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DAMAGE OF ERYTHROCYTES BY ACTIVATED OXYGEN GENERATED IN HYPOXIC RAT LIVER

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The implication of activated oxygen in the interaction between hypoxic rat liver and circulating erythrocytes was investigated.

Reduced species of oxygen generated in hypoxic liver owing to accelerated purine nucleotide degradation via xanthine oxidase initiate alterations of plasma membrane and glutathione system of erythrocytes. Osmotic fragility, hemolysis rate and erythrocytic GSSG:GSH ratio may be considered as appropriate indicators of oxidative load in liver and other tissues.

Addition of erythrocytes to the perfusion medium attenuates the GSSG efflux of hypoxic liver from $2.7 \pm 0.5\,\text{nmol} \times \text{g w.w.}^{-1} \times \text{min}^{-1}$ to $1.4 \pm 0.2\,\text{nmol} \times \text{g w.w.}^{-1} \times \text{min}^{-1}$

Thus, circulating erythrocytes protect the liver against oxidative attack.

KEY WORDS: Oxygen radicals, erythrocytes, rat liver, hypoxia, glutathione, hemolysis, osmotic fragility.

ABBREVIATIONS: GSH, reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid), NEM, N-ethylmaleimide; DDC, diethyldithiocarbamate.

INTRODUCTION

Activated species of oxygen exert a multitude of effects on erythrocytes: perturbation of the glutathione system,[1–5] oxidation of hemoglobin,[3,6–9] lipid peroxidation, increased movement of phosphatidylserine and phosphatidylethanolamine between the bilayer leaflets, cross-linking of proteins, inhibition of enzymes and changes of fluidity of the membrane[10–15] as well as increased osmotic fragility and hemolysis.[1–3,6,9,14,16,17] An overview of studies related to these effects was carried out by Chiu *et al.*[18] Products of radical chain initiation and propagation such as $O_2^{\cdot-}$, H_2O_2 and aldehydes penetrate membranes and may be able, therefore, to act at sites far from their locus of origin.[19,20]

In mixed suspensions of erythrocytes with cellular or subcellular radical-generating systems such as neutrophils,[21] microorganisms,[7] and liver microsomes[22] an oxidative loading of the red cells was observed. Therefore, an oxidative attack on erythrocytes

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Dedicated to Professor em. Dr. mult. S.M. Rapoport on the occasion of his 75th birthday.

may be expected if these cells are circulating through organs producing and liberating oxygen radicals. This effect was shown with perfused liver and lung preparations.[23,24]

The rat liver produces a burst of H_2O_2 and related oxygen species under hypoxia owing to facilitated purine nucleotide breakdown via xanthine oxidoreductase and urate oxidase.[25–27] This model was chosen to elucidate the following problems: (i) Does the oxidative load of hypoxic rat liver have an effect on erythrocytes suspended in the perfusion medium? (ii) Is there, conversely, an effect of circulating erythrocytes on the hypoxic liver?

MATERIALS AND METHODS

Preparation of Red Blood Cells and of the Liver for Perfusion

Blood was collected from several male animals of a Wistar rat H-strain, the red cells were separated by centrifugation, washed, pooled and suspended in Krebs-Henseleit-bicarbonate buffer (pH 7.4, 5 mM glucose, 2 mM lactate, 0.3 mM pyruvate). In order to make the red cells more susceptible to products from the oxidatively loaded liver they were preincubated for one hour in that buffer modified by omission of glucose and addition of 2.5 mM diethyldithiocarbamate in some experiments.

For preparation of livers, animals of the same strain with body weight of 290 ± 12 g and liver weight of 12.8 ± 0.5 g were taken. The rats had free access to standard commercial pellet food and tap water. The procedure of organ preparation was in accord with that in earlier investigations.[25]

Perfusion of the Liver

Krebs-Henseleit solution of the composition presented above and with erythrocytes suspended therein to a hematocrit of 10 ± 1% was used as perfusion medium. The flow-rate of the perfusate was kept constant during each particular experiment at about 2 ml × min^{-1} × g w.w.$^{-1}$ The liver was surrounded by perfusion medium.

