ENZYME DEFENSE AGAINST REACTIVE OXYGEN DERIVATIVES. II. ERYTHROCYTES AND TUMOR CELLS

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Summary

The enzymatic destruction of oxidizing products produced during metabolic reduction of oxygen in the cell (such as singlet oxygen, H_2O_2 and OH radical) involves the concerted action of superoxide dismutase – which removes O_2^- and yields H_2O_2 – and H_2O_2 removing enzymes such as catalase and glutathione peroxidase. A difference in distribution or ratio of these enzymes in various tissues may result in a different reactivity of oxygen radicals.

It was found that in red blood cells superoxide dismutase and catalase are extracted in the same fraction as hemoglobin, while glutathione peroxidase appears to be "loosely" bound to the cellular structure. This suggests that in red blood cells catalase acts in series with superoxide dismutase against bursts of oxygen radicals formed from oxyhemoglobin, while glutathione & peroxidase may protect the cell membrane against low concentrations of H_2O_2. On the other hand, catalase activity is absent in various types of ascites tumor cells, while glutathione peroxidase and superoxide dismutase are found in the cytoplasm. However, the peroxidase/dismutase ratio is lower than in liver cells, and this may provide an explanation for the higher susceptibility of tumor cells to treatments likely to involve oxygen radicals.

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Introduction

It is now well established that in aerobic cells many biochemical events may lead to the production of superoxide anion radicals (O_2^-) through the univalent reduction of O_2, and that this species yields further derivatives which are supposed to be the primary source of oxidative damage in the cell[1]. A likely pattern of reaction is the following[2]: O_2^- dismutates giving H_2O_2 and the highly reactive singlet oxygen: further reaction between O_2^- and H_2O_2 gives the strongly oxidizing OH radicals.

Recently the problem of scavenging the reactive oxygen derivatives from the cellular milieu has received much attention. The discovery of a new enzymatic activity displayed by a long known protein, i.e. the superoxide dismutase activity of erythrocuprein[3], has been followed by a considerable amount of research about its physiological role[4–10]. It has been proposed that the enzymic dismutation of O_2^- into H_2O_2 and O_2 by superoxide dismutase as well as that of H_2O_2 into H_2O and O_2 by catalase prevents the formation of the highly oxidizing singlet oxygen[6,7,10,11] and also the possible reaction of O_2^- with hydrogen peroxide yielding OH radicals[2,7]. It is also well known that H_2O_2 is destroyed by glutathione peroxidase in cells or cell compartments lacking catalase[12].

It appears from these considerations that distribution and levels of superoxide dismutase and H_2O_2-removing enzymes determine the steady state concentration in the cell of all the oxidative species (singlet oxygen, H_2O_2, OH)

and therefore are primary factors in the biological defense against oxidative stresses. In the previous paper of this series[2] we have described the situation existing in the cytoplasm and peroxisomes of rat liver cells, showing that catalase alone is present in the latter compartment, where H_2O_2 appears to be produced by oxidases without the liberation of a free O_2^- intermediate. Furthermore, superoxide dismutase and glutathione peroxidase would have a major role in the cytoplasm, where H_2O_2-producing oxidases appear to give substantial amounts of O_2^- as the reaction intermediate. In the present paper we show, in different cell systems, that there are different patterns of enzyme defense against oxidative damage. This may reflect different requirements and responses. Firstly, we shall report the distribution of superoxide dismutase and H_2O_2- scavenging enzymes in the red blood cell, where the major source of O_2^- and consequently of H_2O_2, appears to be oxyhemoglobin[13] which is the main component of the cell and may give rise, under certain circumstances, to massive liberation of O_2^-. Secondly, we shall discuss the enzyme levels in some tumor cells. The latter investigation was prompted by the well known observation that high energy radiations and hyperbaric oxygenation have injurious effects on the viability of cancer cells. Since irradiation of aqueous solutions[14] or hyperbaric oxygenation[15,16] of many biological systems have been repeatedly shown to cause formation of peroxides, it has been suggested[1] that oxygen radicals, such as superoxide anion radicals (O_2^-), can be involved in the biological effects of these agents. It seemed therefore worthwhile to investigate the state of enzymes involved in detoxification of oxygen radicals also in tumor cells, because of the possible relevance to the biology of the cancer cell and to therapeutic treatments involving oxygen.

Materials and Methods

Chemicals

The chemicals used were reagent grade, purchased from Merck, Darmstadt, W. Germany, phenazine methosulfate and nitro blue tetrazolium were from Sigma, St. Louis, Mo. U.S.A. NADH and the kits for the determination of acethylcholine esterase and of glutathione peroxidase were from Boehringer, Mannheim, W. Germany.

