5 hours in the presence of 500 μM xanthine + 18 mU/ml xanthine oxidase (X/XO) or 23 mM glucose + 45 mU/ml glucose oxidase (G/GO) in a FCS-free culture medium. In some cases, 0.2 mM $FeCl_3$ / 2mM ADP were added to the culture medium to yield $^{\cdot}OH$ radicals. At various time points, aliquots were taken to measure the ^{51}Cr released in the culture medium. After 5 h, cells were lysed and the residual radioactivity was counted. Results are expressed as the percentage of ^{51}Cr released in the medium versus total ^{51}Cr (aliquots + lysate). Results are means ± S.E.M. of 3 independent experiments.

Similar experiments have been performed with isolated liver endothelial cells. Contrary to what is observed for hepatocytes, an important release of ^{51}Cr takes place when endothelial cells are incubated in presence of the xanthine oxidase system (Fig. 3). Identical results have been obtained with LDH (data not shown). Since xanthine oxidase is able to give rise to $O_2^{\cdot-}$ and H_2O_2 under aerobic conditions, we tried to identify the main cytotoxic agent by adding antioxidant enzymes in the medium. As depicted in Fig. 3, exogenous catalase, but not superoxide dismutase prevents the loss of plasma membrane integrity. Simultaneous addition of both catalase and SOD does not afford a better protection (data not shown). Therefore, in agreement with Link and Riley [27], it seems that hydrogen peroxide, or one of its derivatives, is the main cytotoxic agent of the xanthine/xanthine oxidase system. The addition of chelated Fe^{3+} does not accentuate the ^{51}Cr release (Fig.

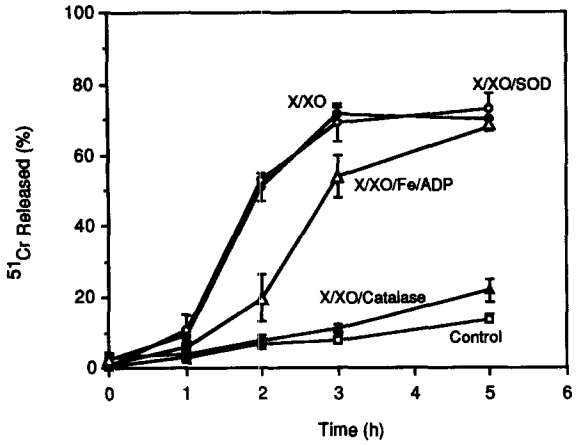

Fig. 3. Viability of endothelial cells exposed to xanthine/xanthine oxidase. The ^{51}Cr-preloaded cells were incubated for a maximum of 5 h in the presence of 500 μM xanthine and 18 mU/ml xanthine oxidase in a FCS-free culture medium. In some experiments, superoxide dismutase (100 U/ml) or catalase (72 mU/ml) was also present. At various time points, aliquots were taken to measure the ^{51}Cr released in the culture medium. After 5 hours, cells were lysed and the residual radioactivity was counted. Results are expressed as the percentage of ^{51}Cr released in the medium versus total ^{51}Cr (aliquots + lysate). Results are expressed as means ± S.E.M. ($n = 3$).

3), suggesting that hydroxyl radicals, produced by the Fenton reaction, are not required for the deleterious process. The absence of protection exerted by 'OH scavenger such as α-tocopherol (10 μM) or by an iron chelator like deferroxamine (5 mM) supports this conclusion (data not shown). The cytotoxicity of H_2O_2 was confirmed by the exposure of endothelial cells to the glucose/glucose oxidase system which yields exclusively hydrogen peroxide (Fig. 4). Here again, catalase prevents cell death.