The perfusate was gassed in a bubble oxygenator with air (normoxia, pO_2 of the influent 19 kPa) or with nitrogen (hypoxia, pO_2 of the influent about 1 kPa; that is due to air contact of perfusate in the liver preservation flask).

In order to check solely the influence of the perfusion system onto erythrocytes without interference of the liver experiments only with red cell suspensions were carried out.

The protective effect of erythrocytes directed on the hypoxic liver was studied by perfusion experiments in presence or absence of red cells. In order to approach the oxygen consumption rate of the hypoxic perfusions with erythrocyte suspensions (about 200–300 nmol × min^{-1} × g w.w.$^{-1}$) in hemoglobin-free perfusions a pO_2 of 5 kPa in the influent was adjusted.

In addition few experiments were performed in a non-recirculating mode to avoid accumulation in the perfusate of products of oxidative load. The temperature was 25° or 37°C, as indicated in the figures.

Analytical Procedures

GSH was assayed according to Beutler *et al.*,[28] GSSG was determined fluorimetrically.[29] The GSH autoxidation was prevented by addition of 50 mM N-ethylmaleimide.

The autoxidation of GSH in the perfusion medium was estimated as previously described.[25] The osmotic fragility of red cells was measured according to Sacher and Miller.[30]

The glutathione status and the osmotic fragility were determined immediately after rapid washing of the cells in saline. In order to exclude errors originating from red cell swelling the data on GSH and GSSG concentrations were corrected by taking into account variations of the hemoglobin concentration.

Lactate and pyruvate were determined with lactate dehydrogenase (E.C. 1.1.1.27). Hemoglobin and methemoglobin were measured as reported.[31]

Reagents

DTNB was purchased from Sigma Chemical Co. (St. Louis, USA), N-ethylmaleimide and o-phthaldialdehyde from Calbiochem (San Diego, USA) and GSH and GSSG from Boehringer (Mannheim, FRG). Sodium hexobarbital, lactate dehydrogenase and allopurinol were obtained from VEB Arzneimittelwerk Dresden (GDR), DDC was from Reanal (Budapest, Hungary).

RESULTS AND DISCUSSION

During 2 hours hypoxic perfusion the concentration of intraerythrocytic GSH decreased from 2.9 mmol $\times$ (l cells)$^{-1}$ to about 75% (Figure 1). This decrease is almost abolished under normoxic conditions and prevented by allopurinol or sodium formate. The most drastic change was observed in erythrocytes pretreated with DDC.

The GSSG:GSH ratio in erythrocytes was higher in experiments with hypoxia of the liver in comparison with normoxia (Figure 2). The initial values are relatively high but this may be due to washing of red cells in glucose free buffer. Since glucose is present during perfusion these ratios decrease as expected. In the experiment with hypoxia there was initially an elevation of the GSSG:GSH ratio which is caused by a burst of activated oxygen production within the first hour.

The dynamics of the glutathione status in erythrocytes results at least from three mechanisms: (i) the attack of activated oxygen and related secondary products formed in liver and perfusate on erythrocytic thiol groups, (ii) antioxidative mechanisms within erythrocytes and eventually in the suspending medium counteracting this attack, (iii) the efflux of GSSG from erythrocytes.

The third mechanism seems to be of minor quantitative importance. It was quantitated in studies with circulation of an erythrocyte suspension in the perfusion apparatus without liver. Within the first hour of perfusion the rate of GSSG egress was 0.26 ± 0.12 nmol $\times$ min^{-1} $\times$ (ml cells)$^{-1}$ and in the second hour it was 0.11 ± 0.06 nmol $\times$ min^{-1} $\times$ (ml cells)$^{-1}$. These values correspond to data found by others.[32,33] The modulation of this efflux by the liver which was not measured was obviously negligible for two reasons: (i) the rate-determining factors of GSSG efflux of erythrocytes[33] changed only to a limited extent, (ii) the total release of GSSG from erythrocytes is very small in comparison with that of the liver.