Preparation of tissues

Freshly drawn bovine blood, collected with citrate, was centrifuged and washed three times with isotonic NaCl. Plasma and buffy coat were removed by suction. 10 ml of packed erythrocytes were hemolysed with ten volumes of ice cold 1 mM acetic acid containing 4 mM $MgSO_4$ [17]. After 5′ at 0° the ghosts from the hemolysed red cells were sedimented at 20,000 × g for 20′. The pellets were resuspended in 100 ml of either 6 mM or 15 mM potassium phosphate buffer pH 6.8 and centrifuged again for 20′ at 20,000 × g. A second extraction was made with 100 ml of potassium phosphate buffer pH 8 of the same molarity. After centrifuging at 20,000 × g, a third washing was made with the same buffer. The final precipitate which appeared colorless was suspended in 10 ml of the pH 8 buffer. The various fractions are referred to as supernatants 1, 2, 3, 3 bis and precipitate.

The precipitate was solubilized in 0.5% Triton X-100 before testing the enzymatic activity.

Ehrlich ascites tumor cells were harvested 6 to 8 days after intraperitoneal transplantation of 4–7 million cells in male Swiss albino mice. Tumor cells from a transplantable methylcholanthrene-induced rabdomyosarcoma, MCl-A, grown in the ascites form[18], were withdrawn from C_3H inbred female mice 12 days after intraperitoneal transplantation of 0.5 million cells. Yoshida ascites tumor cells were collected from male Sprague Dawley rats 8 days after intraperitoneal transplantation of 3–5 million cells. Novikoff hepatoma ascites tumor cells were obtained from male Sprague Dawley rats 7–9 days after intraperitoneal transplantation of 2–4 million cells. Isolated liver cells from male Swiss albino mice were obtained by mechanical separation on an 80 mesh nylon net[19] after exhaustive perfusion of the liver with isotonic NaCl[20].

All cells were washed three times with 0.15 M Krebs Ringer phosphate buffer pH 7.4 and resuspended in the same solution. Cell viability was checked by the dye exclusion test, using trypan blue (1 mg/ml in isotonic NaCl)[21]. Only

nonhemorrhagic cell samples containing more than 90% viable cells were used. Cell counts were made in a Burker chamber and samples were then disrupted by sonication and the debris centrifuged off. The homogenate was then used as such, since dialysis and partial purification of the enzyme proteins by treatment with saturated ammonium sulfate did not significantly improve the enzyme assays. For localization of superoxide dismutase activity in tumors, the cell preparations were disrupted gently with a teflon homogenizer and the whole homogenate was then centrifuged at 105,000 × g for 60 min. The supernatant and the sonicated precipitate were then examined for superoxide dismutase activity.

Determination of enzymes

Superoxide dismutase was determined in red blood cells by the nitroblue tetrazolium method of NISHIKIMI *et al.*[22] and in tumor cells with the epinephrine autoxidation test of MISRA and FRIDOVICH[23]. The data are expressed as μg of enzyme/mg protein. The amount of total protein giving 50% inhibition of the autoxidation of epinephrine was assumed to correspond to 40 ng of enzyme, according to a calibration curve obtained with the pure protein, isolated with the procedure of the MCCORD and FRIDOVICH[3]. Catalase activity was determined with the spectrophotometric method of LÜCK[24]. Glutathione peroxidase activity was measured with the method of PAGLIA and VALENTINE[25]. The units for catalase and glutathione peroxidase activities are μmole of substrate transformed/mg protein/min.

Acetylcholine esterase activity was determined at 25°C using 5.2 mM acetylthiocholine as substrate and estimating the liberation of thiol groups according to ELLMAN and CALLAWAY[26].

The esterase activity of carbonic anhydrase was followed by the method of POCKER and STONE[27].

The protein concentration was determined with a biuret method[28]. In red blood cells the method was applied after removal of heme by the acid acetone procedure[29], since the strong absorption of this chromophore interfered heavily with the determination.

Hemoglobin was determined by the pyridine hemochromogen method[30].