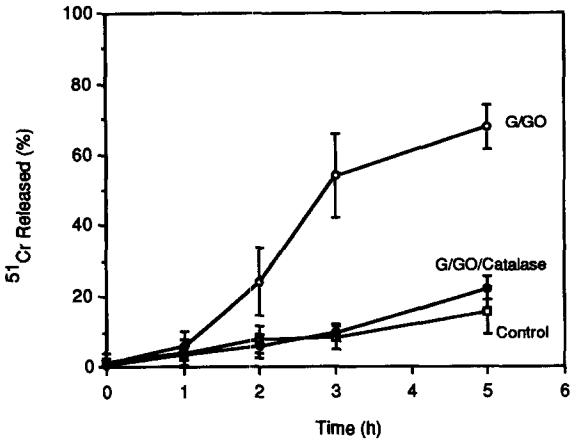

Fig. 4. Viability of endothelial cells exposed to glucose/glucose oxidase. The ^{51}Cr-preloaded cells were incubated for a maximum of 5 h in a FCS-free culture medium containing 23 mM glucose and 30 mU/ml glucose oxidase with or without catalase (72 U/ml). At various time points, aliquots were taken to measure the ^{51}Cr released in the culture medium. After 5 h, cells were lysed and the residual radioactivity counted. Results are expressed as the percentage of ^{51}Cr released in the medium versus total ^{51}Cr (aliquots + lysate). Results are means ± S.E.M. ($n \geq 3$).

Table 3
Specific enzymatic activities in endothelial and parenchymal cells in primary culture

Enzymes	Endothelial cells	Hepatocytes	Ratio EC/PC
Superoxide dismutase	146.0 ± 19.6 (7)	460.8 ± 21.2 (8)	0.32
Catalase	0.78 ± 0.08 (7)	273.2 ± 18.2 (12)	0.003
Glutathione peroxidase	42.4 ± 2.2 (8)	349.9 ± 13.2 (11)	0.12
Glutathione reductase	6.4 ± 0.2 (2)	19.7 ± 1.1 (8)	0.33

Liver cells were isolated and purified as described in Section 2. Enzymatic activities were measured on cellular homogenates at 4°C (for catalase) and room temperature (for the others). Results are expressed in U (superoxide dismutase) or μU (catalase, glutathione peroxidase, glutathione reductase) per μg of protein and correspond to mean ± S.E.M. The number of experiments is indicated in parentheses.

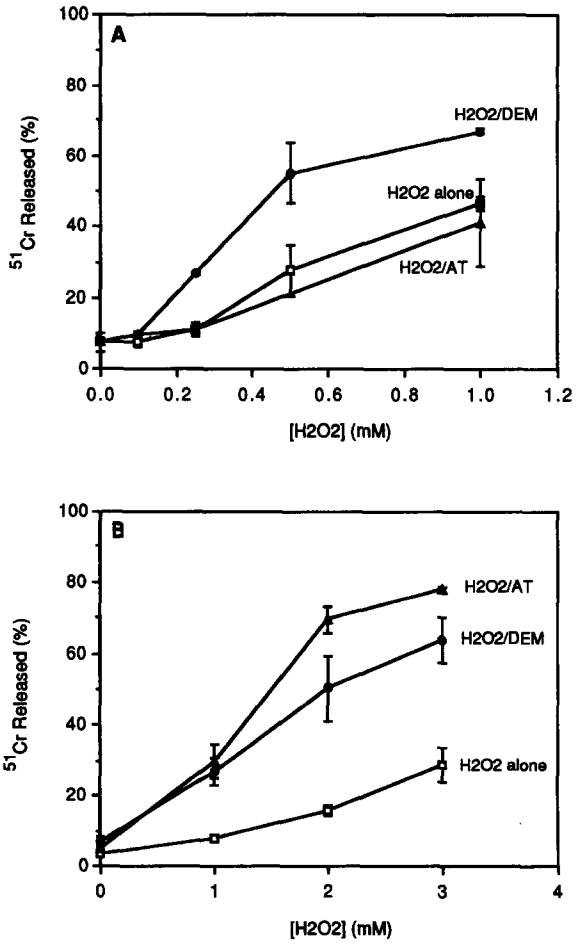

Fig. 5. Viability of endothelial cells (A) or hepatocytes (B) exposed to hydrogen peroxide as a function of H_2O_2 concentration. Influence of catalase inhibition and reduced glutathione depletion. The ^{51}Cr-preloaded cells were incubated in the presence of various H_2O_2 concentrations for 2 h. In some experiments, 2.5 mM 3-amino-1,2,4-aminotriazole (AT) or 1 mM diethylmaleate (DEM) was added to the medium. At the end of the treatment, cells were lysed and the residual radioactivity counted. Results are expressed as the percentage of ^{51}Cr released in the medium versus total ^{51}Cr (aliquots + lysate). Values represent means ± S.E.M. of 3 (EC) or 4 (PC) independent experiments.