In Figure 3 is depicted the extent of hemolysis during perfusions. There is a considerably higher rate in the hypoxia experiments. The data on osmotic fragility are demonstrated in Figure 4. After preincubation of red cells 50% hemolysis is approached at 65–67 mM NaCl. At the end of one hour of hypoxic perfusion this value

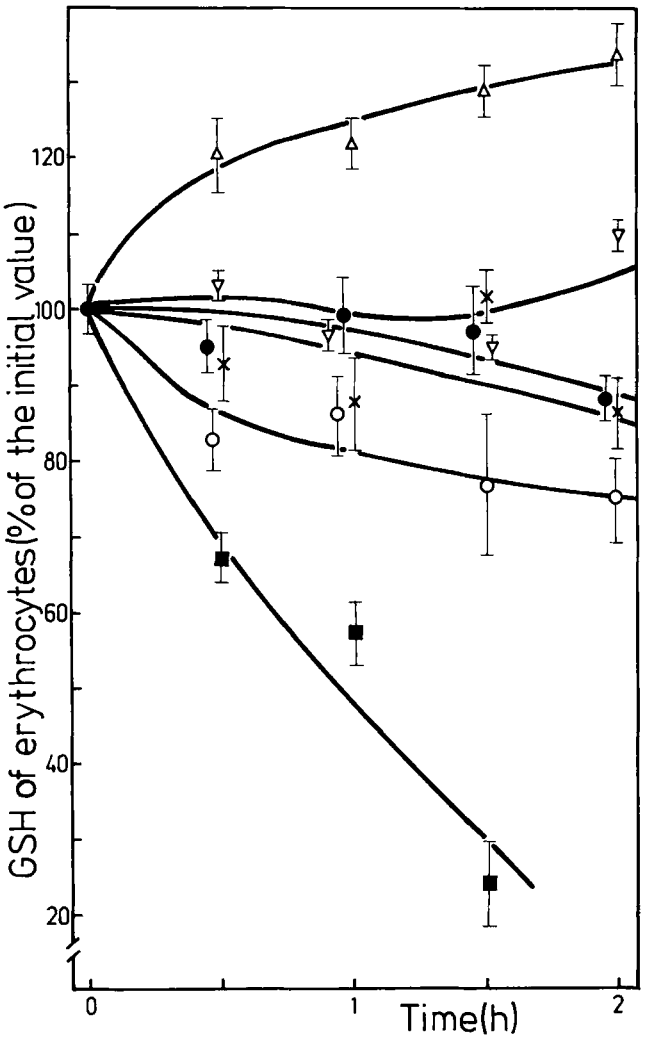

FIGURE 1 GSH concentration of red cells in a liver perfusion system. Initial GSH concentration was 2.85 ± 0.12 mM. Without liver (×), n = 10; normoxic perfusion (●), n = 8; hypoxic perfusion (○), n = 8; hypoxia + allopurinol (▽), n = 2; hypoxia + formate (△), n = 6; diethyldithiocarbamate-preincubated erythrocytes (■), n = 2. Temperature 37°C.

changed to 74 mM and after two hours it was 78 mM NaCl. During normoxic perfusion there were minor changes from 67 to 69 and 70 mM NaCl, resp. When erythrocyte suspensions circulate up to two hours in the perfusion apparatus without liver there was no variation of osmotic fragility.

By summing up the results shown in figures 1 to 4 the following conclusions are derived: (i) hypoxia in the liver deteriorates to some extent the glutathione status of circulating erythrocytes and considerably their plasma membrane structure, (ii) these changes are attenuated by allopurinol or formate.