Results

The distribution patterns of hemoglobin, catalase, superoxide dismutase, glutathione peroxidase, carbonic anhydrase and acetylcholine esterase in red blood cells are presented in Figure 1. The supernatant 3 bis (not reported) contained only trace amounts of proteins and of enzymatic activities. It appears that catalase and superoxide dismutase behave like hemoglobin which is almost completely extracted by the hemolysis medium (supernatant 1). In the final precipitate which contains the erythrocyte ghosts these three proteins are practically absent. Carbonic anhydrase, although largely removed in the various extracting media is still present in significant amounts in the final precipitate, as previously described[31]. On the other hand, glutathione peroxidase is evenly distributed in the three supernatants and is present in the ghosts in little amounts. Only in the case of glutathione peroxidase the two slightly different concentrations of the extraction medium give a different pattern. In fact, the more concentrated phosphate buffer pH 6.8 removes more enzyme from the cellular structure. This is particularly evident in the second supernatant. This observation points out another difference between the "loosely" bound glutathione peroxidase, and the more tightly membrane bound acetylcholine esterase[32] as well as the freely soluble proteins hemoglobin, catalase and superoxide dismutase.

The results concerning tumor cells are reported in Table 1. It appears that the tumor cells examined contain less superoxide dismutase than either liver or red blood cells and considerably lower glutathione peroxidase than that of liver but comparable to the value obtained in red blood cells. Little or no catalase activity was present in the tumor cells.

The superoxide dismutase in Ehrlich ascites cells was found mainly in the soluble particle-free cytoplasmic fraction. The localization of glutathione peroxidase in the same compartment has already been reported[33].

Discussion

In the interpretation of these results, it should be emphasized that the presence of superoxide dismutase together with H_2O_2-removing enzymes are of necessity important to the cells.