3.3. Antioxidant enzymes in isolated liver cells

A possible explanation for the difference between hepatocytes and endothelial cells with respect to their resistance to an oxidative stress could originate from a difference in their content of antioxidant enzymes. This is the reason why we measured these enzymes in the two kinds of cells. As indicated in Table 3, the activity of antioxidant enzymes is strikingly lower in endothelial cells than in parenchymal cells. This is particularly true for the two hydrogen peroxide detoxifying enzymes, catalase and glutathione peroxidase, which exhibit respectively 0.3 and 12% of the activity measured in hepatocytes. The weak activity of catalase is probably related to the small number of peroxisomes present in endothelial cells [28].

To obtain further information about the relative contribution of endogenous catalase and glutathione peroxidase in the defense of endothelial cells and hepatocytes against hydrogen peroxide, we selectively inhibited catalase with 3-amino-1,2,4-triazole (AT) and glutathione peroxidase by depletion of intracellular stores of reduced glutathione with diethylmaleate (DEM) [29]. At the concentrations chosen, AT and DEM completely inhibit both H_2O_2 detoxifying enzymes and cell viability is preserved (data not shown). Inhibition of catalase does not increase ^{51}Cr release induced by the addition of up to 1 mM H_2O_2 to endothelial cells (Fig. 5A). In sharp contrast, the blockade of the glutathione redox cycle dramatically increases the endothelial cells susceptibility to H_2O_2. In hepatocytes, both antioxidant defense systems play a crucial role in detoxifying hydrogen peroxide (Fig. 5B).

4. Discussion

Restoration of blood flow after a period of ischemia is associated with the production of reactive oxygen species. These reactive molecules can be generated by several catalytic activities, including xanthine oxidase, cyclooxygenase and NADPH oxidase/myeloperoxidase of activated leucocytes, or cellular mechanisms such as mitochondrial or microsomal electron transport. In post-ischemic livers, a decrease in oxygen scavenging enzyme activity has been demonstrated [30], aggravating the imbalance between generation and removal of reactive oxygen species.

In the present study, we report a marked increase in xanthine oxidase activity between 2 and 3 hours of ischemia (at the time of no return), resulting from the conversion of xanthine dehydrogenase to xanthine oxidase. This increase is probably accompanied by an important production of oxygen radicals, since hypoxanthine and xanthine accumulate in ischemic tissues [31] and pO_2 is apparently sufficient even during ischemia to allow $O_2^{\cdot-}$ generation by xanthine oxidase [32]. Two additional lines of evidence suggest that xanthine oxidase is involved in liver cell injury induced by ischemia. At normal body temperature, xanthine oxidase activity is enhanced at the same time that cell death occurs and when body temperature falls to or below 35°C, both phenomena are delayed. Of course, cell death is a multifactorial process and probably other parameters must be taken into account also. For example, mitochondria are also a major source of superoxide anions and hydrogen peroxide during ischemia/reperfusion [30]. In particular, ubisemiquinone and NADH-dehydrogenase flavin-semiquinone release a large amount of superoxide anions upon reoxygenation owing to their increased autooxidation rate. The superoxide anions serve as precursors for H_2O_2 which is able to diffuse through mitochondrial membranes to the cytosol and give rise to highly oxidizing species [9].

Our findings reveal that the specific activities of SOD, catalase and glutathione peroxidase do not change significantly during the first four hours of ischemia. In contrast, Gonzalez-Flecha et al. [30] observed a 50% decrease in these antioxidant enzymes after 120 and 180 min of ischemia followed by 30 min of reperfusion. This difference can be explained in two ways. First, reperfusion triggers a burst of oxygen derived species production which can in turn induce a decrease in enzymatic activities. Second, when our results are expressed in mU/g liver as in [30], catalase and glutathione peroxidase activities drop to 70% after 4 h of ischemia (data not shown). This decrease is significant ($p < 0.05$ and 0.01, respectively). But if proteins, which decrease significantly during ischemia ($p < 0.001$), are taken into account, the activities (in mU/g protein) of both enzymes remain unchanged.