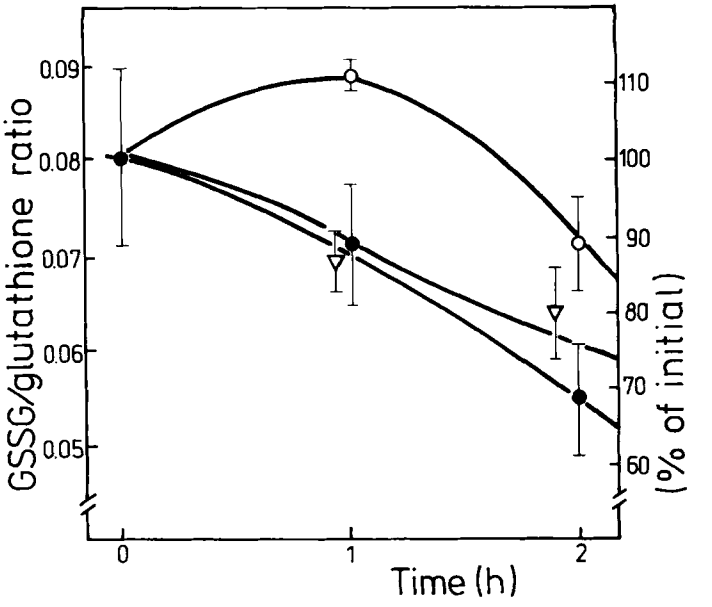

FIGURE 2 GSSG:glutathione ratio in recirculating erythrocytes. Values are given as ratio without dimension and as % of the initial. Normoxia (●), n = 6; hypoxia (○), n = 4; hypoxia + 2 mM allopurinol (▽), n = 2. 37°C.

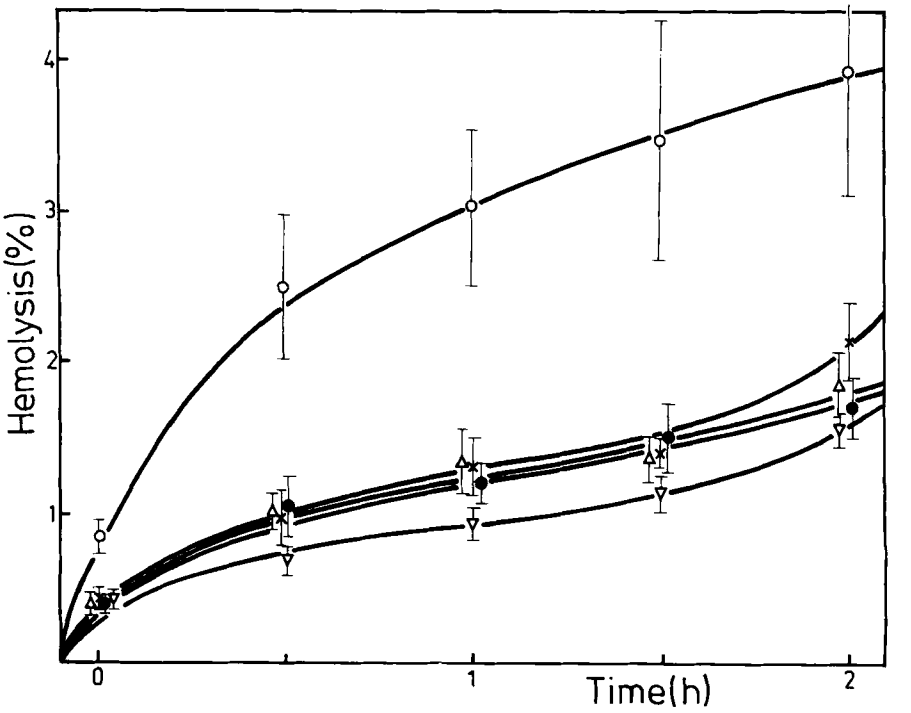

FIGURE 3 Hemolysis in an erythrocyte-containing liver perfusion system. The hemoglobin concentration of erythrocyte at time zero was 4.57 mM. Without liver (x), n = 6; normoxia (●), n = 5; hypoxia (○), n = 5; hypoxia + 2 mM allopurinol (▽), n = 2; hypoxia + 2 mM formate (△), n = 3. Temperature 37°C.