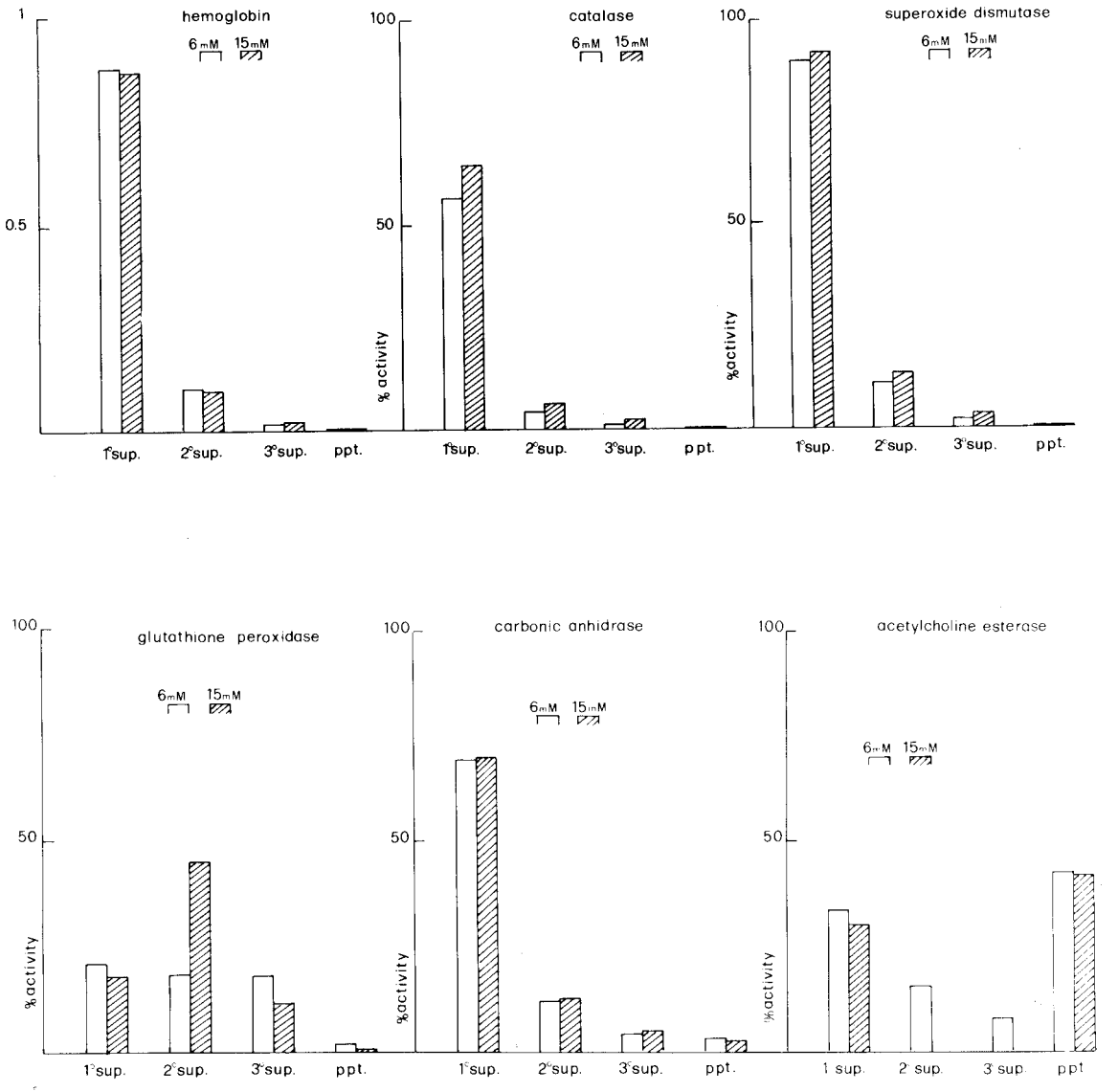

Fig. 1. Distribution of the different enzymatic activities in bovine erythrocytes. For experimental details see text.

The presence of superoxide dismutase alone would result in an increased rate of hydrogen peroxide production with its potential toxic effects. As far as the two H_2O_2-removing enzymes are concerned, it appears from localization studies in liver[2,34] that superoxide dismutase and glutathione peroxidase are both in the soluble fraction together with oxidases which produce H_2O_2 *via* a free O_2^--intermediate. However, catalase is associated in the peroxisome or microbody fraction with H_2O_2-producing enzymes which do not appear to give rise to free O_2^- as an intermediate. By contrast, in red blood cells superoxide dismutase and catalase are present together in the same compartment. This may reflect the fact that direct dissociation of oxyhemoglobin[35], to free O_2^- and subsequent formation of H_2O_2 may come about in bursts induced by interaction of oxyhemoglobin with metabolites or drugs[36]. Since the K_m for

Table 1

Enzyme content of liver and red blood cells and different tumor ascites cells.

	Superoxide dismutase (μg/mg Protein)	Glutathione peroxidase (Units)	Catalase (Units)
Liver cells (normal mouse)	1.0	115	5.0
Liver cells (Ehrlich-ascites tumor-bearing mouse)	1.0	86	2.91
Ehrlich ascites tumor cells	0.25	11	<0.02
MC1A tumor cells	0.50	10	<0.02
Yoshida ascites tumor cells	0.53	36	<0.02
Novikoff hepatoma ascites tumor cells	0.50	13	<0.02
Bovine Erythrocytes	0.82	6	2.0

H_2O_2 is considerably higher for catalase than for glutathione peroxidase[37] the red blood cell catalase may be better suited for scavenging the sudden elevated concentration of H_2O_2 than glutathione peroxidase. The latter in turn would be better suited for the low steady-state levels of H_2O_2 which are more likely to occur in liver cell cytoplasm.

Furthermore, the apparent association of glutathione peroxidase with the erythrocyte membrane suggests that its role could well be to remove the low amounts of H_2O_2 which escape catalase action and diffuse to the membrane. It is also known[38,39] that this enzyme may also act on organic peroxides, mainly those derived from unsaturated fatty acids (linoleate, linolenate) which have an important role in the structure of membranes. Thus this enzyme might also be considered a detoxification enzyme for the protection of the cell membrane.

In the tumor cells investigated in this work, no catalase activity could be detected. This confirms the reported absence of microbodies in rapidly growing tumors[40]. On the other hand, significant glutathione peroxidase activity was detected. Since this enzyme in Ehrlich ascites cells was found to be confined to the microsome-free supernatant fractions[33], where we found superoxide dismutase, it appears that also in these tumor cells superoxide dismutase is associated with glutathione peroxidase as the H_2O_2-removing enzyme. Although the concentration of the dismutase in tumor cells is lower by a factor of two to four as compared to liver, the ratio glutathione peroxidase/superoxide dismutase drops in all tumor cells, except in Yoshida cells, to 20–40% of that found in normal liver cells. This indicates that in the cytoplasm of most of the tumor cells investigated there is an increase of the activity of H_2O_2-producing enzymes relative to those removing H_2O_2. It is interesting to note that a relative deficiency of both H_2O_2-removing enzymes was also observed in the liver of Ehrlich-bearing mice.

It may be that this altered ratio could lead to an altered response of tumor cells to agents which produce a higher steady-state level of O_2^- in the cytoplasm. It is anticipated that these different tissues will be tested against oxidative stresses (such as hyperbaric oxygenation, with or without associated radiations) which are supposed to involve O_2^- as an intermediate. The altered ratio between the H_2O_2-producing (superoxide dismutase) and the H_2O_2-removing (glutathione peroxidase) may have an observable effect on the behavior of the tumor cells.

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