Owing to their high levels, it seems likely that oxygen radical scavenging enzymes are able to remove all the superoxide anions and hydrogen peroxide produced by xanthine oxidase. This is true on condition that production and removal of oxygen free radicals are equally balanced in the different kinds of liver cells. Our results show that this is probably not the case, because: (i) endothelial cells lack critical oxygen reactive scavenging enzymes, mostly catalase and glutathione peroxidase, responsible for H_2O_2 detoxification and (ii) XO appears to be primarily localized in EC [33]. In our experiments, XO activity, measured on cultured cells, was 0.70 and 0.24 mU/mg protein for EC and PC, respectively. The low antioxidant activities, together with the abundance of xanthine oxidase in endothelial cells could account for their susceptibility to oxidative stress. In addition, the especially low catalase activity explains why endothelial cells pretreated with 3-amino-1,2,4-triazole, a specific catalase inhibitor, release ^{51}Cr in a similar way to non-pretreated cells, while depletion of intracellular reduced glutathione stores with diethylmaleate enhances endothelial cell susceptibility to H_2O_2. In marked contrast, in hepatocytes, both defense systems contribute equally to H_2O_2 decomposition.

Recently, it has been demonstrated that activated Kupffer cells are able to produce reactive oxygen species [34].

Unfortunately, Kupffer cells isolated by elutriation do not survive overnight in culture. Under these conditions it is not possible to examine their resistance to an oxidative stress as has been done with hepatocytes and endothelial cells.

The xanthine/xanthine oxidase system is known to yield $O_2^{\cdot-}$ and H_2O_2. Due to its short half – life and poor reactivity in aqueous solution, $O_2^{\cdot-}$ by itself is unable to cause cell death [9]. This is confirmed by the inability of SOD, added in our system, to improve EC survival. In contrast, H_2O_2, produced directly by xanthine oxidase or indirectly by dismutation of $O_2^{\cdot-}$, appears to be cytotoxic to EC, since exogenous catalase completely abolishes cell damage. Hydroxyl radical $^{\cdot}OH$, eventually generated from $O_2^{\cdot-}$ and H_2O_2 in the presence of reducing transition-metal ions, is probably not implicated in cell death, since mannitol and α-tocopherol do not afford any protection to EC. Moreover, it seems unlikely that the highly reactive $^{\cdot}OH$ can reach the cell membrane before its decomposition [9]. Taken together, these results suggest that, under our conditions, H_2O_2 is the major cytotoxic agent and the protective role of catalase in the glucose/glucose oxidase system strengthens this conclusion. In view of its relative stability and its ability to diffuse through cell membranes, H_2O_2 can exert its deleterious action either at the cell surface or on intracellular components. For instance, H_2O_2 can interact with the cytosolic cytochrome *P*-450 and give rise to the ferryl radical (FeO^{2+}), a highly reactive oxidizing species capable of initiating free radical chain reactions [9].

A question that remains to be solved is why there is no decrease in EC survival on addition of SOD to the xanthine/xanthine oxidase system. When EC were directly exposed to H_2O_2, the survival curves resembled each other until the H_2O_2 concentration reached 0.5 mM (data not shown). Hence, it seems possible that in the presence of SOD, the increase of H_2O_2 production was not sufficient to induce a further loss of EC viability.

The primary localization of xanthine oxidase in endothelial cells [33], along with the lack of antioxidant enzymes in these cells, and the production of O_2 reactive species by activated Kupffer cells [34] or by ischemic hepatocytes of the central region [33] suggest that an oxidative stress may arise first in the hepatic sinusoidal space during ischemia or reperfusion. Results showing that lipid peroxidation occurs, during reperfusion after a prolonged liver ischemia, only in the nonparenchymal cell portion of the liver and not in the hepatocyte fractions support this assumption [35]. Subsequently, this oxidative stress might induce the recruitment and activation of leukocytes, extending the insult to the parenchymal cells.

Finally, our results underline the importance of considering a given problem in vivo and in vitro. Indeed, the experiments performed on the whole liver tend to demonstrate that antioxidant enzymes are able to remove all the reactive oxygen species produced by xanthine oxidase during ischemia. In contrast, those performed on isolated liver cells indicate clearly that even though hepatocytes resist oxidative stress, endothelial cells do not survive long under these conditions.