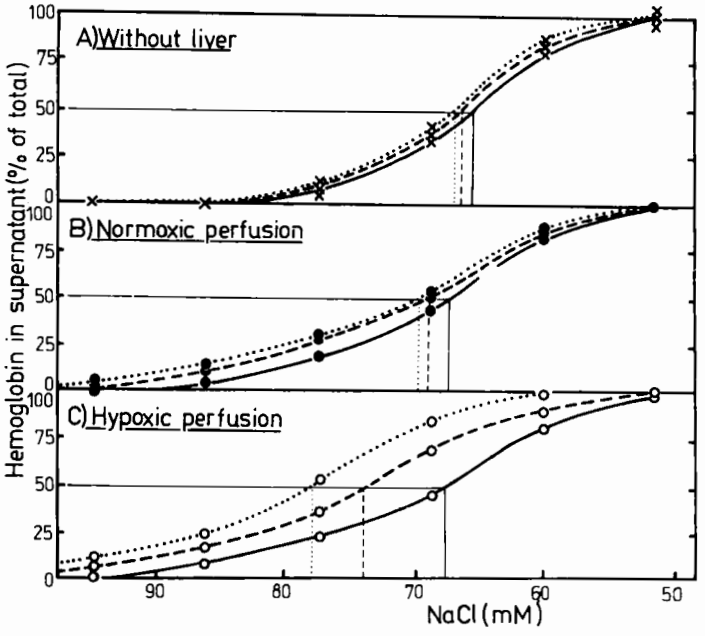

FIGURE 4 Osmotic fragility curves of erythrocytes. The perfusion experiments were carried out in the following manner: A) Without liver (×), n = 12, B) Normoxic perfusion (●), n = 8, C) Hypoxic perfusion (○), n = 8. Osmotic fragility after 0 min —— 60 min - - - - 120 min · · · · Temperature 37°C.

The findings confirm the hypothesis that species of activated oxygen and/or products of lipid peroxidation leave the liver parenchymal cells. Their primary target is apparently the red cell membrane. This conclusion is substantiated by limited variations in the glutathione systems and the absence of methemoglobin accumulation (data not presented). Hemolysis as an irreversible consequence appeared according to Waller *et al.*[1] only after a drastic practically complete GSH exhaustion.

With respect to the role of methemoglobin formation within the sequence of reactions contributing to lipid peroxidation and hemolysis there is controversy in the literature[3,6–9,20] Recently Vercellotti *et al.*[21] reported on a dichotomous production of methemoglobin and erythrocyte lysis by enzymically generated oxygen species and by polymorphonuclear leukocytes. At present one can only speculate with regard to the types of oxygen reduction products and secondary intermediates and their involvement in erythrocytic alterations. H_2O_2 may be mostly favoured owing to its excellent permeability through peroxisomal[34] as well as plasma membranes.[35] $O_2^{\cdot-}$ was shown to enter erythrocytes[7,17,21] using the band 3-protein anion channel,[19] but nevertheless doubts have been expressed with regard to its short lifetime in water.[36] $^{\cdot}OH$ radicals may be generated by Fenton type reactions within or in the vicinity of the erythrocyte membrane. Therefore, activated oxygen species generated in liver initiate lipid peroxidation in liver and in the erythrocyte membrane.