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References

[1] Baker, J.E., Felix, C.C., Olinger, G.N. and Kalyanaraman, B. (1988) Proc. Natl. Acad. Sci. USA 85, 2786–2789.
[2] Parks, D.A., Bulkley, B., Granger, D.N., Hamilton, S.R. and McCord, J.M. (1982) Gastroenterology 82, 9–25.
[3] Mehta, J.L., Nichols, W.W., Donnelly, W.H., Lawson, D.L., Thompson, L., Ter Riet, M. and Saldeen, T.G.P. (1989) Circ. Res. 65, 1283–1295.
[4] Ambrosio, G., Zweier, J.L., Jacobus, W.E., Weisfeldt, M.L. and Flaherty, J.T. (1987) Circulation 76, 906–915.
[5] Jeroudi, M.O., Triana, F.J., Patel, B.S. and Bolli, R. (1990) Am. J. Physiol. 259, H889–H901.
[6] Granger, D.N., Rutili, G., McCord, J.M. (1981) Gastroenterology 81, 22–29.
[7] Bindoli, A., Cavallini, L., Rigobello, M.P., Coassin, M. and Dilisa, F. (1988) Free Rad. Biol. Med. 4, 163–167.
[8] Engerson, T.D., McKelvey, T.G., Rhyne, D.B., Boggio, E.B., Snyder, S.J. and Jones, H.P. (1987) J. Clin. Invest. 79, 1564–1570.
[9] Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 219, 1–14.
[10] Granger, D.N. (1988) Am. J. Physiol. 255, H1269–H1275.
[11] Parks, D.A., Williams, T.K. and Beckman, J.S. (1988) Am. J. Physiol. 254, G768–G774.
[12] Reimer, K.A. and Jennings, R.B. (1985) Circulation 71, 1079–1075.
[13] Lasley, R.D., Ely, S.W., Berne, R.M. and Mentzer, R.M. (1988) J. Clin. Invest. 81, 16–20.
[14] Moorhouse, P.C., Grootveld, M., Halliwell, B., Quinlan, J.G. and Gutteridge, J.M.C. (1987) FEBS Lett. 213, 23–28.
[15] Moriwaki, Y., Yamamoto, T., Suda, M., Nasako, Y., Takahashi, S., Agbedana, O.E., Hada, T. and Higashino, K. (1993) Biochim. Biophys. Acta 1164, 327–330.
[16] Chien, K.R. and Farber, J.L. (1977) Arch. Biochem. Biophys. 180, 191–198.
[17] Della Corte, E. and Stirpe, F. (1972) Biochem. J. 126, 739–745.
[18] Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
[19] Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., Jacques, P. and de Duve, C. (1964) Biochem. J. 92, 179–184.
[20] Flohe, L. and Günzler, W.A. (1984) Methods Enzymol. 105, 114–121.
[21] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[22] Seglen, P.O. (1976) Methods Cell Biol. 13, 29–83.
[23] Nenseter, M-S., Blomhoff, R., Drevon, C.A., Kindberg, G.M., Norum, K.R. and Berg, T. (1988) Biochem. J. 254, 443–448.
[24] Wroblewski, F. and La Due, S.S. (1955) Proc. Soc. Exp. Biol. Med., 90, 210.
[25] Roth, M. (1974) Methods Biochem. Anal. 17, 215.
[26] Wattiaux, R. and Wattiaux-De Coninck, S. (1984) Int. Rev. Exp. Pathol. 26, 85–106.

[27] Link, E.M. and Riley, P.A. (1988) Biochem. J. 249, 391–399.
[28] Blouin, A., Bolender, R.T. and Weibel, E.R. (1977) J. Cell Biol. 72, 441–455.
[29] Harlan, J.M., Levine, J.D., Callahan, K.S., Schwartz, B.R. and Harker, L.A. (1984) J. Clin. Invest. 73, 706–713.
[30] Gonzalez-Flecha, B., Cutrin, J.C. and Boveris, A. (1993) J. Clin. Invest. 91, 456–464.
[31] Kamiike, W., Watanabe, F., Hashimoto, T., Tagawa, K., Ikeda, Y., Nakao, K. and Kawashima, Y. (1982) J. Biochem. 91, 1349–1356.
[32] Rao, K.S. and Müller, H.S. (1983) Adv. Exp. Med. Biol. 161, 347–364.
[33] Frederiks, W.M., Marx, F. and Kooij, A. (1993) Int. J. Exp. Pathol. 74, 21–26.
[34] Rymsa, B., Wang, J.-F. and De Groot, H. (1991) Am. J. Physiol. 261, G602–G607.
[35] Walsh, T.R., Rao, P.N., Makowka, L. and Starzl, T.E. (1990) J. Surg. Res. 49, 18–22.