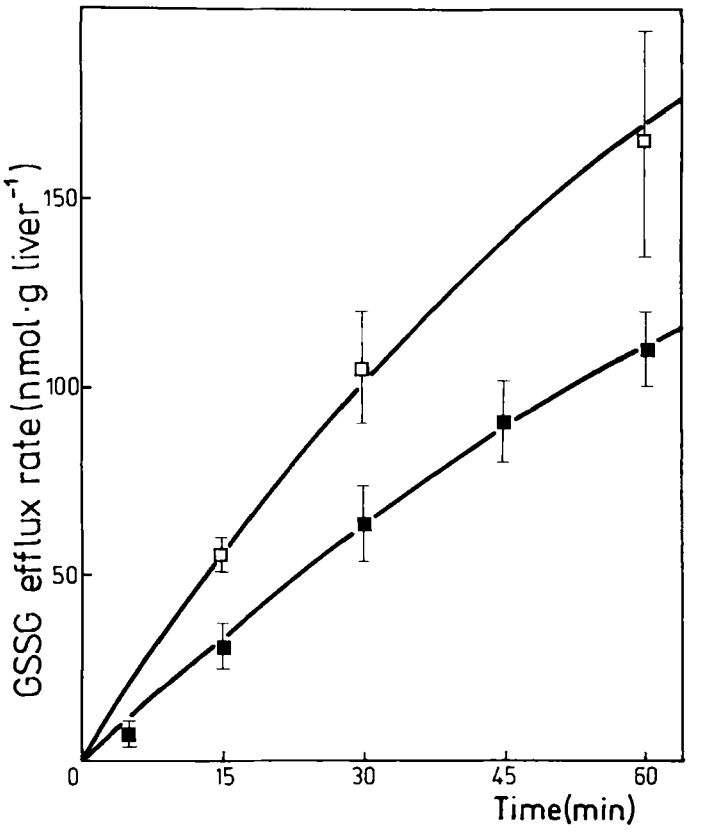

FIGURE 5 GSSG efflux rate of the hemoglobin-free perfused rat liver at hypoxia (pO_2 of the influent 5.2 kPa). Recirculating perfusion (□), n = 7; non-recirculating perfusion (■), n = 4. Temperature 25°C.

Lipid peroxidation results in a wide range of secondary intermediates, such as fatty acid hydroperoxides, malonyldialdehyde and other aldehydes[20,37] as well as several low molecular thiol derivatives. These can also leave the liver[37] and react with constituents of the erythrocyte membrane.[11]

One should expect that their reactions with liver cells may be diminished when the recirculating perfusion is replaced by a nonrecirculating system. That was observed indeed: the rate of GSSG efflux of liver as criterion of oxidative load[38,39] was reduced to about two thirds (Figure 5).

In Figure 6 the protective effect of circulating erythrocytes on the hypoxic liver is demonstrated. During the first hour of incubation the GSSG efflux rate is about twofold in absence of red cells. Such a "scavenging" effect of red blood cells was also postulated for an isolated lung preparation[24] and cultivated Chinese hamster cells,[40] both of them exposed to hypoxanthine plus xanthine oxidase.

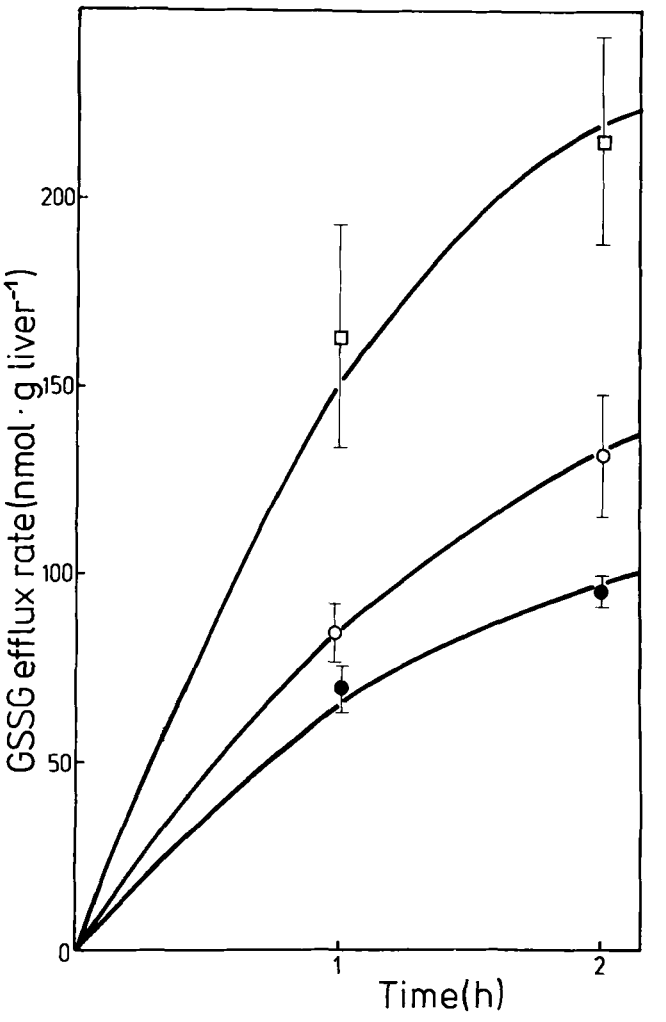

FIGURE 6 GSSG efflux rate of the liver under normoxic and hypoxic conditions in experiments with erythrocyte-containing perfusate and hypoxic hemoglobin-free experiments at 25°C. Normoxia + red blood cells (●), n = 4; hypoxia + red blood cells (○), n = 4; hypoxia without red blood cells (□), n = 7.

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